

World Journal of Gastroenterology®

Volume 17 Number 33
September 7, 2011



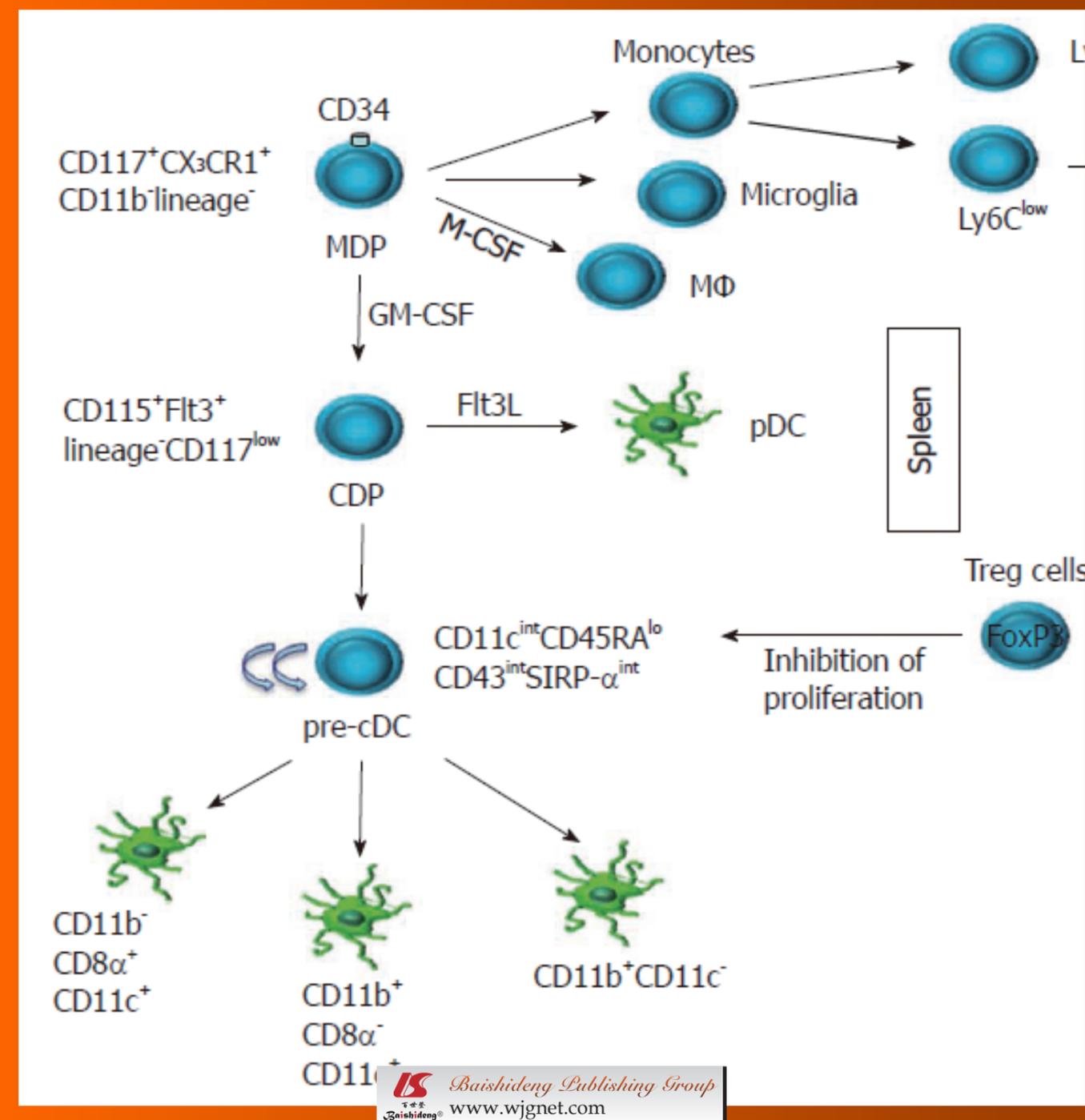
Published by Baishideng Publishing Group Co., Limited,
Room 1701, 17/F, Henan Building,
No. 90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

ISSN 1007-9327



World Journal of *Gastroenterology*

World J Gastroenterol 2011 September 7; 17(33): 3761-3858



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AIM AND SCOPE *World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.
The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

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NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

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

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

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

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

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

PUBLICATION DATE
September 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/1007-9327/office>

Intestinal dendritic cells in the pathogenesis of inflammatory bowel disease

Sergio Rutella, Franco Locatelli

Sergio Rutella, Franco Locatelli, Department of Pediatric Hematology/Oncology, IRCCS Children's Hospital "Bambino Gesù", 00165 Rome, Italy

Sergio Rutella, Catholic University Medical School, 00168 Rome, Italy

Franco Locatelli, University of Pavia, 27100 Pavia, Italy

Author contributions: Rutella S wrote the manuscript draft; Locatelli F gave intellectual input and advice, and contributed to manuscript writing.

Supported by The "Stem Cell Project", Fondazione Roma, Italy and by the Associazione Italiana per la Ricerca sul Cancro, Milan, Italy (AIRC, Grant No. 8556)

Correspondence to: Dr. Sergio Rutella, MD, PhD, Department of Pediatric Hematology/Oncology, IRCCS Children's Hospital "Bambino Gesù", Piazza Sant'Onofrio 4, 00165 Rome, Italy. sergio.rutella@opbg.net

Telephone: +39-66-8592678 Fax: +39-66-8592292

Received: October 21, 2010 Revised: January 18, 2011

Accepted: January 25, 2011

Published online: September 7, 2011

Abstract

The gastrointestinal tract harbors a large number and diverse array of commensal bacteria and is an important entry site for pathogens. For these reasons, the intestinal immune system is uniquely dedicated to protect against infections, while avoiding the development of destructive inflammatory responses to the microbiota. Several models have been proposed to explain how the immune system discriminates between, and appropriately responds to, commensal and pathogenic microorganisms. Dendritic cells (DCs) and regulatory T cells (Treg) are instrumental in maintaining immune homeostasis and tolerance in the gut. DCs are virtually omnipresent and are remarkably plastic, having the ability to adapt to the influences of the microenvironment. Different DC populations with partially overlapping phenotypic and functional properties have been described in different anatomical locations. DCs in the draining mesenteric lymph nodes, in the intestinal lamina propria and in Peyer's patches partake both in

the control of intestinal inflammation and in the maintenance of gut tolerance. In this respect, gut-resident DCs and macrophages exert tolerogenic functions as they regularly encounter and sense commensal bacteria. In contrast, migrating DC subsets that are recruited to the gut as a result of pathogenic insults initiate immune responses. Importantly, tolerogenic DCs act by promoting the differentiation and expansion of Treg cells that efficiently modulate gut inflammation, as shown both in pre-clinical models of colitis and in patients with inflammatory bowel disease (IBD). This article reviews the phenotypic and functional features of gut DC subsets and discusses the current evidence underpinning the DC contribution to the pathogenesis of the major clinical subtypes of human IBD. It also addresses the potential clinical benefit derived from DC targeting either *in vivo* or *in vitro*.

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Key words: Dendritic cell; Tolerance; Gut; Inflammatory bowel disease; Cytokine; Regulatory T cells

Peer reviewer: Dr. John B Schofield, MB, BS, MRCP, FRCP, Department of Cellular Pathology, Preston Hall, Maidstone, Kent, ME20 7NH, United Kingdom

Rutella S, Locatelli F. Intestinal dendritic cells in the pathogenesis of inflammatory bowel disease. *World J Gastroenterol* 2011; 17(33): 3761-3775 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i33/3761.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3761>

INTRODUCTION

The digestive tract has a surface area nearly 200 times greater than that of the skin. Being an important port of entry for microorganisms, the gut must be protected by effective immune responses. However, immune reactivity must be prevented from damaging gut tissues in response

to benign foreign material to which the gut is continuously exposed. T cell immunity relies on the recognition of antigenic peptides processed and presented to T cells by dendritic cells (DCs), which act as initiators, stimulators and regulators of antigen-specific T cell responses, but also play a pivotal role in the maintenance of tolerance towards the commensal microflora^[1,2].

DCs are specialized accessory cells distinguishable from other mononuclear phagocytes (MPs) such as monocytes and macrophages by their unique morphology and ability to capture and process antigens for presentation to effector T cells. Upon encounter with pathogens and activation, DCs undergo rapid maturation characterized by the upregulation of major histocompatibility complex (MHC) and costimulatory molecules and migrate to the draining lymph nodes. The remarkable flexibility of DC functions likely results from their ability to sense the local environment and to shape the ensuing immune response^[3,4]. Intestinal MPs are distributed in organized lymphoid organs, such as Peyer's patches (PP) and mesenteric lymph nodes (MLN), and are highly abundant in the loose connective tissue underlying the epithelium, the lamina propria (LP)^[5].

It is now established that DCs play a crucial role in both immunity and tolerance^[1,6]. In a tolerogenic setting, DC can induce anergy in antigen-specific T cells or generate protective FoxP3⁺ regulatory T cells (Treg) in the lymph nodes. Under steady-state conditions, DCs continuously migrate from peripheral organs *via* the lymph to secondary lymphoid organs, where they present self-antigens or innocuous environmental antigens to maintain peripheral tolerance. The chemokine receptor, CCR7, is a key regulator of the homeostatic and inflammation-induced trafficking of DCs from skin, lung and gut to their respective draining lymph nodes^[7].

Human inflammatory bowel disease (IBD) consists of 2 dominant disease subtypes, Crohn's disease (CD), largely arising from a Th1 response, and ulcerative colitis (UC), largely mediated by interleukin (IL)-5- and IL-13-producing T cells or natural killer T cells^[8]. The immunopathology of human IBD relates to an inappropriate and exaggerated immune response to constituents of the gut flora in a genetically predisposed individual. Amongst other cell types^[9], DCs play a role in IBD pathogenesis, as suggested by mouse models of colitis and by observations in humans. The local microenvironment regulates the function of mucosal DCs through the presence of immune cells, non-immune cells and luminal bacteria^[10]. In principle, DC dysfunction may promote the development of gut inflammation by priming T-cell responses against bacteria, by sustaining T cell reactivity within the inflamed mucosa and by functioning as effector cells releasing pro-inflammatory cytokines^[11].

DC LINEAGE AND SUBSETS IN MICE AND HUMANS

DC origin and precursor-progeny relationships have remained a matter of controversy and debate for decades^[12].

Recent landmark studies have led to a better definition of DC ontogeny in mice (Figure 1), unraveling that a macrophage and DC precursor (MDP) serves as a common bone marrow progenitor for classical or conventional DCs (cDCs), plasmacytoid DCs (pDCs) and monocytes (Table 1). Specifically, Fogg *et al.*^[13] have identified a clonogenic MDP with a CD117⁺CX₃CR1⁺CD11b⁻ lineage⁻ phenotype, representing ~0.5% of total bone marrow cells and giving rise to monocytes, to several macrophage subsets and, ultimately, to steady-state CD11c⁺CD8 α ⁺ and CD11c⁺CD8 α ⁻ DC. Lymphoid organ DCs in the steady state originate from a bone marrow precursor with a Lin⁻CD115⁺Flt3⁺CD117^{lo} phenotype, termed common DC progenitor (CDP)^[14]. Migratory DC precursors (pre-DCs) also exist in the peripheral blood and are in equilibrium with DCs in lymphoid organs and in non-lymphoid tissues, such as skin, lung, kidney and intestine. DCs actively divide *in vivo* and their lifespan varies from 5 to 7 d in the spleen, lymph node, liver and kidney and can be as long as 25 d in the lung^[15].

DCs lack a unique surface marker, but rather express a distinct set of cell surface antigens. The number of DC subsets that have been phenotypically characterized and functionally designated is increasing steadily. In addition to the classical integrin marker CD11c distinguishing DCs, the integrin α E (CD103) recently gained attention and has been used to sub-classify DC subsets based on specific functional activities and anatomic location (see below for a thorough discussion)^[16]. CD103 mediates T cell adhesion to epithelial cells through its binding to E-cadherin, which is expressed on the basolateral side of epithelial cells but not on endothelial cells. Mice with a targeted disruption of *cd103* show a mild reduction in T cell numbers in the intraepithelial and LP compartments, coupled with the inability to reject islet allografts^[17].

MECHANISMS UNDERLYING DC-MEDIATED TOLERANCE IN THE GUT

One of the major functions of tolerogenic DCs may be the differentiation of Treg cells from naïve T cells. Two major subtypes of Treg cells have been described to date, namely, naturally occurring CD4⁺CD25⁺FoxP3⁺ Treg cells (nTreg) and inducible type 1 Treg cells (Tr1).

DCs as inducers of nTreg cells

Naturally occurring Treg cells, a functionally specialized subset of CD4⁺ T cells, have been involved in preventing T cell-mediated and innate immune pathology in a number of disease models^[18]. The transcription factor FoxP3 is expressed by CD4⁺CD25⁺ Treg cells and is fundamental for Treg development and function. nTreg cells mainly suppress effector T cells through a cell contact-dependent and largely contact-independent mechanism. Membrane-bound transforming growth factor (TGF)- β has been implicated in nTreg-mediated inhibition of T cell responses. Moreover, TGF- β 1 acts as a co-stimulatory factor for FoxP3 expression, leading to Treg differen-

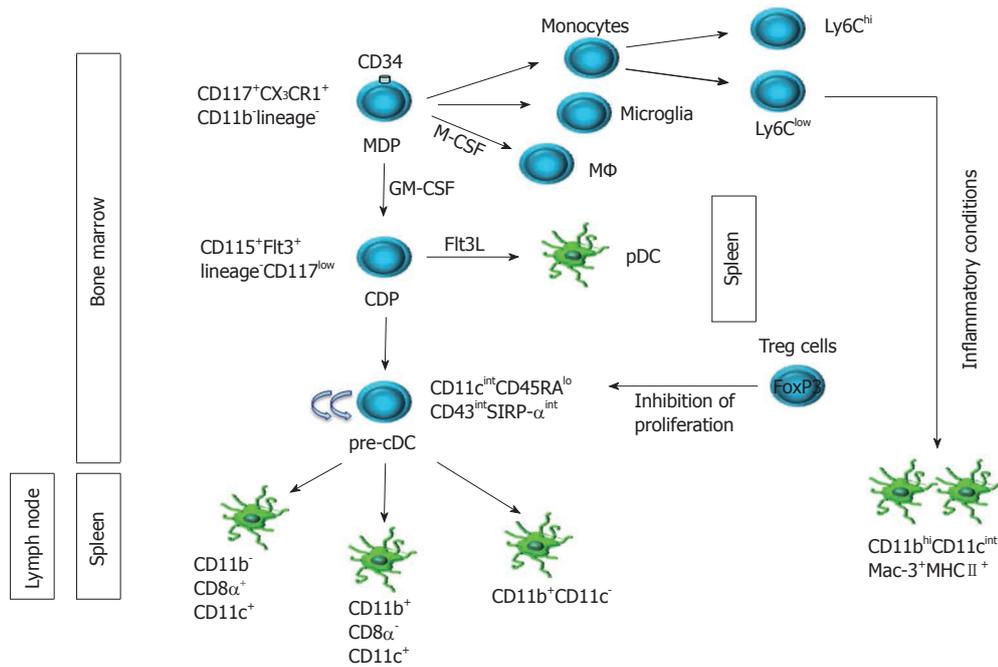


Figure 1 Ontogeny of dendritic cell subsets in mice. The most recent evidence elucidating dendritic cell (DC) ontogeny and unraveling the complexity of the DC compartment in mice is summarized. The curved arrows in cyan denote proliferation potential. Regulatory T (Treg) cells may contribute to DC development and homeostasis in mice, as suggested by studies where Treg depletion has been associated with a 2- and 12-fold increase in precursor conventional DC (pre-cDC) and cDC in spleen and lymph node, respectively^[14]. MDP: Macrophage and dendritic cell progenitor; CDP: Common dendritic cell progenitor; pDC: Plasmacytoid DC.

tiation from CD4⁺CD25⁻ T cells. Interestingly, TGF-β1 production by Treg cells is not required for inhibition of colitis, suggesting that Treg cells may induce TGF-β release by other hematopoietic or stromal cells^[19]. Support for this hypothesis is provided by the observation that suppression of colitis by TGF-β1^{-/-} Treg cells was inhibited by anti-TGF-β antibodies, indicating that TGF-β is central to the function of Treg cells even when they do not synthesize it themselves^[19]. In this respect, DCs remain a key and intriguing candidate for TGF-β production *in vivo*. It is conceivable that Treg cells be required to express TGF-β1 on the cell surface and to present it to pathogenic T cells, as previously shown^[19].

Treg cells are believed to play a crucial role in inhibiting intestinal inflammation and IBD. Notably, Treg cells may contribute differentially to the modulation of experimental autoimmune gastritis and colitis. Protection from colitis, but not from gastric inflammation, has been reported to depend on IL-10 expression by CD4⁺CD25⁺nTreg cells^[20]. The T cell transfer model of colitis allows an understanding of Treg-mediated mechanisms controlling intestinal inflammation. During cure of experimental colitis, Treg cells proliferate and accumulate in MLN and colonic LP, in contact with CD11c⁺ DCs and effector T cells^[21]. Interestingly, IL-10-producing Treg cells selectively enrich within the colonic LP, whereas FoxP3-expressing Treg cells are present in similar frequencies in both the secondary lymphoid organs and LP of colitic animals^[22]. Transfer of CD4⁺CD45RB⁺ T cells into RAG^{-/-} mice causes colitis. Disease development requires β7-integrin-dependent intestinal localization. Importantly, β7-deficient Treg cells prevent colitis, suggesting that Treg

accumulation in the intestine is dispensable for disease suppression^[23]. The presence of Treg cells impacts on CD4⁺CD45RB⁺ T cell accumulation in the intestine, indicating that one major function of Treg cells may involve the inhibition of tissue localization of Th1 effector cells.

Peripheral blood CD4⁺CD25^{high} T cells may be decreased in active human IBD compared with inactive disease^[24]. Notably, Treg cells are increased in mucosal IBD lesions, coincident with an increase in transcripts for IL-8, a hallmark of inflammation in the gut, and for FoxP3^[24]. The higher degree of Treg infiltration in the gut LP of patients with diverticulitis compared with IBD suggests that an insufficient increase of Treg cells in IBD accounts for inflammation and intestinal pathology^[24]. In the LP of human colon, Treg accumulation has been detected in a variety of inflammatory conditions, such as diverticulitis, pseudo-membranous colitis and cytomegalovirus-induced colitis, and may not be a specific feature of CD or UC^[22]. The presence of FoxP3⁺ T cells in the LP of patients with IBD suggests that defects in Treg numbers may not account for the pathology, and that ineffective Treg activity may rather contribute to sustained gut inflammation.

DCs as inducers of Tr1 cells

Tr1 have been described as a CD4⁺ T-cell subset releasing high levels of IL-10, in the absence of measurable IL-2 and IL-4 production, and exerting suppressive functions in an IL-10/TGF-β-dependent but cell contact-independent manner^[25]. The production and release of interferon (IFN)-γ and TGF-β by Tr1 cells are comparable with those of Th0 and Th1 clones, respectively^[25].

Table 1 Intestinal dendritic cell subsets and other mononuclear phagocytes described to date

Subset	Anatomic location	Function, if known	Ref.	
CD11c ^{hi} CD11b ⁺ CD8 α ⁻	Peyer's patches	Localized in the subepithelial dome	[57]	
CD11c ^{hi} CD11b ⁺ CD8 α ⁺		Localized in the interfollicular regions	[57]	
CD11c ⁺ CD11b ⁻ CD8 α ⁻		Localized in both subepithelial dome and interfollicular regions; secretion of IL-10 in response to CD40 cross-linking; induction of T-cell release of IL-4/IL-10; promotion of T-cell proliferation	[58]	
CD11c ^{mid} plasmacytoid DC	Small-intestinal and colonic lamina propria	Low T-cell responses <i>in vitro</i> ; IL-12p40 ^{low} IL-10 ⁺ ; tolerance to OVA (m); secretion of IFN- α ; differentiation of Tr1-like cells that secrete IL-10, IL-4 and IFN- γ	[30,55]	
CD11c ⁺ CD11b ⁺ CD8 α ⁺ DC		Pro-inflammatory activity dependent upon TNF- α production	[101]	
CX ₃ CR1 ⁺ DC	Mesenteric lymph node	Generation of CCR9 ⁺ α ₄ β ₇ ⁺ T cells with gut tropism	[51]	
CD11c ^{hi} class II ^{hi} CD103 ⁺ DC		Conversion of Foxp3 ⁺ T cells to Foxp3 ⁺ T cells; retinoic acid and TGF- β required	[54]	
CD11b ⁺ CD8 α ⁻ CD103 ^{-/-} DC		Promotion of CD4 ⁺ CD25 ⁺ Treg function; differentiation of Tr1-like cells (IL-10 ⁺ IL-4 ⁺ IFN- γ ⁺) from naïve T cells after repeated stimulations	See above	
CD11c ^{lo} B220 ⁺ CD8 α ⁺			See above	
CD11c ^{hi} CD11b ⁺ CD8 α ⁺		~50% of CD11c ⁺ DC in the MLN; conversion of naïve T cells into Treg cells; retinoic acid and TGF- β required; induction of CCR9 on gut-tropic T cells	[16]	
CD11c ^{hi} CD11b ⁻ CD8 α ⁻		Expression of pro-inflammatory cytokines (TNF- α , IL-6, IL-23p19) and genes such as <i>Tbet</i> , <i>tlr2</i> and <i>tbx21</i>		
CD103 ⁺ DC				[16]
CD103 ⁻ DC		Induction of CCR9 gut-homing receptor on CD4 ⁺ T cells	[53]	
CD11c ^{hi} class II ^{hi} CD103 ⁺ DC	Small and large intestine	Induction of Treg cells, secretion of IL-10 but not IL-12p40 or IL-12p70	[105,106]	
CD11b ⁺ CD11c ⁻ macrophages				

DC: Dendritic cell; OVA: Ovalbumin; IFN: Interferon; MLN: Mesenteric lymph node; Tr1: Type 1 Treg cells; TGF: Transforming growth factor; IL: Interleukin; M: Mouse studies.

Colitis in the severe combined immunodeficient (SCID) mouse model involves the development of Th1 cells responding primarily to the intestinal flora. The transfer of ovalbumin (OVA)-specific Tr1 cells in SCID mice with CD4⁺CD45RB^{hi} T cell-induced colitis prevents disease manifestations, an effect that is dependent upon the *in vivo* activation of Tr1 cells by feeding mice with OVA^[25]. This observation indicates that Tr1 cells can inhibit immune responses to unknown antigens by a bystander suppression mechanism. Another report has shown that IL-10^{-/-} mice lack CD4⁺CD45RB^{lo} Treg cells capable of controlling intestinal inflammatory responses, pointing to IL-10 as a crucial mediator of tolerance in the gut^[26]. Similarly, TGF- β is required to suppress Th1-mediated colitis induced by CD4⁺CD45RB^{hi} T cells^[27], indicating that IL-10 and TGF- β play non-redundant roles in the functioning of intestinal Treg cells.

The source of IL-10 which regulates colitis remains to be unequivocally identified. Treg-derived IL-10 was recently shown to be dispensable for suppression of colitis in *Rag1*^{-/-} mice, but host IL-10 was required to inhibit disease development^[28]. Specifically, IL-10 production by myeloid CD11b⁺F4/80⁺ cells, mostly macrophages, was important for the maintenance of Foxp3 expression by Treg cells^[28]. IL-10 acted directly on Treg cells, because Treg cells lacking IL-10R β chain failed to suppress colitis when transferred together with CD4⁺CD45RB^{hi} T cells. In addition, this study demonstrates that IL-10 is not required to maintain Foxp3 expression in non-inflammatory conditions, because Treg development and function are unaffected in *Il10rb*^{-/-} mice^[28]. It is conceivable that the differential requirement for IL-10 for Foxp3 expression and maintenance in inflammatory vs non-inflammatory conditions may reflect the need for an additional signal to

counter inflammatory mediators such as IL-6 or tumor necrosis factor (TNF)- α ^[29]. It remains to be determined whether IL-10-mediated mechanisms are unique to the gut microenvironment or whether IL-10 may be required to maintain Foxp3 expression in other organs.

Other studies pointed to Treg-derived IL-10 as a major contributor to Treg-mediated suppression^[25]. These discrepancies may be attributed to differences in the endogenous flora and/or in the model systems studied. Mucosal CD8 α ⁺ DCs with a CD11c^{lo}B220⁺ phenotype can be isolated from mouse MLN and have been reported to promote the suppressive function of CD4⁺CD25⁺ Treg cells and to promote the conversion of naïve T cells into Tr1-like cells^[30]. At variance with classical Tr1 cells, the Tr1-like cells described in this study released IL-10, IL-4 and IFN- γ and suppressed T helper proliferation^[30]. The CD8 α ⁺ DC were capable of supporting Tr1-like cell differentiation also in the presence of a maturational stimulus, such as CpG, as reported for other tolerogenic, semi-mature DC preparations^[6,31].

DC expression of indoleamine 2,3-dioxygenase 1 and gut tolerance

Indoleamine 2,3-dioxygenase 1 (IDO1) is a tryptophan-catabolizing enzyme implicated in maternal allograft acceptance and in immune tolerance to tumors^[32-36]. IDO1 converts tryptophan into immune suppressive kynurenines that profoundly affect T-cell functions, promoting T-cell unresponsiveness, T-cell apoptosis and differentiation of Treg cells. IDO expression has been associated with CD103⁺ DCs in the gut LP and MLN of mice^[37]. Similarly, human intestinal CD11c⁺CD103⁺ DCs express higher levels of IDO mRNA compared with CD11c⁺CD103⁻ DCs. IDO inhibition of mouse CD103⁺ DCs with the D

isomer of 1-methyl-tryptophan (1MT) reduced the ability of IDO⁺ DCs to convert Treg cells and augmented the generation of IL-17-producing T cells. Mice treated with 1MT concomitant with adoptive transfer of OVA transgenic T cells and oral immunization with OVA led to a reduction in the frequency of Treg cells in the LP, PP and MLN. *Ido1*^{-/-} mice displayed a decreased percentage of Foxp3⁺ Treg cells in the LP and an almost double the proportion of IL-17⁺CD4⁺ and IFN- γ ⁺CD4⁺ T cells in the intestine compared with wild-type animals. Finally, *Rag1*^{-/-} mice injected with colitogenic T cells from C57BL/6 mice experienced more extensive gut inflammation and aggressive disease if treated with 1MT. Similar effects were demonstrated in mice with dextran sodium sulfate (DSS)-colitis, where 1MT administration worsened the mortality rate and colon shortening. Collectively, these experiments indicate that IDO may play a previously unappreciated and fundamental role in regulating gut inflammation through the control of Th1/Th17/Treg balance.

The expression of IDO in the murine gut may increase with age via an IFN- γ -dependent mechanism that involves commensal microorganisms^[38]. IDO-deficient mice have abnormally high levels of both IgG and IgA, a phenomenon driven by the commensal flora. IDO may then physiologically restrict B-cell responses to intestinal commensal bacteria. The elevated levels of IgG and IgA in IDO-deficient mice might in principle confer resistance to enteric pathogens such as *Citrobacter rodentium*, a gram-negative bacillus similar to human enteropathogenic *Escherichia coli*. When infected orally with *Citrobacter*, IDO-deficient mice appeared well throughout the course of the experiment, at variance with wild-type animals that had decreased activity, ruffled fur and hunched posture, and had attenuated gut colonization by the pathogen^[39]. IDO-deficient mice had reduced edema, inflammatory cell infiltration and epithelial damage in colonic tissue sections, associated with lower levels of TNF- α compared with wild-type mice. These observations point to IDO as a novel target to manipulate intestinal inflammation and to control diseases caused by enteric pathogens.

Crosstalk between DCs and intestinal epithelial cells

Intestinal epithelial cells (IECs) are a central component of the immune system of the gut. They express receptors for microbial-associated molecular patterns that activate signaling cascades leading to the production of antimicrobial products and chemokines^[40]. IECs can also recruit leukocytes to complement their barrier function or to participate in the activation of gut adaptive immune responses, including the production of IgA and the differentiation of effector Th1, Th2 and Th17 cells.

IECs are in close contact with LP DCs and have been shown to release molecules that influence DC functions. Thymic stromal lymphopoietin (TSLP) is a cytokine secreted by IECs under steady-state conditions and imparts a Th2-polarizing phenotype to DCs^[41]. IEC-derived factors also stimulate the expression of both chains of TSLP receptor on DCs, namely the common

IL-7 receptor α chain and the TSLP receptor, thus conferring the ability to respond to TSLP and to drive Th2 responses. Importantly, TSLP expression by primary IECs may be deregulated in a proportion of patients with IBD. The same study also showed that mRNA signals for TSLP are readily detected in IECs from healthy controls, although the protein is consistently below the detection limit by immunoprecipitation, unless IECs are challenged with bacteria such as *S. typhimurium*. TSLP has been detected in epithelial cells of the Hassall's corpuscles and activates myeloid CD11c⁺ DCs in the thymic medulla^[42]. These apparently mature DCs promote the development of Treg cells through a mechanism that requires peptide-MHC class II interactions, and the presence of CD80, CD86 and IL-2. Plasmacytoid DCs can be also activated by TSLP and become efficient generators of Treg cells from thymocytes through an IL-10-dependent mechanism^[43]. CD4⁺ T cells triggered through the T cell receptor, but not resting CD4⁺ T cells, respond to TSLP with robust proliferation and acquire sensitivity to low doses of IL-2^[44].

INTESTINAL DCs UNDER STEADY-STATE CONDITIONS AND IN EXPERIMENTAL COLITIS

DCs in the non-inflamed gut

Cells with antigen-presenting function within the intestine and associated lymphoid tissue include macrophages, conventional CD11c-expressing DCs and plasmacytoid DCs. Macrophages belong to a family of tissue cells that includes Kupffer cells in the liver and glial cells in the brain and have predominantly innate immune functions, such as capturing and killing of microbes, scavenging of apoptotic and dead cells, and production of regulatory cytokines^[45]. Macrophages are the most abundant population of phagocytic cells in the intestine. Distinctive characteristics have also been assigned to intestinal macrophages as compared with splenic macrophages or blood monocyte-derived macrophages. Early studies identified macrophages in the small and large intestine in the mouse, based on the expression of the F4/80 glycoprotein in association with CD11b^[46]. LP macrophages are detected in juxtaposition to CD4⁺ T cells and in close contact with the epithelium^[23,47]. CD11b⁺CD11c⁻ macrophages are scattered throughout the villus-tip axis of small and large intestine, express immune regulatory molecules such as programmed death ligand 1 (PD-L1) and PD-L2, and secrete IL-10 but not IL-12p40 or IL-12p70. They are hyporesponsive to Toll-like receptor (TLR) stimulation, suppress the differentiation of Th1 and Th17 cells, and promote the differentiation of Treg cells^[23]. Local macrophages may contribute to colitis development in IL-10^{-/-} mice^[48]. The pharmacological depletion of macrophages in this model of colonic inflammation ameliorated colitis, suggesting that IL-10 deficiency impedes the conditioning of macrophages, leading to macrophage-mediated destructive inflammatory responses.

Under steady-state conditions, the functional properties of the DC subpopulations vary according to their anatomical location. For instance, functional differences among DCs from PP, from MLN and from small intestinal and colonic LP have been reported. Within a single anatomical site, DCs can be distinguished and further subdivided according to their surface membrane phenotype. Under inflammatory conditions, DC recruitment to the intestine occurs, although it is presently unclear whether these DC populations are separate from DCs present in the steady-state or whether DCs arriving in the inflamed intestinal microenvironment acquire the ability to foster pro-inflammatory responses as a result of their exposure to pathogens and local inflammatory mediators.

LP DCs can be isolated in the absence of overt inflammatory stimuli and perform a tolerogenic function by constitutively migrating to the draining MLN, where they present antigen to T cells. The carriage of antigens from commensal bacterial strains to the MLN might be triggered by low-level production of pro-inflammatory cytokines. In this respect, the chemokine receptor CCR9 is crucial for the positioning of plasma cells^[49] and plasmacytoid DCs^[50] to the small intestine, suggesting that the chemokine CCL25/TECK may regulate DC homing during inflammatory processes. After their migration to the MLN, DCs interact with T and B cells and initiate immune responses aimed at maintaining a non-inflammatory state in the intestine. Intestinal DCs have been reported to promote the peripheral induction of FoxP3-expressing Treg cells from naïve T cells. Such Treg cells with specificity for commensal bacteria and dietary antigens may prevent naïve T cells from inducing pathological responses, thus complementing the pool of thymus-derived Treg cells. In this respect, gut-associated lymphoid tissue DCs may synthesize the vitamin A metabolite retinoic acid, that selectively induces CCR9 and $\alpha_4\beta_7$ integrin on CD8⁺ T cells with gut tropism^[51]. This phenomenon occurs more efficiently after oral as compared with intraperitoneal antigen administration, indicating differential DC targeting by the 2 immunization routes^[51].

CD103 is the α chain of the $\alpha E\beta 7$ integrin expressed by most mouse and human intestinal lymphocytes and mediating lymphocyte adhesion to E-cadherin-expressing intestinal epithelial cells. CD103-expressing DCs may also be required to induce gut-tropic effector T cells in the MLN^[51]. Interestingly, TGF- β plays a dominant role in CD103 induction on gut-tropic CD8⁺ T cells, as shown in a mouse model of post-transplantation graft-*versus*-host disease (GVHD) with T-cell infiltration of the intestinal epithelium^[52]. T cells from 2C T cell receptor-Tg mice that express a dominant negative TGF- β type II receptor were incapable of upregulating CD103 upon migration into the intestinal epithelium^[52]. In addition, CD103 expression on host-reactive CD8⁺ T cells was causally related to the development of GVHD pathology and mortality. Although TGF- β activity is present locally within the intestinal milieu, this study did not exclude the possibility that CD8 effectors encounter TGF- β and upregulate CD103 expression before their entry into the intestinal epithelium.

CD103^{-/-} T cells migrate into the host intestine but are retained much less efficiently than wild-type T cells, indicating that CD103 expression may also contribute to T-cell accumulation in the gut^[52]. In a T-cell transfer model of colitis, disease-inducing CD4⁺CD45RB^{high} T cells were shown to promote colitis development irrespective of their expression of CD103^[53]. However, anti-CD103 antibodies abrogated the suppression of colitis mediated by Treg cells. Further experiments suggested that CD103 expression by Treg cells was not essential for their function, indicating the requirement for CD103 on non-T host cells for protection from colitis^[53]. Of interest, ~50% of CD11c⁺ DCs in the MLN co-expressed CD103 at high density, at variance with ~30% of splenic DCs. Sorted CD103⁺ DCs activated the proliferation of allogeneic CD4⁺ T cells to a similar extent compared with the CD103⁻ counterpart but were potent inducers of CCR9 co-expression by day 4 of culture, suggesting their ability to impart gut tropism on T cells. In addition, CD103⁺ DCs were inefficient at inducing IL-10 and IFN- γ production by T cells. Collectively, this study suggested that DC subsets that are primed in the immunosuppressive environment of the gut may be unable to drive the release of pro-inflammatory cytokines such as IFN- γ , thus preventing the development of unwanted effector responses to ingested antigens. Another report by the same investigators has shown that CD103⁺ DCs isolated from the MLN may both induce *de novo* expression of Foxp3 in naïve T cells and maintain pre-existing Foxp3⁺ cells^[16]. The conversion of naïve T cells into Treg cells by CD103-expressing DCs was completely inhibited by anti-TGF- β antibodies, but further enhanced by exogenous TGF- β , so that provision of 1 ng/mL TGF- β to the T-cell/DC co-cultures translated into the expression of Foxp3 by ~50% of T cells^[16]. Even the provision of high concentrations of TGF- β to CD103⁻ DCs did not allow the generation of similar percentages of Treg cells to CD103⁺ DCs, suggesting that CD103⁻ DCs may lack an essential cofactor. Further experiments led the authors to identify retinoic acid as the cofactor for the TGF- β -driven conversion of Treg cells from naïve T cells. Compared with the CD103⁺ DCs, CD103⁻ DCs released higher amounts of pro-inflammatory cytokines (TNF- α , IL-6), and expressed higher levels of IL-23p19 and Tbet^[16]. Collectively, this study showed the existence of functionally distinct DC populations in the MLN of normal mice, with apparent diverging functions. A companion paper by Sun *et al.*^[54] has shown, both in a lymphopenic mouse transfer model and in an immunologically complete setting, that retinoic acid released by LP DCs promotes Treg conversion in the presence of TGF- β . The LP DCs expressed a CD8 α CD11c⁺ phenotype and displayed the morphologic features of conventional DCs, consisting of a stellar shape comparable to freshly isolated splenic DCs^[54].

LP DCs

The extensive phenotypic and functional characterization of mouse LP DCs so far pursued has revealed a greater complexity than previously appreciated. The majority of

LP DCs express a CD11b⁺CD8 α ⁻ phenotype, although CD11b⁻CD8 α ⁺ and CD11b⁻CD8 α ⁻ DCs have also been identified^[55]. Treatment of mice with Flt3 ligand increases the proportion of LP DCs without significantly altering the relative proportion of DC subsets, thus allowing the purification of a higher DC number for detailed functional analyses. Using this approach, some authors have shown that LP DCs are not fully mature *in situ* but they can be induced to differentiate in response to appropriate stimuli^[55]. LP DCs were also less efficient at stimulating OVA-specific T-cell proliferation *in vitro* when compared with splenic DCs, and mediated the development of tolerance when transferred to mice fed with OVA^[55]. LP DCs exhibited a unique cytokine profile, consisting of low levels of IL-12p40 mRNA associated with constitutive IL-10 and type I IFN production^[55]. A specialized subset of LP DCs with a CD8 α ⁺ phenotype has been identified in mice^[30]. Gut-derived CD8 α ⁺ DCs secrete IFN- α and support antigen-specific suppression mediated by CD4⁺CD25⁺ Treg cells. Furthermore, CD8 α ⁺ DCs favor the differentiation of Tr1-like cells that release high quantities of IL-10, IL-4 and IFN- γ upon activation with plate-bound anti-CD3 antibodies^[30]. The ability of CD8 α ⁺ DCs to induce Tr1-like cells was not affected by their exposure to maturation stimuli, as reported for other populations of maturation-resistant, tolerogenic DCs^[31].

Other LP DC subsets identified in mice include CD11b⁻CD103^{hi} and CD11b⁻CD103^{low} DCs. LP DCs can be further subdivided into CD11b⁻CD103^{hi}CX₃CR1⁻ DCs and CD11b⁻ DCs with different CX₃CR1 (fractalkine receptor) expression levels^[56]. The CD103^{hi}CX₃CR1⁻CD11b⁻ LP DCs originate through a DC-committed non-monocytic intermediate from MDP, a differentiation pathway that is driven by Flt3L. Conversely, CD103^{hi}CX₃CR1⁺CD11b⁺ LP DCs derive from Ly6C^{hi} monocytes and their derivation involves an extensive, granulocyte-macrophage colony-stimulating factor (GM-CSF)-driven local expansion in the mucosa. Importantly, mice that were persistently or transiently depleted of LP DCs neither developed spontaneous intestinal inflammation nor were susceptible to colitis development. In contrast, mice that harbored predominantly CD103^{hi}CX₃CR1⁺CD11b⁺ LP DCs developed severe colitis in response to a DSS challenge, as evaluated by colonoscopy and histological examination. This pro-inflammatory activity was dependent on TNF- α secretion with ensuing epithelial damage, and might also be regulated through IL-10/TGF- β production by the CD103^{hi}CX₃CR1⁻CD11b⁻ LP DC subset^[23]. This study highlighted the importance of a critical balance between LP DC subsets for tissue repair and gut homeostasis.

PP DCs

PP are the primary sites for the induction of immune responses in the intestinal mucosa and are representative of lymphoid follicles present in diffuse mucosal tissues. DCs from PP possess a unique capacity to induce T-cell responses that regulate systemic immunity through the release of IL-4 and IL-10 and that provide help for IgA B-cell differentiation. It has been shown that PP DCs

reside in different anatomical sites, with CD11b⁺CD8 α ⁻ DCs being localized in the subepithelial dome, CD11b⁻CD8 α ⁺ in the interfollicular regions and CD11b⁻CD8 α ⁻ [double-negative (DN) DCs] in both compartments^[57]. The DN DCs constitute approximately 30% of PP DCs, are interspersed within the follicle-associated epithelium, with processes extending to the luminal surface, and occasionally associated with M cells within the M-cell pocket^[58]. DN DCs express intracellular MHC molecules, indicating their immaturity, and secrete IL-12p70, suggesting functional similarity to the lymphoid DC subset^[58]. Collectively, these studies indicate that DN DCs should be able to induce Th1 differentiation, at variance with myeloid DC subsets that have been implicated in IL-10 release and in skewing the immune response towards a Th2 profile. It has been proposed that orally delivered antigens may initially encounter the myeloid and DN DCs located underneath or within the follicle-associated epithelium. As a result of feeding with low-dose antigen, antigen uptake by the DCs would not result in DC activation and migration but rather in the differentiation of Th2 or Th3 cells with regulatory properties. The T cells interacting with antigen would then secrete IFN- γ in the absence of activation signals by the DN DCs, thus becoming anergized. If soluble protein antigen is given at high dose, T-cell activation would occur in the PP and LP as a result of DC stimulation and migration to the interfollicular regions or the MLN. In this scenario, IL-10 produced by the myeloid DCs may serve to control detrimental inflammation induced by microbial antigens, whereas DN DCs and lymphoid DCs may be acting as the primary source of IL-12 for the induction of Th1 responses^[58].

DCs in experimental colitis

The availability of mice expressing the diphtheria toxin receptor under the control of the *Cd11c* promoter has allowed the selective depletion of DCs and the study of DC role in the development of intestinal inflammation. DC ablation has been correlated with the amelioration of DSS-induced colitis^[59]. DSS-stimulated bone marrow-derived DCs release high quantities of proinflammatory cytokines and chemoattractants *in vitro*. Furthermore, DC adoptive transfer exacerbated disease manifestations, whereas DC ablation attenuated disease severity as shown by histological examination of tissue sections. However, DC activation with TLR9 ligands before colitis induction with DSS exacerbated disease manifestations. Since DSS injures the colonic epithelium, it is conceivable that, at least in this model, DCs exerted protective effects through stimulating repair of colonic epithelial cell layers rather than modulating the immune response.

There is evidence that DCs may play both protective and detrimental roles in intestinal pathology. In DSS colitis, an experimental model resembling acute colitis, DC ablation during DSS administration ameliorated disease manifestations^[60]. Conversely, colitis was exacerbated if DCs were ablated before DSS treatment, suggesting that DCs are protective in initial phases of colitis but play a pathogenic role during the disease course^[60].

In a T-cell transfer model of colitis induced by CD45R-B^{hi}CD4⁺ T cells, transplanted T cells formed aggregates with sub-epithelial CD11c⁺ DCs in the MLN^[61]. Blocking OX40-OX40L interactions prevented the development of colitis. DC activation *via* CD40 has been reported to cause colitis in the absence of T and B cells and through a cytokine-dependent mechanism^[62].

Intestinal inflammation is correlated with significant changes in the cellular composition of the colonic LP. Gut inflammation in mice is accompanied by a marked infiltration of CD11c⁺ DCs within the LP^[63]. From a phenotypic standpoint, these DCs express high levels of CD80 and resemble mature activated DCs, while secreting low levels of IL-10 and IFN- α . Of interest, CD103⁺ DCs were dramatically reduced in the LP of colitic mice but were detectable in the spleen, suggesting that intestinal CD103⁺ DCs may migrate to lymphoid organs during inflammation.

The observation that IL-10-deficient and TGF- β -deficient mice develop spontaneous colitis point to IL-10 and TGF- β as important determinants of DC function in the gut. In a T-cell transfer model of colitis, Treg production of IL-10 was dispensable for disease suppression but IL-10 secreted by LP CD11b⁺ macrophages was crucial to maintain FoxP3 expression in Treg cells^[28]. It is conceivable that intestinal bacteria are a fundamental trigger of IL-10 production by LP macrophages through the activation of TLR signaling. It is presently unknown whether IL-10 signals are also required to maintain FoxP3 expression in Treg cells from other organs during inflammation^[29]. Serum IL-10 is reportedly normal in patients with IBD^[64]. However, LP mononuclear cells are impaired in the ability to release and respond to IL-10^[64], suggesting that IL-10 provision might be beneficial in human IBD through effects on the DC compartment^[65].

DCs IN HUMAN IBD

DCs accumulate at sites of inflammation in patients with IBD, whereas both myeloid DC and pDC populations are depleted in the peripheral blood of patients with active disease. DC recruitment to the gut may be the result of an increased expression of chemokines such as CCL20 or of addressins, such as mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1). CCL20 is a nuclear factor- κ B-regulated molecule that mediates the CCR6-dependent recruitment of DCs and T cells to mucosal surfaces. CCL20 has been detected at increased levels in the mucosal epithelium covering lymphoid follicles in patients with both types of IBD, in proximity to CCR6-expressing cell types, such as langerin⁺ DC, B cells and memory T cells^[66]. Phenotypically heterogeneous populations of DCs have been identified in colonic tissues and MLN from patients with IBD^[67]. One population consists of immature DCs expressing DC-specific intercellular adhesion molecule-grabbing non-integrin (SIGN) and mainly located at antigen-capturing sites in the mucosa and medullary cords. A second population expresses CD141 and has a similar localization as that of DC-SIGN⁺ DCs. The third DC subset consists of mature DCs expressing S-100 and

CD83 and is located in the T-cell areas in both the colonic lymphoid follicles and the MLN. In contrast, pDCs were hardly detected in the colon and MLN. The fraction of circulating DC precursors has been found to correlate with established IBD activity indices^[68]. Specifically, higher percentages of pDC and myeloid DCs were measured during disease remission compared with acute flares, suggesting DC migration to secondary lymphoid organs. In line with this hypothesis, DC precursors from patients with IBD expressed α 4 β 7, a gut-homing integrin marker and receptor for MAdCAM-1 also detected on LP T cells^[69]. Importantly, immature DCs are significantly reduced in active IBD, indicating that potentially tolerogenic DC subsets may be defective during disease reactivation.

M-DC8⁺ DCs have been detected in the subepithelial dome of ileal PP from 3 patients with untreated CD^[70]. In one of these patients, an ileal biopsy performed 6 mo after glucocorticoid-induced clinical remission documented the complete disappearance of M-DC8⁺ DCs from the ileal mucosa. The observation that M-DC8⁺ DCs secrete large amounts of TNF- α but not IL-10 upon stimulation with lipopolysaccharide (LPS) suggests that these cell types might contribute to the pathogenesis of IBD^[70].

Colonic CD11c⁺ DCs from patients with either CD or UC express higher levels of TLR2 (interacting with peptidoglycan and bacterial lipoproteins), TLR4 (a receptor for LPS) and CD40 compared with non-inflamed CD tissues and tissues from healthy controls^[71]. This may lead to enhanced recognition of bacterial products and an increased response to them. Importantly, treatment with TNF- α blocking antibodies translated into the downregulation of CD40 expression on DCs, irrespective of resolution of inflammation at the tissue level. Also, production of IL-6 and IL-12 at the single-cell level was increased in DCs from patients with CD but not with UC compared with healthy controls.

Although the DC abnormalities documented in UC generally resemble those evidenced in CD, differences may exist when comparing these 2 major forms of IBD. Epstein-Barr virus-induced gene 3 (EBI3) encodes a secreted protein that shares 27% amino acid sequence identity with IL-12p40. EBI3 can substitute for p40 to form a heterodimer with IL-12p35, and is an IL-27 subunit^[72]. EBI3 expression is upregulated by macrophage/DC-like cells within the LP of patients with active UC but not CD^[73]. These data are consistent with a scenario in which EBI3 opposes the IL-12p40/p35 heterodimer and downregulates the cytotoxicity promoted by IL-12. In addition, this study reinforced the view that macrophages and DCs serve more than one role in the pathogenesis of IBD, being either protective or detrimental.

DCs AS TOOLS AND TARGETS FOR THERAPY IN IBD

Different approaches have been proposed to restore and/or enhance the tolerogenic properties of DCs, including *in vitro* treatment with growth factors and use of drugs that target DC number and/or function (Figure 2)^[74,75].

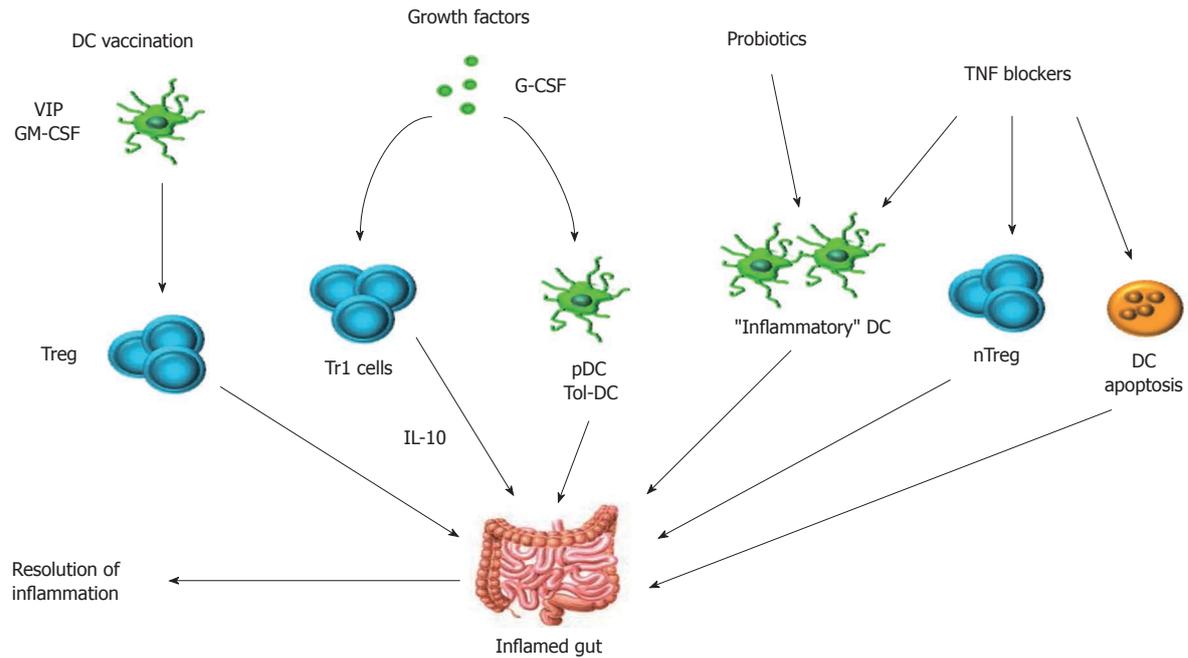


Figure 2 Potential strategies to modulate dendritic cell functionality in human inflammatory bowel disease. *In vitro* differentiated tolerogenic dendritic cell (DC) have been administered to mice with inflammatory/autoimmune disorders. Vasoactive intestinal peptide (VIP) has a unique ability to skew DC function towards a tolerogenic profile and has been used to vaccinate animals with colitis, rheumatoid arthritis and post-transplantation graft-versus-host disease^[76,77]. Selected growth factors have shown to modulate immune reactivity *in vivo*. For instance, granulocyte colony-stimulating factor (G-CSF) has been successfully given to patients with Crohn's disease, leading to accumulation of pDC in the lamina propria and increase in IL-10 production, with favorable repercussions on disease manifestations^[89]. GM-CSF: Granulocyte-macrophage colony-stimulating factor; IL: Interleukin; TNF: Tumor necrosis factor.

Adoptive transfer of cytokine-modulated DCs

DCs have been targeted in animal models of intestinal inflammation. Regulatory DCs differentiated with vasoactive intestinal peptide (VIP) and GM-CSF (DC-VIP) have been transferred to BALB/c mice with colitis induced by trinitrobenzene sulfonic acid (TNBS), a Th1-mediated disease requiring T-cell activation with subsequent macrophage recruitment and activation^[76]. Mice received the DC preparations either 8 h after TNBS instillation or 6 d after colitis induction in order to assess their therapeutic effects both on colitis induction and on established disease. DC infusion ameliorated disease severity and histopathology, being associated with inhibited Th1 responses and with the *in vivo* differentiation of IL-10-producing Treg cells. DC-VIP decreased the production of proinflammatory cytokines both systemically and locally, and deactivated spleen macrophages, blunting the *in vitro* production of TNF- α and IL-12 in response to LPS challenge. Importantly, DC-VIP augmented the number of TGF- β /IL-10-secreting CD4⁺ T cells within LP mononuclear cells cultured in the presence of colonic proteins extracted from colitic mice. Also, CD4⁺ T cells obtained by the MLN of DC-treated mice suppressed proliferation and IL-2 production by autoreactive CD4⁺ T cells in response to colonic proteins. These CD4⁺ T cells were also capable of reversing the body weight loss which is characteristic of TNBS-induced colitis when transferred to colitic mice, suggesting the acquisition of a potent regulatory activity after their *in vivo* encounter with DC-VIP. Finally, the therapeutic effect of CD4⁺ T cells was dependent on TGF- β

and IL-10 production, being reversed by *in vivo* blocking of these cytokines. This elegant study suggests that *in vitro* conditioning of DC preparations with VIP and self-antigens might be pursued as therapeutic strategy in colitis and possibly other inflammatory disorders^[77,78], also to minimize patients' dependence on non-specific immune suppressive drugs currently in use for human IBD.

TGF- β 1 gene-modified immature DCs with enhanced tolerogenicity undergo efficient transport to inflamed colonic tissues and delay the progression of murine IBD induced by DSS^[79]. DC injections in mice with established colitis alleviated weight loss and reduced intestinal bleeding, translating into a lower disease activity index compared with control DC or untreated mice. DC vaccination was associated with increased Treg numbers in the MLN and with increased TGF- β 1 levels in mouse colon tissues^[79].

Probiotic bacteria

Probiotics, mainly belonging to the lactic acid bacteria (LAB) family, exert beneficial effects in human or animal health and are presently considered as peace-keepers in the gut^[40,80]. The regular intake of probiotic bacteria may contribute to immune homeostasis by altering microbial balance or by interacting with intestinal immune cells. Dysbiosis, namely, an imbalance between pro-inflammatory and anti-inflammatory bacteria in favor of the former, may have a causative role in patients with IBD. Probiotics have been tested both in animal models of colitis and in patients with IBD. The potential mechanisms of action of probiotic bacteria include their interaction with

TLR and DCs in the gut. The demonstration of anti-inflammatory effects after systemic administration of probiotics suggests that regulatory cell populations may be induced distant from the site of inflammation^[81].

Importantly, probiotics may present strain-specific *in vitro* immune modulating actions, that are strictly correlated with their *in vivo* anti-inflammatory effects. For instance, *L. salivarius* Ls33 and *L. rhamnosus* Lr32 possess high immunoregulatory capacities and efficiently protect from murine TNBS-induced colitis, at variance with other strains such as *L. acidophilus* NCFM and *L. lactis* MG1363 that exhibit an opposite immunological profile^[82]. The protective effect of probiotic-treated DCs was attributed to a downregulation of proinflammatory mediators such as IL-12 and IL-17, paralleled with an acute overexpression of IFN- γ and IDO^[82]. Of interest, pre-formed naturally occurring Treg cells were required for the protective effect of probiotic-treated DCs, as shown by experiments with an anti-CD25 rat monoclonal antibody.

The probiotic mixture designated IRT5 contains 5 different probiotic strains. IRT5 has been shown to induce T-cell and B-cell hyporesponsiveness when administered for 20 d to mice by the oral route^[83]. Even more intriguingly, IRT5 increased FoxP3 expression in MLN as a result of the enhanced conversion of naïve T cells into Treg cells and the augmentation of the suppression function of pre-existing natural Treg cells. These effects were mediated through the promotion of DC tolerogenic activity, with high expression of IL-10, TGF- β , IDO and COX-2 mRNA. IRT5 retarded the progression of TNBS-induced colitis and was also efficacious in other immune-mediated disorders, such as atopic dermatitis and collagen-induced arthritis. Both the atopic ear and the inflamed colon of IRT5-treated mice were enriched with FoxP3-expressing Treg cells, likely as a result of increased tissue levels of CCL1 and CCL22, chemokines involved in Treg attraction.

The probiotic mixture VSL#3, which contains 8 different bacterial strains and is clinically beneficial in human IBD and pouchitis, has been reported to downregulate IL-12 and upregulate IL-10 production by human blood and colonic LP DCs in a dose-dependent fashion^[84]. This change in DC functional polarization translated into the inhibition of *in vitro* generation of Th1 cells from allogeneic CD4⁺ T cells.

A comprehensive analysis of previously published studies detailing the activity of different probiotics in animal models of colitis suggests that the colitis model used may affect the results^[85]. An interesting study has evaluated the ability of 3 *Lactobacilli* strains (*plantarum*, *LGG* and *paracasei* B21060) to activate DCs either directly or indirectly through epithelial cells. While inducing similar degrees of DC phenotypic maturation, the different strains elicited differential cytokine release, with *L. paracasei* inducing lower levels of IL-12p70, TNF- α and IL-10. The lactobacilli also affected epithelial cell function, and supernatants of *L. paracasei*-treated epithelial cells drastically reduced the ability of DCs to activate T cells and drive their polarization towards a Th1 pheno-

type. Finally, the *in vitro* activity of probiotics was predictive of their *in vivo* efficacy in an acute model of colitis. Taken together, these studies indicate that probiotics interact both with immune cells and with non-immune cells and that the clinical use of individual bacterial strains should be proposed and recommended only after taking into account *in vitro* immunostimulatory or immunoregulatory activity.

Immune modulating drugs

Granulocyte-CSF (G-CSF) has remarkable immune modulating activities^[86]. Indeed, G-CSF mobilizes DC2, differentiates tolerogenic DCs *in vitro* through IL-10 and IFN- α , and polarizes naïve T cells to a Tr1-like functional profile^[31,87,88]. G-CSF has been administered to patients with CD in order to modulate immune reactivity and induce potential clinical benefit^[89]. Nine patients with active CD received subcutaneous G-CSF for 28 d at 5 $\mu\text{g}/\text{kg}$ of body weight. Six patients reported improvement in the CD activity index (CAI) and achieved either a clinical response (4 patients) or remission (2 patients). The 3 non-responding patients had a longer duration of disease, had had bowel resections and one was the only CD patient with active fistulae. In responder patients, IL-10 production by isolated memory CD4⁺ T cells was significantly higher at the end of G-CSF treatment compared with non-responders. Conversely, IFN- γ production in post-G-CSF peripheral blood samples was significantly higher in non-responders. G-CSF also affected the relative proportion of circulating myeloid DCs and pDCs, inducing a decrease in the myeloid DC-to-pDC ratio in responding patients. Notably, 4 patients in the responder group showed an increase in LP CD123⁺ DCs^[89]. In sharp contrast, accumulation of CD123⁺ pDCs could not be evidenced in the LP of non-responders. Finally, the percentage of FoxP3-expressing cells within LP CD25⁺ cells decreased significantly in non-responders at the end of treatment. In line with this, the fraction of CD25^{hi}FoxP3⁺ cells increased in the LP of responding patients at the end of treatment, although these differences failed to achieve statistical significance. Collectively, this study provided proof-of-principle in favor of IL-10-mediated immune regulation by G-CSF in patients with IBD and suggested that treatment with this cytokine may translate into disease control. G-CSF at 3 $\mu\text{g}/\text{kg}$ of body weight was also highly effective at controlling an UC-like syndrome in a 23-year-old patient with glycogen storage disease Ib^[90]. G-CSF therapy was maintained for 16 years, with good control of gastrointestinal symptoms and dramatic improvement of colon histology.

There is evidence that TNF- α antagonism translates into changes in DC function. Although this has been primarily shown in patients with rheumatoid arthritis, it is likely that modifications of DC functions by TNF blockers may also impact on the clinical manifestations of IBD. Both etanercept and adalimumab were shown to downregulate CD83, CD80 and CD86 expression on monocyte-derived DCs and to reduce their T-cell stimula-

tory capacity^[91]. Anti-TNF-treated DCs polarize T-cell responses *in vitro* and favor T-cell release of IL-10, IL-4 and IL-17. Although no correlation was found between the clinical response to TNF blockade and the functional modulation of DCs *in vitro*, DCs derived from patients with rheumatoid arthritis given TNF blocking agents enhanced T-cell production of IL-10, while decreasing the release of IL-4, IL-17 and IFN- γ . Infliximab may also suppress the antigen-presenting capacity of DCs derived from patients with psoriasis by reducing the expression of CD1a and costimulatory molecules, an effect that is not reversed by LPS^[92].

Mesenchymal stromal cells

Mesenchymal stromal cells (MSC) are cells endowed with multi-lineage differentiation capacity and have been isolated from bone marrow, adipose tissue, amniotic fluid, placenta and umbilical cord blood. MSC affect both innate and adaptive immune responses and have reduced immunogenicity, thus being a promising therapeutic tool for inflammatory, autoimmune and degenerative diseases^[93]. Adipose tissue-derived MSC have been shown to ameliorate experimental colitis through the promotion of IL-10 release with subsequent inhibition of activated macrophages and differentiation of Treg cells^[94]. Importantly, the intrafistular injection of *in vitro*-expanded MSC (median number: 64×10^6 for each patient) has resulted into sustained complete closure of fistula tracks, reduction of perianal disease activity index, and with rectal mucosal healing in 10 patients with CD^[95]. Intriguingly, the percentage of mucosal and circulating Treg cells significantly increased during treatment and remained stable until completion of the 12-mo follow-up period. T cells isolated from the inflamed mucosal areas released higher amounts of IL-10 when co-cultured with MSC *in vitro*. Based on previously published data on the ability of IL-10 to skew DC differentiation towards a tolerogenic profile^[96,97], it is tempting to speculate that MSC therapy may target pro-inflammatory DCs *in vivo*, through the promotion of IL-10 release by colitogenic T cells.

Based on the experience reported in patients with GVHD^[98], MSC have also been infused intravenously, at a dose of $1-2 \times 10^6$ cells/kg of body weight, in 10 patients with chronic active CD, refractory to all currently available medical therapeutic options^[99]. Although MSC-based therapy did not induce clinical remissions as defined by a CDAI < 150, reductions of 70 points in CDAI were recorded in 3 patients. The biological effects of MSC intravenous infusion included a trend towards higher percentages of CD4⁺CD127⁺ *bona fide* Treg cells. *In vitro*, patient-derived MSC inhibited the proliferation of autologous peripheral blood mononuclear cells and decreased their production of TNF- α . Collectively, the studies published so far demonstrate that MSC from patients with CD can be expanded *in vitro* and may induce favorable therapeutic effects *in vivo*, including differentiation of Treg cells, and possibly functional inhibition of DCs within the inflamed gut.

CONCLUSION

It is now clear that DC activation is a contributing factor in generation of IBD, as indicated both by mouse models of gut inflammation and by human disease. The recent advances in the phenotypic and functional characterization of DC populations in humans have unveiled a remarkable and previously unappreciated heterogeneity within the DC compartment but have also led to the identification of potential targets for therapeutic manipulation. A thorough understanding and knowledge of DC subsets and functionality in humans is a prerequisite for delivering interventions aimed at correcting DC malfunctioning. Several other cell types with APC function cooperate to ensure appropriate immune responses in the gut. They include IEC, basophils, MSC and other non-immune cells^[10,41]. Theoretical strategies to interfere with DC activity include vaccination with gene-modified DCs or cytokine-treated DCs to restore tolerance, growth factor administration, therapy with DC-modulating drugs, and use of probiotics. The impact of TNF blocking antibodies on DC functions needs to be further investigated. Some of these approaches have been successfully applied to animal models of gut inflammation and other autoimmune/inflammatory disorders such as multiple sclerosis and arthritis. In this respect, “tolerogenic vaccination” with cytokine-modulated DCs may hold promise for the treatment of intestinal inflammation. However, there is a theoretical concern that tolerogenic DCs suppress beneficial anti-infective and anti-tumor responses, in addition to unwanted immune reactivity. These issues must be carefully addressed before this approach is translated into the clinic. Studies in murine GVHD are somehow reassuring, having clearly indicated that the injection of cytokine-treated DCs preserves CD8⁺-mediated cytotoxic responses against leukemia while blunting GVH reactivity^[100,101]. Finally, the patient categories that may benefit from DC-based therapeutic approaches need to be identified. It should be emphasized that other cell-based interventions such as the intravenous infusion of MSC have not induced any clinical remission in severe refractory CD^[99]. Conceivably, patients with IBD should be offered DC-centered treatments earlier in the disease course, following patient profiling and stratification on the basis of molecular predictors for complicated disease (genetic markers such as NOD2 homozygous or compound heterozygous, and anti-microbial antibodies) as well as clinical features at diagnosis^[102]. There is evidence from both pediatric and adult IBD that treatment of short-duration CD with TNF antagonists is associated with better response and remission rates^[103,104]. Whether DC-based approaches have the potential to slow disease progression and alter the natural history of IBD will hopefully be determined in the near future.

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



Giovanni Tarantino, MD, Professor, Series Editor

Spleen: A new role for an old player?

Giovanni Tarantino, Silvia Savastano, Domenico Capone, Annamaria Colao

Giovanni Tarantino, Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, Via S. Pansini 5, 80131 Naples, Italy

Silvia Savastano, Annamaria Colao, Department of Molecular and Clinical Endocrinology and Oncology, Division of Endocrinology, Federico II University Medical School of Naples, Via S. Pansini 5, 80131 Naples, Italy

Domenico Capone, Department of Neurosciences, Unit of Clinical Pharmacology, Federico II University Medical School of Naples, 80131 Naples, Italy

Author contributions: Tarantino G conceived the study, critically analyzed the research articles and wrote the manuscript; Capone D revised the manuscript concerning the neurotransmitter; Savastano S and Colao A contributed to the section on hormone/vitamin D.

Correspondence to: Giovanni Tarantino, MD, Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, Via S. Pansini 5, 80131 Naples, Italy. tarantin@unina.it

Telephone: +39-81-7462024 Fax: +39-81-5466152

Received: October 30, 2010 Revised: December 13, 2010

Accepted: December 20, 2010

Published online: September 7, 2011

Abstract

The spleen could be considered a neglected organ. To date, it has been deemed an ancillary organ in portal hypertension or an organ localization in lymphoproliferative diseases, even though it has had significant attention in infectious diseases for some time. Now, it is thought to be central in regulating the immune system, a metabolic asset and involved in endocrine function with regard to nonalcoholic fatty liver disease. The main mechanisms involved in this complex network will be critically discussed in this article.

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Key words: Endocrine function; Immune system; Metabolic asset; Nonalcoholic fatty liver disease; Spleen

Peer reviewer: Weekitt Kittisupamongkol, MD, Hua Chiew Hospital, 665 Bumrungruang Road, Bangkok 10100, Thailand

Tarantino G, Savastano S, Capone D, Colao A. Spleen: A new role for an old player? *World J Gastroenterol* 2011; 17(33): 3776-3784 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i33/3776.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3776>

SCENARIO

Due to the view that the “spleen is useless”, research on this organ has lagged behind that of other organs. Since 1952, when King and Schumacker reported overwhelming post-splenectomy infection^[1], there has been a growing recognition of the importance of the spleen in the human body. On the other hand, physicians often encounter spleen enlargement, i.e., splenomegaly which is almost always a consequence of other disorders. Hypersplenism is a secondary process that can arise from splenomegaly of almost any cause. In recent years, following in-depth studies of spleen organization and structure, cell function, secretion and innervations, a better understanding of the function of the spleen has been gained. It was initially accepted that the spleen not only filters blood but is an important regulation center of the body’s immune-metabolic-endocrine network. However, a number of questions have arisen: Is the spleen a player or a bystander, and what are the roles of some cytokines, adipokines/growth factors and neurotransmitters in this complex mechanism? In other words, what is the contribution of the spleen to non-alcoholic fatty liver disease, is it a further expression of Metabolic Syndrome^[2]?

ANATOMY

The spleen, in healthy adult humans, is approximately 11 cm (4.3 in) in length. It usually weighs 150 g (5.3 oz)

Anatomy	Composition
Red pulp	“Sinusoids” which are filled with blood “Splenic cords” of reticular fibers “Marginal zone” bordering on white pulp
White pulp	Nodules, called Malpighian corpuscles, containing “lymphoid follicles” rich in B-lymphocytes “periarteriolar lymphoid sheaths”, plenty of T-lymphocytes

and lies beneath the 9th to the 12th thoracic ribs. The spleen is an intraperitoneal organ with a smooth serosal surface and is attached to the retro-peritoneum by fatty ligaments that also contain its vascular supply. The splenic surfaces are described relative to their locations and are termed the diaphragmatic (phrenic) and visceral surfaces. The visceral surface is divided into an anterior or gastric ridge and a posterior or renal portion. The splenic hilum is directed antero-medially. The splenic artery and vein emerge from the splenic hilum in the form of six or more branches; the splenic artery is remarkable for its large size and tortuosity. The splenic artery is slightly superior to the vein. The spleen is part of the lymphatic system. The germinal centers are supplied by arterioles called penicilliary radicles. The spleen is derived from mesenchymal tissue (Table 1).

SPLEEN FUNCTION

Immune function (through phagocytosis, but also through T cell-mediated immunity and B cell-mediated humoral immunity) is the most important function of the spleen (Table 2). A current paradigm states that monocytes circulate freely and patrol blood vessels but differentiate irreversibly into dendritic cells (DCs) or macrophages upon tissue entry. Recently, it was shown that bona fide undifferentiated monocytes reside in the spleen and outnumber their equivalents in the circulation. The reservoir monocytes assemble in clusters in the cords of the subcapsular red pulp and are distinct from macrophages and DCs. In response to ischemic myocardial injury, splenic monocytes increase their motility, exit the spleen en masse, accumulate in injured tissue, and participate in wound healing. These observations uncover a role for the spleen as a site for storage and rapid deployment of monocytes and identify splenic monocytes as a resource that the body exploits to regulate inflammation^[3]. The spleen plays a complex role in tumor immunity, which changes in the different periods of cancer^[4]. The initiation of T-cell immune responses requires professional antigen-presenting cells. Emerging data point towards an important role for macrophages (Mphi) in the priming of naïve T cells. In this study we analyzed the efficiency and the mechanisms by which Mphi derived from spleen (Sp-Mphi) or bone marrow (BM-Mphi) present lymphocytic choriomeningitis virus antigens to epitope-specific T cells. It was demonstrated that because of phagosomal

Red pulp	Extramedullary hematopoiesis if required Facilitating an environment wherein erythrocytes rid themselves of solid waste material Blood filter for foreign material and damaged and senescent blood cells Storage site for iron, erythrocytes, platelets, plasmablasts and plasma cells Rapid release of antigen-specific antibodies into the circulation produced by red pulp plasma cells Defense against bacteria using iron metabolism by its macrophages
White pulp	T cell zone (periarterial lymphatic sheath) and B cell zone (follicles) Storage site for B and T lymphocytes Development of B and T lymphocytes upon antigenic challenge Release of immunoglobulins upon antigenic challenge by B lymphocytes Production of immune mediators involved in clearance of bacteria such as complement, opsonins, properdin and tuftsin
Marginal zone	Phagocytosis of circulating microorganisms and immune complexes by MZ macrophages Development of marginal zone B lymphocytes upon TI-2 antigenic challenge Blood trafficking of B and T lymphocytes Release of immunoglobulins upon antigenic challenge by splenic B lymphocytes

maturation, Sp-Mphi downregulate their ability to cross-present cell-associated, but not soluble, antigens, as they are further differentiated in culture without altering their capacity to directly present virus antigens after infection. Authors proposed that Sp-Mphi are extremely efficient at direct and cross-presentation. However, if these cells undergo further M-CSF-dependent maturation, they will adapt to be more scavenger and phagocytic and concurrently reduce their cross-presenting capacity. Accordingly, Sp-Mphi can have an important role in regulating T-cell responses through cross-presentation depending on their differentiation state^[5]. The spleen is one of the centers of activity of the reticulo-endothelial system and can be considered analogous to a large lymph node, as its absence leads to a predisposition toward certain infections. Other functions of the spleen are the production of opsonins^[6], properdin^[7], and tuftsin^[8], as well as the creation of red blood cells. While the bone marrow is the primary site of hematopoiesis in the adult, the spleen has important hematopoietic functions up until the fifth month of gestation. After birth, erythropoietic functions cease, except in some hematologic disorders. As a major lymphoid organ and a central player in the reticuloendothelial system, the spleen retains the ability to produce lymphocytes and, as such, remains a hematopoietic organ. In horses, roughly 30% of red blood cells are stored in the spleen. These red blood cells can be released when needed^[9]. In humans, the spleen does not act as a reservoir for red blood cells but it can store platelets in case of an emergency. Platelets are major carriers of serotonin (5-HT) in the blood^[10]. 5-HT has been reported to modulate T cell and natural killer (NK) cell proliferation. This aspect

was clearly elucidated by studies on cultures of mouse and rat spleen cells. Results showed that serotonin up-regulates mitogen-stimulated B lymphocyte proliferation through 5-HT_{1A} receptors, thus providing an important link between this neurotransmitter and the immune system^[11]. Another study using RT-PCR methods to examine the mRNA expression of 5-HT receptors in the cells of lymphoid tissues of the rat (*ex vivo* isolated spleen, thymus, and peripheral blood lymphocytes) confirmed 5-HT receptors (5-HT_{1B}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT₆, and 5-HT₇) in mitogen-stimulated spleen cells. In contrast, 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2C}, 5-HT₄, 5-HT_{5A}, and 5-HT_{5B} mRNAs were not detected in any of the examined cell populations^[12]. The role of platelets and serotonin was recently highlighted as novel contributors in the mechanisms of liver regeneration after partial hepatectomy^[13]. Furthermore, platelets are attracted to the liver following systemic inflammatory stimuli^[14].

ASSESSMENT OF SPLEEN FUNCTION

Patients with impaired splenic function are difficult to identify^[15]. IgM memory B cells are a potential parameter for assessing splenic function^[16]; however, more studies are necessary for its validation. The detection of Howell-Jolly bodies does not reflect splenic function accurately^[17], whereas determining the percentage of pitted erythrocytes is a well-evaluated method and seems a good first-line investigation for assessing splenic function^[18]. When assessing spleen function, (99m)Tc-labeled, heat-altered, autologous erythrocyte scintigraphy with multimodality single photon emission computed tomography (CT)-technology is the best approach, as all facets of splenic function are evaluated^[19].

THE BLOOD-SPLEEN-BARRIER

The blood-spleen-barrier (BSB) is a barrier composed of macrophages and endothelial cells of the marginal sinus. Their basement membrane is composed of reticular tissue (reticular cells and reticular fibers) and collagen fibers. It can regulate splenic filtration and its intrasplenic consequences including blood flow, cell homing and migration, hematopoietic and immune responses, and clearance of infectious organisms. Here, the cells of the barrier can trap circulating infectious organisms and monocytes on their cell surfaces, clearing them from the blood and providing a selective environment for monocyte differentiation into macrophages and subsequent phagocytosis of the microorganisms. The interactions between the circulating lymphocytes and the macrophages may regulate the entry of lymphocytes into the white pulp. Thus, the functions of the BSB are to filter antigens, to keep the microenvironment of the white pulp stable, and to present antigen information to white pulp through the effects of the mechanical barrier, which depends on the connection between cells and the phagocytosis of macrophages. Compared to other biological barriers in the human body, such as the blood-brain barrier and the blood-thymus barrier, the structure of the BSB is relatively

loose without the tight junction between cells; however, the BSB has more constituents and ability to stop and phagocytize more xenobiotic materials than other barriers^[20,21]. As compared to the normal spleen, the density of macrophages in the portal hypertension (PH) spleen was decreased, but the macrophages were mainly located in the marginal zone and distributed around the splenic corpuscle, with many villi and pseudopodium-like protrusions on the cell surface. The accretion of collagen fibers was obvious around the splenic corpuscle and central artery. The increased reticulate fibers encircled the splenic corpuscle with more connection between the fibers. The vascular endothelial cells were in diffused distribution, without any regionality in PH spleen, but the vessel with enlarged lumina increased in red pulp^[22].

THE OLD PLAYER

Except for malaria and genetic metabolic diseases (e.g., Gaucher disease), splenic enlargement can be caused by diseases such as PH, lymphoma and leukemia. PH is considered the most common cause of splenomegaly in Western countries. Previous findings showed that splenomegaly is secondary to PH with associated liver cirrhosis. In fact, the increase in the width of the celiac axis in cirrhotic patients with PH was closely related to the increased width of the splenic artery which in turn was related to enlargement of the spleen, and increased blood flow through the spleen. The increased size of the spleen is due partly to venous engorgement and partly to reticulo-endothelial cell hyperplasia, and is accompanied by an increased total blood supply, although flow per 100 g tissue is often reduced. An increase in blood flow can not occur without dilatation of the entire splenic arterial tree (Pousselle's law) and in keeping with this are the studies of^[23], using injected spleen casts, which showed an increased number of peripheral arterioles of 100 mm diameter. In addition to local factors, circulating vasodilator substances may sometimes have an additional effect. The cardiac output is often raised, the blood flow through skin and muscle is also increased, and there is evidence of an increased number of small peripheral arterioles in the lungs. All these findings show that a generalized vasodilatation may occur in some patients with cirrhosis. Increasing tortuosity of peripheral vessels is a well-known accompaniment of aging, but in cirrhotic patients there was no relationship between tortuosity and either age or length of history. The increased length and tortuosity of the splenic artery is probably a secondary effect of arterial dilatation, although there was no direct relationship either to total splenic blood flow or size of the spleen. This was particularly striking in patients with tropical splenomegaly who had enormously enlarged spleens, increased blood flows, but splenic arteries of normal length^[24]. Currently, there is controversy on the immune function of enlarged spleen in patients with PH and hypersplenism. As compared to the normal spleen, the density of macrophages in the PH spleen was decreased, but the macrophages were mainly located in the marginal zone and distrib-

uted around the splenic corpuscle, with many villi and pseudopodium-like protrusions on the cell surface. The “accrementation”, i.e., growth by addition of similar collagen fibers, was obvious around the splenic corpuscle and central artery. The increased reticulate fibers encircled the splenic corpuscle with more connection between the fibers. The vascular endothelial cells were in diffused distribution, without any regionality in PH spleen, but the vessel with enlarged lumina increased in red pulp. Those morphological changes of the BSB may be one of the pathological fundamentals for the abnormality of immune function and the increased destruction of blood cells located in the spleens of patients with PH^[22].

Lymphoma is the commonest malignant tumor of the spleen. Although a number of lymphomas and leukemias can involve the spleen and may present clinically with splenomegaly, only the B cell disorders SMZL and hepato-splenic γ/δ T cell lymphoma can be considered true primary splenic lymphomas^[25]. It is important to detect splenic involvement because it can alter the management and for this reason Gadolinium-enhanced sequences are sensitive.

LIVER CIRRHOSIS, SPONTANEOUS SPLENORENAL SHUNT AND HYPERSPLENISM

Although significant advances are expected to be made in the assessment of PH-related complications, the prognostic role of spleno-renal shunts (SRS) has not been fully explored so far. Clarifying this aspect could help tackle the life-threatening events occurring in patients suffering from liver cirrhosis. A recent study on SRS^[26] focused on the role of the spleen and showed a strict link between spleen size and the presence of SRS and the development of hepatocarcinoma.

An up-to-date study evaluated the effect of liver transplantation on spleen size, spontaneous SRS function, and platelet counts in patients with hypersplenism in 462 adult patients who underwent orthotopic liver transplantations (OLT). Of these patients, CT or magnetic resonance imaging information was reviewed retrospectively in 55 patients. Volume measurements of the spleen and liver, spleen/liver volume ratio (S/L ratio), presence and size of SRS, and platelet counts were evaluated before and after OLT. Spleen size and SRS size were significantly smaller after OLT. However, patients with postoperative S/L ratio > 0.35 tended to have lower platelet counts after OLT^[27].

THE NOVEL PLAYER

Nonalcoholic fatty liver disease (NAFLD), the most common cause of steatosis, is associated with obesity, mainly visceral and insulin resistance. In the presence of more severe risk factors (major obesity, diabetes mellitus, metabolic syndrome, MS), simple hepatic steatosis or fatty liver (FL) may be complicated by liver inflammation

(nonalcoholic steatohepatitis or NASH). NASH can lead to perisinusoidal fibrosis and cirrhosis. Fat-laden hepatocytes are swollen, and in steatohepatitis, further swelling occurs due to hydropic change (ballooning) of hepatocytes to cause sinusoidal distortion, as visualized by *in vivo* microscopy, reducing intrasinusoidal volume and microvascular blood flow. Involvement of other cell types (sinusoidal endothelial cells, Kupffer cells, stellate cells) and recruitment of inflammatory cells and platelets lead to dysregulation of microvascular blood flow. In animal models, the net effect of such changes is a marked reduction of sinusoidal space (approximately 50% of control), and a decrease in the number of normally perfused sinusoids. Such microvascular damage could accentuate further liver injury and disease progression in NASH. Hepatic steatosis is also exquisitely sensitive to ischemia-reperfusion injury, at least partly due to the propensity of unsaturated fatty acids to undergo lipid peroxidation in the face of reactive oxygen species. This has important clinical consequences, particularly limiting the use of fatty donor livers for transplantation^[28]. NASH is a progressive liver disease characterized by Kupffer cell dysfunction which contributes to its pathogenesis. It is noteworthy that the reticular-endothelial system also plays a key role in the spleen. Colloid scintigraphy is a good method of reflecting Kupffer cell activity. A study on 22 patients with biopsy-proven NASH who underwent colloid liver scintigraphy, after intravenous injection of 185 MBq Tc tin colloid, showed that liver right/left lobe ratio was altered in all of these patients. Colloid shift to the spleen was observed in 55% of patients as well as prolonged blood pool clearance time^[29].

The first group of researchers^[30] who aimed to determine if there was an association between NAFLD and spleen enlargement, measured spleen volume using CT. The values were compared with the patient's demographic data, the liver-to-spleen (L/S) ratio of CT Hounsfield unit measurements, and the results of liver function tests. Diagnosis of fatty liver was made if the L/S ratio was less than 1.0. The mean spleen volume was $73.0 \pm 24.4 \text{ cm}^3$ (range, 21.1-106.1) in normal subjects and $141.2 \pm 54.1 \text{ cm}^3$ (range, 44.1-267.3) in patients with fatty liver ($P < 0.0001$). Multivariate linear regression analysis identified that only the L/S ratio ($P < 0.0001$) and age ($P < 0.01$) were significantly correlated to spleen volume. Using forward selection stepwise regression, the L/S ratio entered first ($\beta = -0.634$) and age second ($\beta = -0.293$).

Obesity and insulin resistance are strongly associated with systemic markers of inflammation. Focusing on this aspect, authors have attempted to find a noninvasive method that could likely assess the presence of NASH and help to decide liver biopsy performance. Using histology as a gold standard to diagnose NAFLD, 43 patients with NASH and 40 with fatty liver were consecutively studied, their data were compared with those of 48 healthy control participants. The outcomes evaluated were ultrasonographic spleen longitudinal diameter coupled with the splenic artery resistive index, serum interleukin (IL)-6 and vascular endothelial growth factor

concentrations. The NASH group had higher spleen longitudinal diameter values ($P = 0.0001$) as well as significantly higher IL-6 and vascular endothelial growth factor concentrations than the other groups ($P = 0.0001$). The optimal cut-off value for spleen longitudinal diameter that best discriminated NASH from fatty liver patients was 116 mm (specificity 95% and sensitivity 88%); the sensitivity and specificity of this parameter was better than both IL-6 and vascular endothelial growth factor in the same setting (area under the receiver operating characteristic curve 0.920 *vs* 0.817 and 0.678, respectively). Splenic artery resistive index was similar between patients with NASH and those with fatty liver, but differed when compared with controls ($P = 0.0001$). IL-6 was highly specific in confirming the absence of NASH at normal values. In that series of patients, normal values of spleen longitudinal diameter and IL-6 were strongly associated with fatty liver^[31]. Further confirmation of these findings comes from another study which highlighted that spleen enlargement may be a distinct feature of NASH, especially early-stage NASH^[32].

A subsequent study^[33] showed that spleen enlargement was found at significant levels (38%) in obese patients as determined by Cavalieri stereologic volume calculation, an unbiased stereological method. Finally, recent results clearly indicated that high fat diet caused splenomegaly *via* sinusoidal dilatation and intracellular or intercellular deposits in obese female rats^[34]. Although in patients with NAFLD, liver biopsy remains the only reliable method to differentiate simple steatosis from NASH, the objective of the study was to evaluate the efficacy of non-invasive (^{99m}Tc-phytate scintigraphy in the diagnosis of NASH. Thirty-seven patients with suspected NAFLD at the time of liver biopsy also underwent (^{99m}Tc-phytate scintigraphy. Signal intensities of regions of interest in the liver and spleen were measured. The same authors also examined scintigraphic features in a nutritional model of NASH in rats fed a methionine- and choline-deficient (MCD) diet. The liver/spleen uptake ratio determined by scintigraphy was significantly decreased in patients with NASH in comparison with patients with simple steatosis. The liver/spleen ratio was an independent predictor distinguishing NASH from simple steatosis. The decrease was observed for all stages of NASH, including the early stage (stages 1 and 0). In animal studies, the liver/spleen uptake ratio was significantly decreased in rats after 8 wk of a MCD diet in comparison with control diet-fed rats. These authors concluded that non-invasive (^{99m}Tc-phytate scintigraphy is a reliable tool to differentiate NASH from simple steatosis^[35]. The frequency of ischemic heart disease observed after splenectomy for trauma and the low cholesterol levels found in patients with hypersplenism are observations that suggest a possible role for the spleen in lipid metabolism and in the etiology of atherosclerosis^[36,37]. Previous studies showed that obese subjects are more susceptible to cardiovascular disease, hypertension, cerebrovascular disease, and diabetes mellitus than are non-obese subjects. They have a higher incidence of infection and some types of cancer, suggesting impaired

immune function. In humans, only a few studies have directly compared specific immune responses in obese and non-obese subjects. It is known that obesity induces decreases in both T lymphocyte response to concanavalin A and B lymphocyte response to pokeweed mitogen^[38]. In addition, a negative correlation between percentage body fat and natural killer cell activity was found in both elderly women^[39], and adult men^[40]. Elderly people (> 60 years of age) are also at risk of an increased incidence of infection. Their peripheral blood lymphocytes show an impaired proliferative capacity and a decreased reactivity to mitogens^[41]. Researchers found and reported that obesity suppresses lymphocyte functions, natural killer cell activity, and lymphocyte mitogenesis in men and women > 60 years of age^[42]. This suggests that obesity is a risk factor for deteriorating cellular immune functions. However, the mechanism by which obesity decreases cellular immune functions remains to be elucidated. Expression of glucose transporter 1 (GLUT-1), analyzed by Western blot analysis, was lower in the splenic lymphocytes of obese compared with lean Zucker rats. In obese subjects it is associated with the decreased uptake of glucose into immune cells, which in turn is associated with the decreased expression of GLUT-1. This suggests that decreased proliferation of splenic lymphocytes in obese Zucker rats is associated with the impairment of glucose uptake, which is due to the decreased expression of GLUT-1^[43]. An up-to-date study assessed the magnitude of antigen-specific immunity in a murine model of NAFLD. Because antigen-specific immunity was diminished in NAFLD mice, the underlying mechanisms were evaluated through analysis of the functions of antigen-presenting DC and other immunocytes. For 12 wk, NAFLD mice received a high-fat and high-calorie diet. NAFLD mice and control mice were immunized with hepatitis B vaccine containing hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg). Antibody to HBsAg (anti-HBs), HBsAg and HBcAg-specific cellular immune response and functions of whole spleen cells, T lymphocytes, B lymphocytes and spleen DCs of NAFLD and control mice were assessed *in vitro*. Levels of anti-HBs and the magnitude of proliferation of HBsAg and HBcAg-specific lymphocytes were significantly lower in NAFLD mice than control mice. The spleen cells of NAFLD mice produced significantly higher levels of inflammatory cytokines and exhibited significantly increased T cell proliferation compared with control mice. However, the antigen processing and presenting capacities of spleen DCs were significantly decreased in NAFLD mice compared with control mice. Palmitic acid, a saturated fatty acid, caused diminished antigen processing and presenting capacity of murine DCs^[44]. Liver fat represents a balance between input, secretion, and oxidation of fatty acids. As humans spend the majority of a 24-h period in a postprandial state, dietary fatty acids make an important contribution to liver fat metabolism. Oxidation of dietary fatty acids, hepatic desaturation and elongation of palmitic acid occurs to a greater extent in abdominally obese men.

NEUROTRANSMITTER, HORMONES, VITAMIN D AND THE SPLEEN

Increasing evidence has placed hormones and neurotransmitters among potent immunomodulators, in both health and disease. 5-HT functions as a neurotransmitter in the nervous systems of simple as well as complex animals. 5-HT diffuses to serotonin-sensitive neurons, which control the animal's perception of nutrient availability. This system has been partially conserved during the 700 million years of evolution which separates *C. elegans*, a transparent nematode, from humans. When humans smell food, dopamine is released to increase the appetite. However, unlike that in worms, serotonin does not increase anticipatory behaviour in humans; instead the serotonin released while consuming activates 5-HT_{2C} receptors on dopamine-producing cells. This halts their dopamine release, and thereby serotonin decreases appetite. Drugs which block 5-HT_{2C} receptors make the body unable to shut off appetite, and are associated with increased weight gain^[45], especially in people who have a low number of receptors^[46]. The expression of 5-HT_{2C} receptors in the hippocampus follows a diurnal rhythm, just as the 5-HT release in the ventromedial nucleus, which is characterized by a peak in the morning when the motivation to eat is strongest^[47]. In humans, serotonin levels are affected by diet. An increase in the ratio of tryptophan to phenylalanine and leucine will increase serotonin levels. Fruits with a good ratio include dates, papaya and banana. Foods with a lower ratio inhibit the production of serotonin. Research also suggests that eating a diet rich in carbohydrates and low in protein will increase serotonin by secreting insulin, which helps in amino acid competition^[48]. However, increasing insulin for a long period may trigger the onset of insulin resistance, obesity, type 2 diabetes, and lower 5-HT levels. Researchers showed that expression of 5-HT(2A) receptors was up-regulated in hypertrophic 3T3-L1 adipocytes, which exhibited decreased expression of adiponectin and increased expression of PAI-1. 5-HT(2A) receptor antagonists and suppression of 5-HT(2A) receptor gene expression enhanced adiponectin expression. Activation of Gq (the G protein-coupled receptor is activated by an external signal in the form of a ligand or other signal mediator) negatively regulated adiponectin expression, and inhibition of mitogen-activated protein kinase reversed the Gq-induced effect. Moreover, the 5-HT(2A) receptor blockade reduced PAI-1 expression^[49]. As food intake and energy balance are among the functions regulated by 5-HT in the brain, it would be interesting to discover its link with some adipokines. Recent studies have shown an interaction between the serotonergic system and leptin, a protein released from adipose tissue that inhibits feeding behavior and increases fuel expenditure. An up-to-date study found low brain serotonin immunoreactivity in all animals with high neuronal leptin accumulation in the raphe nucleus, independently of their age. In contrast, high brain serotonin immunoreactivity was accompanied by a low neuronal accumulation of leptin. These findings indi-

cate that serotonin regulates leptin uptake by neuronal cell bodies of the dorsal raphe and hypothalamus, suggesting that at least part of the effects of serotonin may be mediated by the regulation of neuronal trafficking in the brain^[50]. 5-HT promotes the release of growth hormone (GH) by a hypothalamic site of action^[51]. Exogenous GH enhances thymic microenvironmental cell-derived secretory products such as cytokines and thymic hormones. Moreover, GH increases thymic epithelial cell (TEC) proliferation *in vitro*, and exhibits a synergistic effect with anti-CD3 in stimulating thymocyte proliferation, which is in keeping with data showing that transgenic mice overexpressing GH or GH-releasing hormone exhibit overgrowth of the thymus. GH also influences thymocyte traffic: it increases human T-cell progenitor engraftment into the thymus; augments TEC/thymocyte adhesion and the traffic of thymocytes in the lymphoepithelial complexes, the thymic nurse cells; modulate *in vivo* the homing of recent thymic emigrants, enhancing the number of fluorescein isothiocyanate positive cells in the lymph nodes and diminishing them in the spleen. In keeping with the effects of GH on thymic cells, is the detection of GH receptors in both TEC and thymocytes. Insulin-like growth factor (IGF)-1 is a potent hormone that stimulates growth and differentiation and inhibits apoptosis in numerous tissues. Preliminary evidence suggests that IGF-1 exerts differentiating, mitogenic and restoring activities in the immune system, however, the sites of synthesis of local IGF-1 are unknown. Identification of these sites would allow the functional role of local IGF-1 to be clarified. The presence of IGF-1 in non-immune cells suggests that it acts as a trophic factor, while its occurrence in subtypes of lymphocytes or antigen-presenting cells indicates paracrine/autocrine direct regulatory involvement of IGF-1 in the human immune response. Additionally, data indicate that IGF-1 is involved in several effects of GH in the thymus, including the modulation of thymulin secretion, TEC proliferation as well as thymocyte/TEC adhesion. This is in accordance with the demonstration of IGF-1 production and expression of IGF-1 by TEC and thymocytes. Also, it should be seen as an intrathymic circuitry, involving not only IGF-1, but also GH itself, as intrathymic GH expression is seen both in TEC and in thymocytes, and that thymocyte-derived GH could enhance thymocyte proliferation^[52]. With regard to the implication of the IGF family in immune physiology and development, a recent study has focused on type 1 IGF receptor, a transmembrane tyrosine kinase homologous to the insulin receptor that mediates most of the biological effects of IGF-1 and IGF-2. Normal development and *ex vivo* activation of T and B cells are observed in chimeric *Rag2*-deficient C57BL/6 mice reconstituted with fetal liver cells from *Igf1r*^{-/-} mice. However, this model revealed an unexpected decrease in the T-independent B cell response which is important in bacterial defense mechanisms^[53]. The major role of IGF-2 is as a growth promoting hormone during gestation. To date, very few studies have investigated the function of IGF-2 in immune development and physiology.

This growth factor is the dominant peptide of the insulin family expressed in the thymus epithelium of different species. Thymic IGF-2 influences thymic development and T cell differentiation as evidenced by the analysis of IGF-2 transgenic dwarf mice, which develop thymic hyperplasia with an increased number of thymocytes (and CD4⁺ T lymphocytes in particular). This increase in T cells is also observed in the spleen compartment of IGF-2 transgenic mice, but there is no significant effect on B cell development^[54]. There is further evidence that IGF-2 may intervene in the control of T cell differentiation^[55]. A recent study investigated the location of IGF-1 messenger RNA and protein on archival human lymph node samples by *in situ* hybridization, immunohistochemistry and double immunofluorescence staining using an IGF-1 probe and antisera specific for human IGF-1 and CD3 (T lymphocytes), CD20 (B lymphocytes), CD68 (macrophages), CD21 (follicular DCs), S100 (interdigitating DCs) and podoplanin (fibroblastic reticular cells). Numerous cells within the B- and T-cell compartments expressed the IGF-1 gene, and the majority of these cells were identified as macrophages. Solitary follicular DCs exhibited IGF-1. A few T lymphocytes, and no B lymphocytes, contained IGF-1 immunoreactive material. Furthermore, IGF-1 immunoreactive cells outside the follicles that did not react with CD3, CD20, S100 or podoplanin markers were identified as high-endothelial venule cells^[56]. GH was used to counteract the catabolic metabolism in critically ill patients until it was demonstrated that administration of GH was associated with increased morbidity due to uncontrolled infections and sepsis^[57]. The immunomodulatory effect of GH and its main mediator IGF-1 during systemic inflammation remain to be established. Authors investigated the effect of GH and IGF-1 on cellular immune functions in a murine model of sepsis and found that GH did not affect cellular immune functions or the survival rate in that model. In contrast, IGF-1 improved splenocyte proliferation and cytokine release independently of GH but did not affect the determined clinical parameters of septic mice^[57]. Aging is under the control of a small number of regulatory genes. Mice genetically selected for high immune responses, in most cases, exhibit a longer life span and lower lymphoma incidence than do mice selected for low responses. The link between immunity and aging is further evidenced by the age-related alterations in the immune system, mostly of the T-cell population, in terms of replacement of virgin by memory cells, accumulation of cells with signal transduction defects, and changes in the profile of Th1 and Th2 type cytokines^[58]. Also, B cells exhibit intrinsic defects, and NK cell activity is profoundly depressed by aging. *In vitro* experiments indicate that the production of IL-2, interferon (IFN)- γ , and IL-4 by mouse spleen cells changes with aging and may be up-regulated by recombinant cytokines. These findings suggest possible cytokine interventions to prevent or treat age-related immune disorders, as they may affect the duration and the biological quality of life^[59]. Excessive alcohol consumption continues to be a major public health

problem, particularly in the adolescent and young adult populations. Generally, such behavior tends to be confined to the weekends, resulting in frequent binge drinking. Various authorities have emphasized the strict link between mechanisms inducing alcoholic and nonalcoholic liver diseases, thus it could be of interest to ask questions about alcohol toxicity, such as: is there a link between alcohol abuse and impaired immune system and what is the link? A study in peri-pubertal male rats compared the effect of the discontinuous feeding of a liquid diet containing a moderate amount of ethanol (6.2% wt/vol) to that of continuous ethanol administration or a control diet, taking as end points the 24-h variations in plasma prolactin levels and mitogenic responses and lymphocyte subset populations in the spleen. Animals received the ethanol liquid diet starting on day 35 of life, the diet being similar to that given to controls except that maltose was iso-calorically replaced by ethanol. Ethanol provided 36% of the total caloric content. Each week, the discontinuous ethanol group received the ethanol diet for 3 d and the control liquid diet for the remaining 4 d. After 4 wk the rats were killed. A significant decrease in splenic cell response to concanavalin A, and of splenic cell response to lipopolysaccharide was found in rats under the discontinuous ethanol regime, when compared with control- or ethanol-chronic rats. Under discontinuous ethanol feeding, mean values of splenic CD8(+) and CD4(+)-CD8(+) cells decreased, whereas splenic T cells, and splenic B cells were augmented. In rats chronically fed with ethanol, splenic mean levels of CD8(+) and CD4(+)-CD8(+) cells were augmented. Both modalities of ethanol administration disrupted the 24 h variation in immune function seen in controls. Mean plasma prolactin levels increased by 3.6-fold and 8.5-fold in rats chronically or discontinuously fed with alcohol, respectively. These results supported the view that the discontinuous drinking of a moderate amount of ethanol can be more harmful for the immune system than continuous ethanol intake, presumably by inducing greater stress as indicated by the augmented plasma prolactin levels observed^[60]. Numerous studies have focused their attention on the role played by vitamin D in obesity, MS and NAFLD. The hormonal form of vitamin D, 1,25-dihydroxyvitamin D3, is well known for its immunosuppressive, anti-proliferative and pro-apoptotic activities. In a recent work, authors studied the effect of 1,25-dihydroxyvitamin D3 on *Toxoplasma gondii*-infected mice. They observed that 1,25-dihydroxyvitamin D3 reduces the survival rate of infected mice by up to 37% at day 10 post-infection compared to untreated infected mice ($P < 0.0001$). IFN- γ and IL-12p40 levels were significantly reduced by 1,25-dihydroxyvitamin D3 in infected mice sera indicating an inhibition of Th-1-type cytokines. CD4⁺ T lymphocyte and splenocyte counts were also reduced following 1,25-dihydroxyvitamin D3 treatment and a marked induction of apoptosis, accompanied by down-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-X(L), was observed. The above results indicate that 1,25-dihydroxyvitamin D3 induces splenocyte apoptosis and enhances host suscepti-

bility to toxoplasmosis^[61]. Bone components participate in the regulation of hematopoietic stem cells (HSC) in the adult mammal. Vitamin D regulates bone mineralization and is associated with pleiotropic effects in many cell types including putative roles in hematopoietic differentiation. Researchers reported that deletion of the vitamin D receptor (VDR) in hematopoietic cells did not result in cell autonomous perturbation of HSC or progenitor function. However, deletion of VDR in the microenvironment resulted in a marked accumulation of HSC in the spleen that could be reversed by dietary calcium supplementation. These data suggest that VDR participates in restricting splenic hematopoiesis through maintenance of bone calcium homeostasis and are consistent with the concept that calcium regulation through VDR is a central participant in localizing adult hematopoiesis preferentially to bone marrow^[62].

CONCLUSION

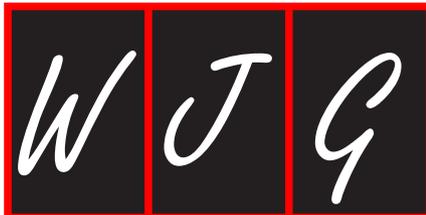
This special organ should be taken into account when interpreting the mechanisms of NAFLD and in its diagnosis, mainly when dealing with the more severe form, i.e., NASH, although recent research has challenged the benignity of FL^[63].

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Giovanni Tarantino, MD, Professor, Series Editor

JNKs, insulin resistance and inflammation: A possible link between NAFLD and coronary artery disease

Giovanni Tarantino, Armando Caputi

Giovanni Tarantino, Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, Via Sergio Pansini, 580131 Naples, Italy

Armando Caputi, Department of Clinical Medicine, Cardiovascular & Immunological Sciences, Federico II University Medical School of Naples, Via Sergio Pansini, 580131 Naples, Italy

Author contributions: Tarantino G conceived the research, analyzed the literature data and wrote the paper; Caputi A critically revised the part concerning the cardiovascular risk.

Correspondence to: Giovanni Tarantino, Professor, MD, Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, Via Sergio Pansini, 580131 Naples, Italy. tarantin@unina.it

Telephone: +39-81-7462024 Fax: +39-81-5466152

Received: December 21, 2010 Revised: February 19, 2011

Accepted: February 26, 2011

Published online: September 7, 2011

Abstract

The incidence of obesity has dramatically increased in recent years. Consequently, obesity and associated disorders such as nonalcoholic fatty liver disease constitute a serious problem. Therefore, the contribution of adipose tissue to metabolic homeostasis has become a focus of interest. In this review, we discuss the latest discoveries that support the role of lipids in nonalcoholic fatty liver disease. We describe the common mechanisms (c-Jun amino-terminal kinases, endoplasmic reticulum stress, unfolded protein response, ceramide, low-grade chronic inflammation) by which lipids and their derivatives impair insulin responsiveness and contribute to inflammatory liver and promote plaque instability in the arterial wall. Presenting the molecular mechanism of lipid activation of pro-inflammatory pathways, we attempt to find a link between nonalcoholic fatty liver disease, metabolic syndrome and cardiovascular diseases. Describing the common mechanisms by which lipid derivatives, through modulation of macrophage function, promote plaque instability in the arterial wall, impair

insulin responsiveness and contribute to inflammatory liver and discussing the molecular mechanism of lipid activation of pro-inflammatory pathways, the key roles played by the proliferator-activated receptor and liver X receptor α , nuclear receptors-lipid sensors that link lipid metabolism and inflammation, should be emphasized. Further studies are warranted of anti-inflammatory drugs such as aspirin, anti-interleukin-6 receptors, immune-modulators (calcineurin inhibitors), substances enhancing the expression of heat shock proteins (which protect cells from endoplasmic reticulum stress-induced apoptosis), and anti-c-Jun amino-terminal kinases in well-designed trials to try to minimize the high impact of these illnesses, and the different expressions of the diseases, on the whole population.

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Key words: Non-alcoholic fatty liver disease; c-Jun amino-terminal kinase; Cardiovascular disease

Peer reviewer: Ching Chung Lin, MD, MMS, Division of Gastroenterology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei 111, Taiwan, China

Tarantino G, Caputi A. JNKs, insulin resistance and inflammation: A possible link between NAFLD and coronary artery disease. *World J Gastroenterol* 2011; 17(33): 3785-3794 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i33/3785.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3785>

INTRODUCTION

The rapid increase in the prevalence of obesity is a major global health problem. Its associated complications are burdened by an increased risk of death by 20%-40% in overweight individuals and by 2-3-fold in obese individuals compared to normal controls, even though the

strength of the association between body mass index and heart failure events declines with age^[1]. Obesity is a known risk factor for non-alcoholic fatty liver disease (NAFLD)^[2], hypertension, stroke, gallbladder disease, osteoarthritis, obstructive sleep apnea, and other breathing problems as well and some forms of cancer (breast, colorectal, endometrial and kidney). Type 2 diabetes (T2D) and obesity, now collectively referred to as “diabesity”, are interrelated, in that obesity is known to exacerbate the pathology of T2D and greater than 60% of diabetics are obese. Low grade chronic inflammation, strictly linked to overweight/obesity, causes insulin resistance (IR) that interacts with other complex mechanisms such as hypercholesterolemia, smoking, hypertension, hyperglycemia, type A behavioral patterns, hemostatic factors, hereditary differences in such diverse aspects as lipoprotein structure and that of their associated receptors, homocysteine processing/metabolism, and high levels of lipoprotein(a) to increase the risk of coronary heart disease (CHD). CHD is present not only in nondiabetic and normotensive obese adult subjects^[3], but also in obese children^[4]. IR is a condition in which normal amounts of insulin are inadequate to produce a normal insulin response from fat, muscle and liver cells. Indeed, lipolysis, which is normally inhibited by insulin, is overstimulated in insulin-resistant states leading to an increase in free fatty acid (FFA) flux^[5]. IR in muscle reduces glucose uptake whereas IR in liver reduces glucose storage, both effects serving to elevate blood glucose. High plasma levels of insulin and glucose due to IR often leads to metabolic syndrome (MS) and T2D. Plasma FFAs from white adipose tissue lipolysis have been shown to be the major contributor to triglyceride accumulation^[6] observed in NAFLD, further expression of MS, which ranges from simple fatty liver (FL) through the more severe form, non alcoholic steatohepatitis (NASH) to cryptogenic cirrhosis. Sharing the same mechanisms of NASH, FL is no longer considered completely benign, accordingly to recent data^[7]. Activation of the endoplasmic reticulum (ER) by stress has been reported in most models of hepatic steatosis in rodents and its contribution to hepatic fat deposition has been recently documented, with lipogenesis being the main metabolic pathway affected. ER stress-related activation, observed in adipose tissue of obese humans^[8], could have metabolic consequences and participate in fat deposition in the liver. Activation of ER could directly induce an insulin-resistant state in adipocytes. Indeed, it has been shown that activation of the ER stress sensor kinase/endonuclease inositol-requiring protein 1 (IRE1), a component of the unfolded protein response (UPR) could stimulate c-Jun amino-terminal kinase [JNKs or stress-activated protein kinases (SAPKs)]^[9], which, by phosphorylating serine residues of insulin substrate receptor 1, is a key player in the development of IR^[10]. The IRE1/box binding protein 1, a branch of the ER stress signaling pathway, has been recently shown to regulate and be regulated by innate immune signaling pathways in both the presence and absence of ER stress^[11]. Disruption of ER homeostasis has been observed in liver and

adipose tissue of humans with NAFLD and/or obesity. Importantly, the signaling pathways activated by disruption of ER homeostasis, the UPR, has been linked to inflammation and apoptosis, lipid biosynthesis, insulin effects, all of which are involved in the initiation/evolution of NAFLD. The ER is a crucial organelle for cellular homeostasis, in which the synthesis and the post-translational modifications of membrane and secreted proteins take place, as well as the synthesis of lipids and cholesterol for membranes formation. However, the ER quality control system can be compromised under a variety of conditions such as accumulation of unfolded protein, alteration of calcium homeostasis or disruption of the redox state. The UPR activates JNKs^[9,12]. Up-to-date results provide evidence that heat shock proteins protect cells from ER stress-induced apoptosis. Obesity and associated disorders constitute a serious problem, for example NAFLD can lead to hepatocarcinoma, and the contribution of adipose tissue to metabolic homeostasis has become a focus of interest. Adipose tissue secretes FFAs and hormones, known as adipokines, and thus seems to play a major role in the development of NAFLD. Apoptotic cell death is a prominent feature in NASH. Indeed, toxic FFAs can activate the intrinsic apoptosis pathway in hepatocytes *via* c-JNK. JNK activates the proapoptotic protein Bim, resulting in Bax activation^[13] and enhanced apoptosis, termed “lipoapoptosis”.

JUN AMINO-TERMINAL KINASES

Jun N-terminal kinases (JNKs), also named SAPKs, are one of 3 members of the mitogen-activated protein kinase (MAPK) superfamily, which also includes the extracellular signal-regulated kinases (ERKs) or classical MAPKs and the p38 MAPK. JNKs bind and phosphorylate c-Jun on Ser63 and Ser73 within its transcriptional activation domain. MAPK kinases (MKK) are responsive to stress stimuli, mainly inflammatory signals, but also to a lesser extent, to ultraviolet irradiation, heat and osmotic shock, and are involved in apoptosis and T cell differentiation. This latter immunological aspect should not be overlooked.

JNKs consist of 10 isoforms derived from 3 genes: JNK1 (4 isoforms), JNK2 (4 isoforms), and JNK3 (2 isoforms). JNK1 and JNK2 are found in all cells of every tissue. JNK3 is found mainly in the brain, but is also found in the heart and the testes. JNK1 is involved in apoptosis, neurodegeneration, cell differentiation and proliferation, inflammatory conditions and cytokine production mediated by activation protein-1 (AP-1) such as regulated upon activation, normal T-cell expressed, and secreted cytokine, interleukin-8 and granulocyte-macrophage colony-stimulating factor. Recently, JNK1 has been found to regulate Jun protein turnover by phosphorylation and activation of the ubiquitin ligase Itch (polyubiquitination marks proteins for degradation by the proteasome). JNKs can associate with scaffold proteins, JNK-interacting proteins as well as their upstream Jun N-terminal kinase kinase 1 and Jun N-terminal kinase

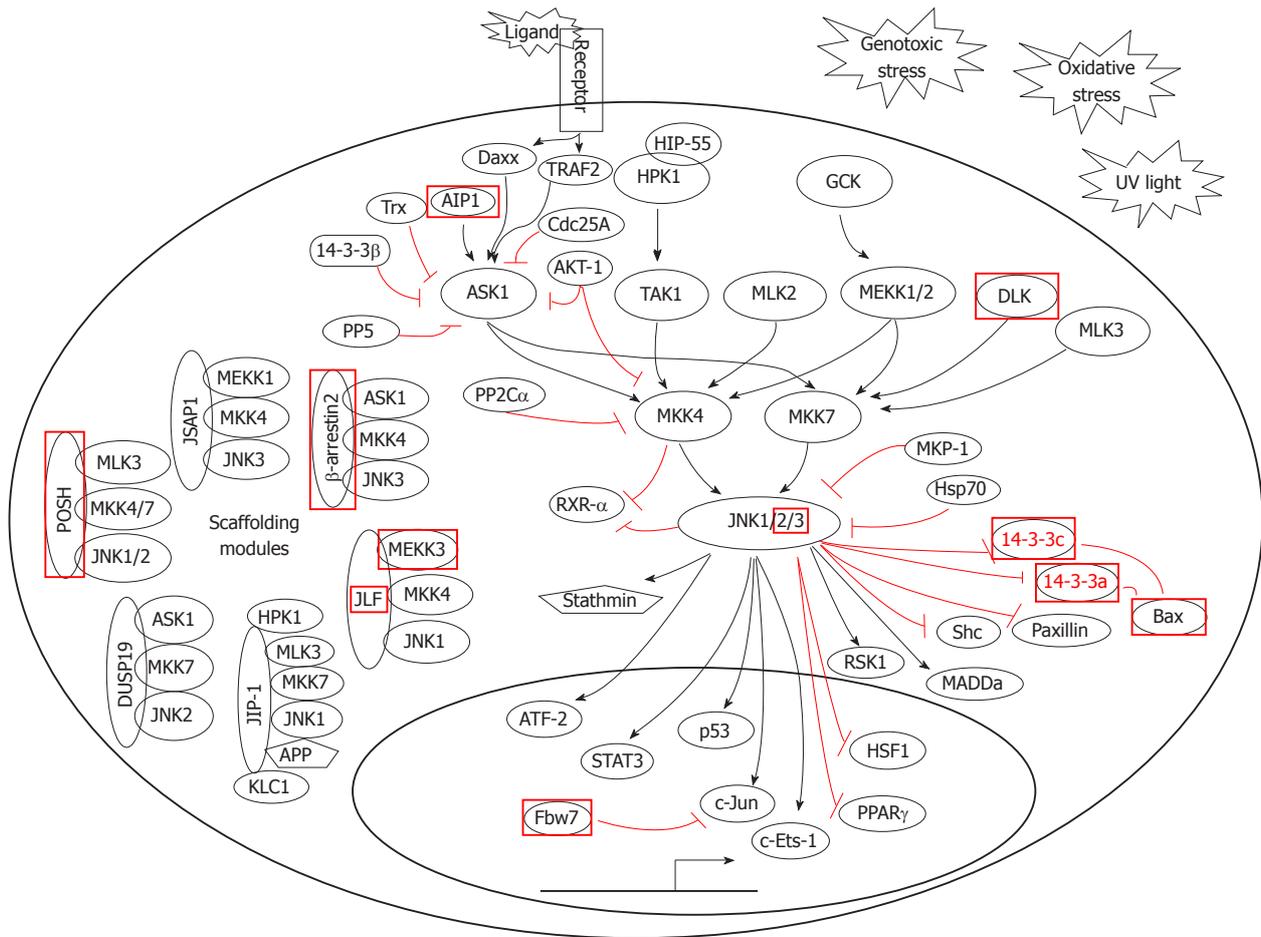


Figure 1 Jun N-terminal kinase signaling pathway. ERK: Extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinase; MKK: MAPK kinase; MEKK: MAPK/ERK kinase kinase; MLK: Mixed lineage kinase; JNK: Jun N-terminal kinase; ASK: Apoptosis signal-regulating kinase; HSP: Heat shock protein; RSK: p90 ribosomal S6 kinase; HPK: Hematopoietic progenitor kinase; PPAR: Peroxisome proliferator-activated receptor; DLK: Dual leucine zipper-bearing kinase; RXR: Retinoid X receptor; ATF: Activating transcription factor; STAT: Signal transducers and activators of transcription; MADD: Mitogen-activated kinase activating death domain protein; TRAF: Tumor necrosis factor receptor-associated factor; MKP: MAPK phosphatase; TAK: Transforming growth factor-activated kinase; KLC: Kinesin light chain; DAXX: Death domain-associated protein.

kinase 2 following their activation. JNK, by phosphorylation, modifies the activity of numerous proteins that reside at the mitochondria or act in the nucleus. Thus, JNK activity regulates several important cellular functions. One way this activation may occur is through disruption of the conformation of sensitive protein phosphatase enzymes; specific phosphatases normally inhibit the activity of JNK itself and the activity of proteins linked to JNK activation. The JNK proteins lead to varied and seemingly contradictory cellular responses. In particular, JNKs have been reported to have a role in the induction of apoptosis, but have also been implicated in enhancing cell survival and proliferation. The opposing roles of JNKs have been attributed to the observation that JNKs activate different substrates based on a specific stimulus, cell type or temporal aspects^[14].

JNK signaling pathway

JNKs are activated by MAPK kinases such as MKK-4, MKK-6 and MKK-7 (Figure 1). These kinases are in turn activated by the MAP3 kinases, such as apoptosis signal-

regulating kinase 1 (ASK1) also known as mitogen-activated protein kinase kinase 5 (MAP3K5), mixed lineage kinases (MLKs) (MLK1, MLK2 and MLK3), MAP/ERK (extracellular signal-regulated kinase) kinase kinase 1 (MEKK1), MEKK4 and transforming growth factor (TGF)-β-activated kinase 1 (TAK1). JNK MAP3 kinase pathways are activated by MAP4 kinases that link to a variety of cell receptors which sense stress and inflammation, including death receptors (Fas), inflammatory cytokine receptors of tumor necrosis factor alpha (TNF-α) and TGF-β, G-protein-coupled receptors (GPCRs) and antigen receptors. Signals are communicated to JNK pathway MAP4 kinases by tyrosine kinase receptor associated adapter and effector molecules and/or by G-protein mediated signaling. GPCRs signal the JNK pathway through trimeric G-proteins to monomeric p21RhoGTPases, Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homolog (CDC42). ASK1 links to stress receptors such as TNF receptor (TNFR) and Fas, and is activated by reactive oxygen species (ROS)-mediated dissociation

of thioredoxin, binding to TNFR associated factor 2 (TRAF2) or death domain-associated protein (DAXX) and oligomerization. ASK1 activates the JNK MAP2Ks, MKK4 (SEK1) and MKK6 (MKK3/MAPKK6). The JNK MAP3 kinases: TAK1, MEKK1 and MLK3 are activated by the tyrosine kinase (TK) receptor-activated MAP4 kinase, hematopoietic progenitor kinase 1 (HPK-1). HPK-1 associates with TK receptors through adaptor proteins, such as CT10-regulated kinase (Crk), Crk-like (CrkL) and growth factor receptor-bound 2 (Grb2). Activation of HPK1 requires multiple phosphorylation events including autophosphorylation and protein kinase phosphorylation by protein kinase D1 (PKD1). The JNK MAP3 kinases, MEKK1, MEKK4 and MLK3 are also activated by the p21Rho-GTPases, Ras-related C3 botulinum toxin substrate 1 also known as Rac1 and Cdc42 through the MAP4K, P21-activated kinase-1 (PAK-1). This links the JNK pathways to a wide variety of GPCR, integrin and receptor pathways. In addition to participating in the stress response, the MAPKs c-Jun N-terminal Kinases JNK1 and JNK2 regulate the proliferation of normal and neoplastic cells. JNKs contribute to these processes largely by phosphorylating c-Jun and thus contributing to the activation of the AP-1 complex. Furthermore, JNKs control entry into mitosis. It has been observed that JNK activity and phosphorylation of c-Jun become elevated during the G/M transition of the cell cycle in immortalized fibroblasts and ovarian granulosa cells. Pharmacological inhibition of JNK causes a profound cell cycle arrest at the G/M transition in both cell types. This effect is specific as it occurs with 2 distinct small molecular compounds. Inactivation of JNK prior to mitosis prevents expression of aurora B and phosphorylation of histone-H3 at Ser 10. Silencing of JNK1 and 2 causes a similar effect, whereas overexpression of JNK1 and 2 causes the opposite effect. Inhibition of JNK delays activation of Cdc2 and prevents downregulation of cyclin B1, whereas Ras controls the activation of MAPKs. Authors have recently observed that in certain cells, the small guanosine triphosphate (GTP)-binding proteins Rac1 and Cdc42 but not Rho regulate the activity of JNKs^[15]. Furthermore, because Rac1 and Cdc42 but not Rho, bind and activate a P21-activated kinase 1 (Pak1), it has been suggested that Pak1 is the most upstream component of the pathway linking these GTPases to JNK. However, in mammalian cells, Rho1p, a Rho homologue, and RhoA directly interact with a number of proteins, including kinases related to protein kinase C. Exploring the ability of Ras and Rho-related GTP-binding proteins to activate MAPK or JNK in a variety of cell lines, it was found that in the human kidney epithelial cell line, 293T, Cdc42 and all Rho proteins, RhoA, RhoB, and RhoC, but not Rac or Ras can induce activation of JNK. Furthermore, other researchers provided evidence that signaling from Rho proteins to JNK in 293T cells does not involve Pak1^[16]. c-JNK activity is abnormally elevated in obesity. Furthermore, an absence of JNK1 results in decreased adiposity, significantly improved insulin sensi-

tivity and enhanced insulin receptor signaling capacity^[17]. As previously mentioned, elevated FFAs and hepatocyte lipoapoptosis are the main features of NAFLD. However, the mechanism by which FFAs mediate lipoapoptosis is unclear. Recently, data have indicated that saturated FFAs induce JNK-dependent hepatocyte lipoapoptosis by activating the pro-apoptotic Bcl-2 family Bim and Bax-mediated apoptosis, which triggers the mitochondrial apoptotic pathway^[18]. Additional support for involvement of JNK1 overactivation in conditions associated with IR and MS has been provided^[19]. A positive correlation was found between the expression intensity of JNK1 and IR^[20], and JNK1 contributes to the development of liver fibrosis by inducing chronic inflammation as ascertained in a mouse NASH model^[21]. Methionine-choline-deficient feeding causes NASH coincident with the activation of c-JNK and caspase-12 in a murine model^[22].

Further data indicated that the increased oxidative stress and its associated JNK activation, as well as an imbalance in pro- and anti-apoptotic proteins in the Bcl-2 family all contribute to marked hepatocyte apoptosis in a rat NASH model^[23]. Examining fat biopsy samples from obese insulin-resistant nondiabetic individuals, UPR activation in subcutaneous adipose tissue was demonstrated, with JNK being a link between obesity, IR, and inflammation^[8]. ER stress activates the proteolytic cleavage of the lipogenic transcription factor sterol regulatory element binding protein-1c leading to the induction of lipogenic enzyme expression. A role for X box-binding protein 1, an ER stress-activated transcription factor, has also recently emerged. ER stress, by inhibiting apoB100 secretion, has associated with impaired very low density lipoprotein (VLDL) secretion. In rodents, treatment with molecular or chemical chaperones that reduce ER stress markers have demonstrated effectiveness in the treatment of hepatic steatosis^[24].

INSULIN RESISTANCE

SAPK/JNKs are activated by inflammatory cytokines, and JNK signaling is involved in IR and β -cell secretory function and survival. An up-to-date study suggested that FFAs stimulate functional autophagy of β cells, possibly through the RNA-dependent protein kinase (PKR)-JNK1 pathway independent of the ER or oxidative stress^[25].

Post-transcriptional modifications altering activity of insulin signaling molecules are the most proposed mechanism for inhibition of the insulin pathway. Various kinases including stress activated protein kinase, c-JNK, and protein kinase C (PKC) can phosphorylate insulin receptor substrate (IRS) 1-2 at specific serine and threonine residues, leading to inhibition of insulin signaling^[26]. A high fat diet is an established cause of systemic and adipose tissue IR. Activated JNK is a major contributor to FFA-induced cellular IR, and TNF- α is an autocrine/paracrine downstream effector of activated JNK that can also mediate IR^[27]. TNF- α is over-expressed in adipose tissue of obese rodents and humans, and its concentra-

tion is reduced after weight loss. TNF- α inhibits insulin signaling in the liver by mechanisms which include the activation of serine kinases such as JNK-1 and induction of suppressor of cytokine signaling proteins^[28]. An important consequence of IR in adipose tissue is to reduce the anti-lipolytic effect of insulin that in turn leads to elevated plasma FFA in obese and diabetic patients. Released FFAs from adipose tissue is taken up by liver and muscle cells. In the liver, FFAs induce gluconeogenesis and VLDL overproduction. Increased FFAs, especially metabolites such as acyl-CoAs, ceramides, and diacylglycerol, have been shown to inhibit insulin signaling by activating protein kinases such as PKC, JNK, and the inhibitor of nuclear factor- κ B (IKK- β)^[29].

Chronic high glucose concentrations and leptin induce IL-1 β secretion from pancreatic islets, an event that is possibly promotes β -cell dysfunction and death. A recent study provided evidence that chronically elevated concentrations of leptin and glucose induced β -cell apoptosis through activation of the JNK pathway in human islets and in insulinoma (INS 832/13) cells. JNK inhibition by the dominant inhibitor JNK-binding domain of IB1/JIP-1 (JNKi) reduced JNK activity and apoptosis induced by leptin and glucose. Exposure of human islets to leptin and high glucose concentrations led to a decrease of glucose-induced insulin secretion, which was partly restored by JNKi. An interplay between the JNK cascade and the caspase 1/IL-1 β -converting enzyme in human islets has been found. The *caspase 1* gene, which contains a potential activating protein-1 binding site, is upregulated in pancreatic sections and in isolated islets from T2D patients. Similarly, cultured human islets exposed to high glucose- and leptin-induced caspase 1 and JNK inhibition prevents this upregulation. Therefore, JNK inhibition may protect β -cells from the deleterious effects of high glucose and leptin in diabetes^[30].

Obesity is closely associated with IR and is established as a leading risk factor for T2D, yet the molecular mechanisms of this association are poorly understood. JNKs can interfere with insulin action in cultured cells and are activated by inflammatory cytokines and FFAs, molecules that have been implicated in the development of T2D. As previously highlighted, it has been shown that JNK activity is abnormally elevated in obesity. Furthermore, an absence of JNK1 results in decreased adiposity, significantly improved insulin sensitivity and enhanced insulin receptor signaling capacity in 2 different models of mouse obesity. Thus, JNK is a crucial mediator of obesity and IR and a potential therapeutic target^[31]. Chronic oxidative stress results in decreased responsiveness to insulin, eventually leading to T2D and CHD. Activation of the JNK signaling pathway can mediate many of the effects of stress on IR through inhibitory phosphorylation of IRS-1. In contrast, exercise, which acutely increases oxidative stress in muscle, improves insulin sensitivity and glucose tolerance in patients with T2D. Authors used a cellular model of insulin-resistant muscle to induce either chronic or acute oxidative stress and investigate their contrasting effects on insulin and JNK signaling. Chronic oxidative stress

resulted in increased levels of phosphorylated (activated) JNK in the cytoplasm, whereas acute oxidative stress led to redistribution of JNK-specific phosphatase MKP7 from the nucleus into the cytoplasm, a reduction in cytoplasmic phospho-JNK, and concurrent accumulation of phospho-JNK in the nucleus. Acute oxidative stress restored normal insulin sensitivity and glucose uptake in insulin-resistant muscle cells, and this effect was dependent on MKP7^[32].

Finally, it is likely that JNK activity modulates pancreatic islet function and/or survival in numerous ways. First, there is convincing evidence for the involvement of JNK in islet cell inflammation and death mediated by cytokines^[33]. Second, JNK activation may generate a state of β -cell dysfunction and defective insulin production, thereby contributing to the development of overt diabetes^[34]. Third, administration of SP600125, a synthetic inhibitor of JNK, results in improved glucose-stimulated insulin production in isolated islets in the *db/db* model of obesity and diabetes^[35]. Hence, there is a strong possibility that JNK may integrate defects in insulin secretion with peripheral IR in T2D through its actions in pancreatic β -cells as well as peripheral sites of insulin action. If this is the case, it is also likely that JNK may be important in the pathogenesis of type 1 diabetes, and recent studies have provided evidence to support a role for the JNK-2 isoform in this disease^[36].

LIPOLYTIC MACHINERY, ADIPOCYTES AND NAFLD

Several lines of evidence implicate an inadequate response to lipid storage/catabolism of cellular fat stores as being important in NAFLD. Subcutaneous adipose tissue (AT) is composed mostly of small, differentiated adipocytes that absorb circulating FFAs due to their insulin-sensitivity. They form triglycerides (lipogenesis) and store them in cellular lipid droplets (LD) or lipid bodies (surrounded by a monolayer of lipase-regulating proteins) until FFAs are needed during fasting. They also secrete adiponectin, which by opposing hepatic lipogenesis and stimulating long chain fatty acid β -oxidation, protects the liver from harmful effects of lipid accumulation, such as IR^[37]. In MS, failure of subcutaneous AT to store energy leads to swollen adipocytes that are stressed and de-differentiated. They continually release FFAs from triglycerides (lipolysis). Lipolysis is the biochemical pathway responsible for the catabolism of triacylglycerol (TAG) stored in cellular LD. The hydrolytic cleavage of TAG generates FFAs, which are subsequently used as energy substrates, essential precursors for lipid and membrane synthesis, or mediators in cell signaling processes. Consistent with its central importance in lipid and energy homeostasis, lipolysis mostly occurs in white and brown adipose tissue. Over the last few years, important enzymes and regulatory protein factors involved in lipolysis have been identified. These include an essential TAG hydrolase named adipose triglyceride lipase (ATGL) [a

patatin-like phospholipase domain-containing protein A2 (PNPLA2)], the ATGL activator comparative gene identification-58 (an α/β hydrolase containing protein 5), and the ATGL inhibitor G0/G1 switch gene 2. ATGL catalyzes the first step in adipocyte and muscle triglyceride hydrolysis. Together with the established hormone-sensitive lipase (lipase E) and monoglyceride lipase, these proteins constitute the basic “lipolytic machinery”. Additionally, a large number of hormonal signaling pathways and lipid droplet-associated protein factors regulate substrate access and the activity of the “liposome”^[38]. Activation of β -(AR) in adipocytes triggers acute changes in metabolism that can alter patterns of gene expression. A recent work examined the mechanisms by which activation of hormone sensitive lipase induces expression of inflammatory cytokines in adipocytes *in vivo* and model adipocytes *in vitro*. β 3-adrenergic receptor (AR) activation in mice triggered expression of inflammatory genes CCL2, IL-6, and PAI-1, as well as ER stress markers GRP78 and CHOP^[39]. Recent findings suggest that genetic variants in PNPLA3 predispose towards hepatic steatosis and, in the context of other environmental stressors, progression to irreversible liver failure. PNPLA3 is predominantly expressed in human liver and adipose tissue, possesses both lipolytic and lipogenic activity *in vitro*, and is localized on the surface of lipid droplets in hepatocytes. The 148M mutant protein has reduced lipolytic activity, with attendant increased cellular triglycerides^[40], only recently confirmed^[41]. Studies in animal models of NAFLD demonstrate that inhibition of acyl-coenzyme A:diacylglycerol acyltransferase (DGAT)-1, the enzymes that catalyze the final step in triglyceride synthesis, results in improvement in hepatic steatosis and insulin sensitivity. Researchers recently confirmed that hepatic-specific inhibition of DGAT-1 with antisense oligonucleotides improved hepatic steatosis in obese, diabetic mice but, unexpectedly, exacerbated injury and fibrosis in that model of progressive NAFLD. When hepatocyte triglyceride synthesis was inhibited, FFA accumulated in the liver, leading to induction of fatty acid oxidizing systems that increased hepatic oxidative stress and liver damage. These findings suggest that the ability to synthesize triglycerides may, in fact, be protective in obesity^[42]. This is a key point. MEK1/2 inhibition significantly increased both cellular and microsomal triglycerides mass, and mRNA levels for DGAT-1 and DGAT-2. In contrast to ERK, modulation of the phosphatidylinositol 3-kinases pathway or inhibition of the p38 MAP kinase, had no effect on lipoprotein density profile^[43]. The biogenesis of LD induced by serum depends on group IVA phospholipase A(2) [cPLA(2) α /GIVA PLA(2)], a regulatory enzyme that releases arachidonic acid for production of prostaglandins and leukotrienes. Recent data suggest that cPLA(2) α regulates the transport of tight junction and adherens junction proteins through Golgi cell-cell contacts in confluent endothelial cells. Expression of specific activators of different MAP kinases show that phosphorylation of cPLA(2) α at Ser-505 is due to JNK. This was confirmed by pharmacological inhibition and

expression of a dominant-negative form of the upstream activator MEKK1. LD biogenesis was accompanied by increased synthesis of ceramide 1-phosphate. Overexpression of its synthesizing enzyme ceramide kinase increased phosphorylation of cPLA(2) α at Ser-505 and the formation of LD, and its downregulation blocked the phosphorylation of cPLA(2) α and LD biogenesis. These results demonstrate that LD biogenesis induced by serum is regulated by JNK and ceramide kinase^[44].

The effect of glucose and palmitate on the phosphorylation of proteins is associated with cell growth and survival. Fresh results suggest that short-term changes in MAPK and AKT signaling pathways, and c-fos and c-JNK expressions induced by glucose are abolished by palmitate through phosphatidylinositol 3-kinase inhibition *via* ceramide synthesis^[45].

Activation of β -AR in mouse adipocytes triggered expression of inflammatory genes CCL2, IL-6, and PAI-1, as well as ER stress markers GRP78 and CHOP. Pharmacological inhibition of hormone sensitive lipase (HSL) blocked induction of inflammatory genes, but not ER stress markers. Promoting intracellular accumulation of FFAs in 3T3-L1 adipocytes increased the expression of inflammatory cytokines, whereas inhibiting ceramide synthesis partly blocked PAI-1 expression, but not IL-6. Induction of inflammatory markers *in vivo* and *in vitro* was preceded by phosphorylation of p38 and JNK, and inhibition of HSL prevented activation of these kinases. Together, these results demonstrate that FFAs liberated by HSL activate p38 and JNK, and p38 mediates pro-inflammatory cytokine expression in adipose tissue^[39].

As previously emphasized, accumulation of lipid metabolites within non-adipose tissues can induce chronic inflammation by promoting macrophage infiltration and activation. Oxidized and glycated lipoproteins, FFAs, free cholesterol, triacylglycerols, diacylglycerols and mainly ceramides have long been known to induce cellular dysfunction through their pro-inflammatory and pro-apoptotic properties. Emerging evidence suggests that macrophage activation by lipid metabolites and further modulation by lipid signaling represents a common pathogenic mechanism underlying lipotoxicity in atherosclerosis, obesity-associated IR and inflammatory diseases related to MS such as NAFLD and chronic kidney disease.

The sphingolipid ceramide is an important second signaling molecule that regulates diverse signaling pathways involving apoptosis, cell senescence, the cell cycle and differentiation. For the most part, effects of ceramide are antagonistic to growth and survival. Interestingly, ceramide and the pro-growth agonist, diacylglycerol (DAG) appear to be regulated simultaneously but in opposite directions in the sphingomyelin cycle. While ceramide stimulates signal transduction pathways that are associated with cell death or at least are inhibitory to cell growth (SAPK), DAG activates the classical and novel isoforms of the PKC family. These PKC isoforms are associated with cell growth and cell survival. Furthermore, DAG activation of PKC stimulates other signal transduction

pathways that support cell proliferation, e.g., MAPK pathways. Thus, ceramide and DAG generation may serve to monitor cellular homeostasis by inducing pro-death or pro-growth pathways, respectively. The production of ceramide is emerging as a fixture in programmed cell death. Ceramide levels are elevated in response to diverse stress challenges including treatment with pro-death ligands such as TNF- α , chemotherapeutic drug treatment or irradiation. Consistent with this notion, ceramide itself is a potent apoptogenic agent. Ceramide activates c-JNK and thus affects its transcription pathways. Ceramide activates protein phosphatases such as protein phosphatase 1 and PP2A. Ceramide activation of protein phosphatases has been shown to promote inactivation of a number of pro-growth cellular regulators including the kinases PKC α and Akt, Bcl-2 and the retinoblastoma protein. A new role has recently emerged for ceramide in the regulation of protein synthesis. Ceramide-induced activation of PKR, a protein kinase important in anti-viral host defense mechanisms and recently implicated in cellular stress pathways, results in the inhibition of protein synthesis as a prelude to cell death^[46].

ENDOPLASMIC RETICULUM, CHAPERONES AND LONGEVITY

Heat shock proteins (HSPs) have proven to be effective tools for extending invertebrate lifespan, and in *C. elegans* daf-2 mutants, longevity resulting from loss of insulin/insulin-like signals is at least partly dependent upon elevated HSP expression. In mice, inhibition of the orthologous growth hormone/insulin-like growth factor I (GH/IGF- I) pathway has similar pro-longevity effects. A recent study, however, suggested that loss of GH/IGF- I signaling in long-lived mice did not broadly elevate HSP expression, but in fact decreased HSP expression in many tissue types, such as liver and kidney. The contribution of chaperones to the longevity of long-lived mice with altered GH/IGF- I signals may therefore differ from that described in *C. elegans* daf-2 mutants. This result, in combination with other recent findings, underscores the possibility that systemic overexpression of chaperones will have dissimilar effects on longevity in vertebrate and invertebrate systems^[47].

MS AND CARDIOVASCULAR DISEASE RISK

MS is a constellation of common metabolic disorders that is strictly linked to CHD. It is now commonly accepted that low-grade chronic inflammation associated with obesity induces IR in the liver. Low-grade chronic inflammation is characterized by the production of abnormal cytokines and adipokines such as IL-6, TNF- α , IL-1, leptin and resistin. These factors inhibit insulin signaling in hepatocytes by activating SOCS proteins, several kinases such as JNK, IKK- β and PKC and protein

tyrosine phosphatases such as PTP1B and PTEN that in turn impair insulin signaling at the insulin receptor and IRS level. Hepatic IR in turn causes impaired suppression of glucose production by insulin in hepatocytes leading to hyperglycemia. An important and early complication of hepatic IR is the induction of hepatic VLDL production, *via* changes in the rate of apoB synthesis and degradation and *de novo* lipogenesis, or increased FFA flux from adipose tissue into the liver. IR also stimulates the production of C-reactive protein (CRP) and plasminogen activator inhibitor-1 (PAI-1), both markers of an inflammatory state. All these subsequent metabolic abnormalities related to hepatic IR have been shown to directly or indirectly promote atherosclerosis. Hyperglycemia induces a series of alterations including endothelial dysfunction, cellular proliferation, changes in extracellular matrix conformation and impairment of low density lipoprotein (LDL) receptor-mediated uptake, decreasing the *in vivo* clearance of LDL. Small dense LDLs associated with high circulating VLDL levels have higher affinity for the intimal proteoglycans leading, to the penetration of more LDL particles into the arterial wall. CRP can also accelerate atherosclerosis by increasing the expression of PAI-1 and adhesion molecules in endothelial cells, inhibiting nitric oxide formation and increasing LDL uptake into macrophages.

Recently it has been shown that a small molecule pan-JNK inhibitor, dosed orally and compared to rimonabant and rosiglitazone, significantly impacted parameters such as adiposity, glucose levels, and insulin sensitization without any effect on liver enzymes, thus establishing the role of JNK as a useful target for metabolic syndrome linked to the pre-diabetic state^[48]. A JNK1 specific antisense oligonucleotide was studied in ob/ob and diet-induced obese mouse models. Profound improvement in insulin sensitivity, glucose levels, plasma cholesterol level, and adiposity without a negative impact on liver function was observed. Decreased body weight and lowered adiposity were attributed to increased food combustion/metabolic rate and decreased lipogenesis^[49].

Describing the common mechanisms by which lipid derivatives, through modulation of macrophage function, promote plaque instability in the arterial wall, impair insulin responsiveness and contribute to inflammatory liver and discussing the molecular mechanism of lipid activation of pro-inflammatory pathways [JNK, nuclear factor (NF) κ B], the key roles played by the proliferator-activated receptor and liver X receptor α , nuclear receptors-lipid sensors that link lipid metabolism and inflammation, should be emphasized^[50]. Atherosclerosis begins as local inflammation of artery walls at sites of disturbed blood flow. JNK is thought to be among the major regulators of flow-dependent inflammatory gene expression in endothelial cells in atherosclerosis. Researchers have shown that JNK activation by both onset of laminar flow and long-term oscillatory flow is matrix-specific, with enhanced activation on fibronectin

compared to basement membrane protein or collagen. Flow-induced JNK activation on fibronectin requires new integrin ligation and requires both MKK4 and p21-activated kinase. *In vivo*, JNK activation at sites of early atherogenesis correlates with the deposition of fibronectin. Inhibiting p21-activated kinase reduces JNK activation in atheroprone regions of the vasculature *in vivo*. These results identify JNK as a matrix-specific, flow-activated inflammatory event. These data elucidate a network of matrix-specific pathways that determine inflammatory events in response to fluid shear^[51].

Visceral AT is known to confer a significantly higher risk of T2D and CHD. Epicardial AT has been shown to be related to cardiovascular disease and myocardial function. Epicardial AT expresses an inflammatory profile of proteins. Authors studied key mediators of the NF κ B and c-JNK pathways in paired epicardial and gluteofemoral (thigh) AT from CHD and investigated circulating endotoxin levels in CHD and control subjects. Serums and AT biopsies (epicardial and thigh) were obtained from CHD and non-CHD patients. Inflammation was assessed in tissue and serum samples through western blot, real-time PCR, ELISAs, and activity studies. Western blotting showed epicardial AT had significantly higher NF κ B, inhibitory- κ B kinase (IKK)- γ , IKK- β , and JNK-1 and -2 compared with thigh AT. Epicardial mRNA data showed strong correlations between CD-68 (again the impaired immunity function) and toll-like receptor-2, toll-like receptor-4, and TNF- α . Circulating endotoxin was elevated in patients with CHD compared with matched controls. Epicardial AT from patients with CHD shows increased NF κ B, IKK- β , and JNK expression compared with both CHD thigh AT and non-CHD epicardial AT, suggesting a depot-specific as well as a disease-linked response to inflammation^[52].

METHODS TO DETECT JNK

The detection of protein kinases is possible in biological liquids such as blood serum or cell lysate. Sandwich ELISAs for detecting phosphoproteins have commonly been used to quantify kinase function and can be performed in 2 configurations. In the first configuration, polyclonal antibodies directed against the structural part of the protein and away from the phosphorylation site (panprotein) are coated onto the bottom of a microwell plate. A cell lysate containing the phosphorylated target protein is added to the well, allowed to bind and the excess lysate is removed by washing. A monoclonal antibody of either mouse or rabbit origin, specific for the phosphorylated form of the protein, is added followed by an enzyme-labeled secondary antibody specific for the monoclonal antibody species. A chromagen is added and the color is quantified spectrophotometrically. In the second configuration, the capture antibody is directed against the phosphor-antibody and the detection antibody is an antibody directed against the panprotein. The latter configuration is sometimes preferred as the amount of phosphor-protein present may be small compared to the total amount of

the panprotein.

In this situation, the large amount of non-phosphorylated panprotein can outcompete the phosphorylated protein for binding to the microwells. This decreases the overall sensitivity of the assay for the phosphor-protein. Using a phosphor-specific capture antibody enriches for the desired target and significantly increases the sensitivity of the assay. Phospho-ELISAs can be used to assess kinase activity in cell lysates or, alternatively, to screen drug candidates targeting a purified kinase. Quantitative measurement of protein phosphorylation has become essential for the development of kinase-inhibiting drugs aimed at therapy of various metabolic diseases. Since kinases are a major source of drug targeting, complex but reliable assay technologies that quantify phosphorylation will continue to be in demand. Biochemical assays that rely on antibodies for assay function are limited by the availability of phosphor-specific antibodies with high affinity and specificity. While many phosphospecific antibodies exist, most are unsuitable for use in quantitative assays due to poor sensitivity or nonspecificity. Alternative methods such as mobility shift, IMAP (a variation of fluorescence polarization that employs nanoparticles bearing immobilized trivalent metal co-ordination complexes that bind specifically to phosphate groups), IQ (using as signal a peptide comprised of an amino acid sequence recognized by the desired kinase that is synthesized with a fluorophore end-label) and light-speed assays (the signal is generated from a polystyrene microsphere that is coated with a modified fluorescent polyelectrolyte) do not rely on antibodies and allow assessment of targets for which no suitable antibodies exist. As a result, these formats will find wider use in the future^[53].

CONCLUSION

As repeatedly emphasized, inflammation is the common mechanism underlying obesity, MS, NAFLD^[54], longevity, CHD and perhaps some cancers. The chicken-and-egg dilemma of IR being cause or effect of inflammation is unsolved at present. Further studies are warranted of anti-inflammatory drugs such as aspirin, anti IL-6 receptors, immune-modulators (calcineurin inhibitors)^[55], substances enhancing the expression of HSPs (which protect cells from ER stress-induced apoptosis), and anti-JNKs in well-designed trials to try to minimize the high impact of these illnesses, and the different expressions of the diseases, on the whole population^[56].

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S- Editor Sun H L- Editor Cant MR E- Editor Xiong L

Natural orifice transluminal endoscopy surgery: A review

João Moreira-Pinto, Estevão Lima, Jorge Correia-Pinto, Carla Rolanda

João Moreira-Pinto, Estevão Lima, Jorge Correia-Pinto, Carla Rolanda, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal; ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

João Moreira-Pinto, Pediatric Surgery division, Centro Hospitalar do Porto, 4099-001 Porto, Portugal

Estevão Lima, Department of Urology, Hospital de Braga, 4709-057 Braga, Portugal

Jorge Correia-Pinto, Department of Pediatric Surgery, Hospital de Braga, 4709-057 Braga, Portugal

Carla Rolanda, Department of Gastroenterology, Hospital de Braga, 4709-057 Braga, Portugal

Author contributions: All authors contributed equally to this work.

Correspondence to: Carla Rolanda, MD, PhD, Surgical Sciences Research Domain, Life and Health Sciences Research Institute, Universidade do Minho, Campus de Gualtar, 4709-057 Braga, Portugal. crolanda@ecsau.de.uminho.pt

Telephone: +351-253604910 Fax: +351-253604809

Received: October 4, 2010 Revised: December 1, 2010

Accepted: December 8, 2010

Published online: September 7, 2011

Abstract

Minimally invasive surgery started spreading worldwide in 1987, when the first laparoscopic cholecystectomy was performed. Meanwhile, improvement of endoscopic equipment and instruments allowed gastroenterologists to attempt more aggressive endoluminal interventions, even beyond the wall barrier. The first transgastric peritoneoscopy, in 2004, brought to light the concept of natural orifice transluminal endoscopic surgery (NOTES). The idea of incisionless surgery is attractive and has become a new goal for both surgeons and other people interested in this field of investigation. The authors present a review of all developments concerning NOTES, including animal studies and human experience.

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Key words: Transesophageal; Transgastric; Transvesi-

cal; Transvaginal; Transcolonic; Natural orifice transluminal endoscopic surgery; Minimally invasive techniques

Peer reviewer: Akihito Nagahara, Associate Professor, Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo Bunkyo-ku, Tokyo 113-8421, Japan

Moreira-Pinto J, Lima E, Correia-Pinto J, Rolanda C. Natural orifice transluminal endoscopy surgery: A review. *World J Gastroenterol* 2011; 17(33): 3795-3801 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i33/3795.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3795>

INTRODUCTION

Surgery has experienced a huge development in the past three decades after Dr. Philippe Mouret performed the first laparoscopic cholecystectomy in 1987. Since then, minimally invasive surgery has begun to spread worldwide^[1]. This was largely in part due to patient demands for laparoscopic surgery's advantages - shorter hospital stays, less pain, and smaller, less disfiguring scars^[2]. The improvement of available equipment and instruments allowed more and more surgical procedures to be made through a minimally invasive approach, rapidly becoming a standard practice in most procedures.

At the same time, progresses in gastrointestinal endoscopy have made it an indispensable and multifaceted instrument for diagnosis and therapy. Besides endoluminal procedures, gastroenterologists attempted some interventions beyond the wall barrier, such as pseudocyst drainage^[3] and percutaneous endoscopic gastrostomy^[4]. However, it was not until 2004 that Kalloo *et al*^[5] published the first report of a true transluminal procedure, a transgastric peritoneoscopy in a porcine model, which brought to light the concept of natural orifice transluminal endoscopic surgery (NOTES). The idea of incisionless surgery was attractive and has now become a new goal for both surgeons and other people interested in this field of investigation.

The term NOTES describes novel endoscopic interventions on internal organs performed through natural orifices^[6]. In this new approach, endoscopes enter the abdominal and thoracic cavities *via* any single or combination of natural orifices - mouth, urethra, vagina, and anus. Depending on the orifice, rigid or flexible equipment can be used. The lower “short-ways” (bladder, colon or vagina) allow the easy passage of rigid or flexible instruments into the abdominal cavity, but the upper “long-ways” (esophagus and stomach) require flexible equipment^[7] (Figure 1).

The main goal for NOTES is avoiding skin incisions. Other theoretical advantages include: decreased post-operative pain, reduction/elimination of general anesthesia, performance of procedures in an outpatient or even office setting, and possibly cost reduction. Moreover, eliminating skin incision avoids associated complications such as wound infections and hernias, as well as reduction in hospital stay, faster return to bowel function, improved cosmetic outcomes, and increased overall patient satisfaction^[2].

WHAT DID THE INVESTIGATION ACHIEVE SO FAR?

The first challenge in NOTES is getting good and clean access to the cavity we want to “scope” (Table 1). The first mention of natural orifice procedure dates back to the 1940s, when culdoscopies were performed using an endoscope passed through the recto-uterine pouch to view pelvic organs, as well as to perform sterilization procedures^[8]. At that time, these procedures did not gain much popularity and were restricted to some gynecological procedures. Recently, however, they were recovered by NOTES development. In 2002, Gettman *et al*^[9] published one pure transvaginal nephrectomy along a series of hybrid transvaginal nephrectomy in a porcine model.

Taking advantage of the great developments in gastrointestinal endoscopy, some pioneers began working on the transgastric approach to the abdominal cavity. The first published description of transgastric peritoneoscopy was in 2004 by Kalloo *et al*^[5], in a porcine model. Since then, a number of successful transgastric procedures have been attempted and performed^[10-20]. These initial studies also identified major limitations of the isolated transgastric approach, mainly in more complex procedures such as cholecystectomy, first described in 2005 by Park *et al*^[21]. Lack of triangulation and platform stability were the main problems identified. Searching for solutions to these problems, researchers tried other ways of entering the abdominal cavity. Fong *et al*^[22-24] published the first transcolonic peritoneoscopy followed by a series of transcolonic procedures. The access from below gives a good, direct view of the upper abdominal cavity. Having that in mind, Lima *et al*^[25] published the first transvesical endoscopic peritoneoscopy. And subsequently our group used a combination of transgastric and transvesical approaches to solve the problem of

triangulation, and managed to do a series of cholecystectomies and nephrectomies in porcine models^[26,27].

To accomplish NOTES procedures in the thorax, Sumiyama *et al*^[28] proposed the transesophageal access. Transvesical-transdiaphragmatic thoracoscopy^[29], transgastric-transdiaphragmatic thoracoscopy^[30], and transtracheal thoracoscopy^[31] have been suggested as well. Although the transesophageal method has been preferred as a direct entry to the thorax and posterior mediastinum, this permitted several simple thoracic procedures in porcine models^[32-38].

CURRENT CHALLENGES

Despite the enthusiasm for NOTES, there are still some hurdles to be overcome. The initial concern is the potential for intra-abdominal infection and spillage from the viscerotomy. Infection must first be prevented by using a clean access site. Most transgastric protocols also follow a 24 h liquid formula diet, intravenous antibiotics and stomach irrigation with sterile water and antibiotic solution. Despite these precautions, even a sterile overtube used to protect the endoscope from oral contamination becomes contaminated on oral insertion and can transport bacteria to both the stomach and the peritoneal cavity^[2]. Surprisingly, Narula *et al*^[39] reported no infections after gastrotomy in patients undergoing diagnostic transgastric peritoneoscopy without previous gastric decontamination. The authors considered that the same degree of contamination of the peritoneal cavity is expected as in any operation performed with an open viscus.

There is also some controversy about the need for endoscope sterilization. In a recent literature review, Spaun *et al*^[40] concluded that, although difficult, it is possible to terminally sterilize flexible endoscopes. Steris System 1™ that uses 0.2% peracetic acid was the cheapest and fastest sterilization method and scored second in the risk of recontamination. Ethylene oxide gas sterilization has the lowest risk of recontamination, but is the slowest and most expensive method. The authors recommend sterile instrumentation for clinical NOTES until well-designed and randomized clinical trials are available and guidelines are published.

Concerning viscerotomy closure, gastrotomy has been the most studied and the methods under investigation could also be applied to the colon, esophagus or bladder, depending on the circumstances. Several methods have been proposed for stomach closure, including: conventional endoscopic clips, over-the-scope clip (OTSC) system, septal occluders, T-tags, T-bars for tissue opposing, as well as more complex suturing devices such as the Eagle Claw VII, NDO Plicator, USGI Endosurgical Operating System, and linear endoscopic staplers. Most of these devices still have limitations that need improving, but OTSC shows the most promising results^[41]. More recently, the Padlock-G clip have been described as also showing promising results^[42]. Colonic closure in animal studies has been performed using the

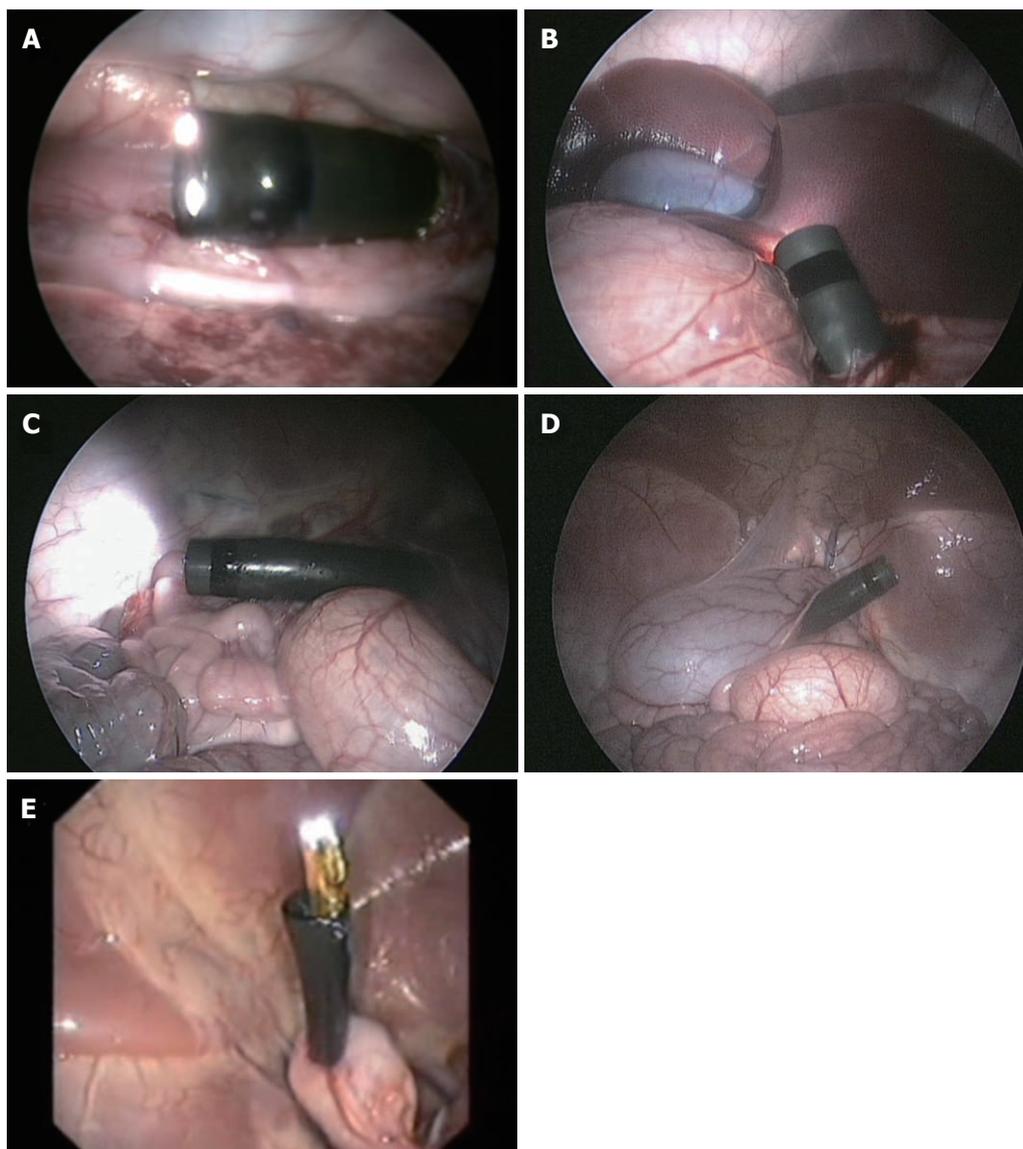


Figure 1 Internal view of natural orifice transluminal endoscopic surgery access (porcine model). A: Trans-thoracic view of transesophageal access; B: Transabdominal view of transgastric access; C: Transabdominal view of transcolonic access; D: Transabdominal view of transvaginal access; E: Transgastric view of transvesical access.

same techniques and devices as those used for gastrotomy closure. Transanal endoscopic microsurgery has been used for a long time, and has been useful for colonic closure in hybrid NOTES procedures in humans^[43]. For vesicotomy closure, Lima *et al*^[44] recently reported the first successful endoscopic closure using a suturing kit (T-fasteners with a locking clinch). Easy and safe closure has been the main advantage for transvaginal route acceptance. Closure after transvaginal access is readily and routinely performed by using standard surgical techniques. Even if closure were to fail, there would be little, if any, clinical significance, because of the extremely low risk of infection or hernia.

Concerning adequate exposure and visualization, pneumoperitoneum is a key component. Air insufflated in an uncontrolled manner through the endoscope results in wide fluctuations in intraperitoneal pressures, overdistension of the abdomen, and adverse hemodynamic effects. Insufflated air can also leak around the endoscope resulting in bowel overdistension^[2]. Many authors are now using a Veress needle to inject carbon

dioxide and safely control its pressure inside the abdomen^[45]. Despite this, new insufflators are being adapted to both deliver and monitor carbon dioxide through the endoscope^[46]. There is a great debate whether CO₂ or room air should be used. The effect of CO₂ with respect to laparoscopy has suggested an overall attenuated inflammatory response that may provide a further immunologic benefit. The acidic environment created has been the main contributing factor believed to facilitate this physiologic result. Conversely, “room air” laparoscopy has been shown to generate a greater inflammatory response, but a recent case-control study did not find a significant difference between the peritoneal inflammatory response of NOTES *vs* laparoscopy with carbon dioxide and air pneumoperitoneum^[47].

As previously stated, maintaining spatial orientation and triangulation of instruments is challenging when using a flexible endoscope. Moreover, flexible endoscopes are difficult to stabilize inside the abdominal cavity and can only pass flexible instruments which are too flaccid for retraction. This challenge can be overcome with

Table 1 Major features of the different natural orifice transluminal endoscopic surgery access for thoracic and abdominal cavities

	Transesophageal	Transgastric	Transvesical	Transvaginal	Transcolonic
Rigid instruments	No	No	Yes	Yes	Yes
Available in both genders	Yes	Yes	Yes	No	Yes
Sterility	No	No	Yes	No	No
Size	Wide	Wide	Up to 6 mm	Wide	Wide
Closure	Endoscopic (in study)	Endoscopic (in study)	Endoscopic (in study)	Direct suture	Endoscopic (in study)
Specimen retrieval	Not reported	Possible	Not reported	Possible	Possible

adequate training, a combination of different routes, and with the constant development of new instruments. Transvaginal, transcolonic and transvesical routes allow the introduction of rigid equipment, and except in the transvesical route, the instruments can be used either through a rigid endoscope or in parallel with a flexible endoscope. Additionally, these access routes coming from the lower abdomen permit a good direct visualization of the upper abdomen. In some cases, one can use an additional transabdominal port. This has been named hybrid NOTES and has been seen as an intermediate step of great help in the training and development of NOTES^[48]. Recently, magnets are being managed to provide the vigorous traction and countertraction required to advance NOTES procedures^[49]. A new magnetic anchoring and guidance system allows concurrent use of multiple working instruments and control of an intra-abdominal camera. It has been used to perform transvaginal, single-port cholecystectomy^[50]. Finally, one of the hurdles of NOTES is getting solid organs out of the thoracic and abdominal cavities. Excision of larger organs such as a kidney, or a gallbladder filled with stones through a small trocar orifice is a huge challenge. The transvaginal access has a big advantage in this matter and has been used for specimen retrieval in most NOTES procedures. On the other hand, transvaginal access is only an option in female patients.

HUMAN EXPERIENCE

In 2003, Rao and Reddy^[51] performed the first NOTES procedure in humans. The authors carried out a transgastric appendectomy in a male patient presenting severe burn lesions in his abdominal wall using a conventional flexible endoscope with two working channels. Only in 2007, was there the first published human NOTES procedure. Marks *et al*^[52] performed a transgastric rescue of a prematurely dislodge gastrostomy tube. The authors advanced a standard gastroscope through the previous gastrostomy, a performed peritoneoscopy, and suctioned away intra-abdominal free fluid. In that same year, another case reported the first human transvesical peritoneoscopy using a flexible ureteroscope during a standard laparoscopic robot-assisted prostatectomy^[53].

The first natural orifice transluminal cholecystectomy in humans was performed in Strasbourg, France^[54]. A

30-year-old woman with symptomatic cholelithiasis was submitted to cholecystectomy using a standard double-channel flexible gastroscope and standard endoscopic instruments. A 2-mm transabdominal needle port was used to insufflate carbon dioxide, to monitor the pneumoperitoneum, and to retract the gallbladder. Colpotomy was closed using conventional instruments. The patient had no post-operative pain and no scars, and was discharged on the second post-operative day. Shortly after that, the same technique was used by a team in Brazil, and by another in Italy^[55,56].

In 2007, a group of investigators from Ohio, United States used transgastric peritoneoscopy after standard laparoscopy to diagnose pancreatic masses^[57]. In 9 out of 10 patients, transgastric abdominal exploration corroborated the decision to proceed to open exploration made during traditional laparoscopic exploration. The average time of diagnostic laparoscopy was 12.3 min, compared to the 24.8 min taken for the transgastric route. Closure of the gastrotomy was obviated through its integration into the primary operation, whether that involved a resection with curative intent or palliation. No cross-contamination of the peritoneum or infectious complications was noted.

Other procedures using exclusively natural orifice transluminal procedures in humans have been performed - transgastric and transduodenal pancreatic necrosectomy^[58], transvaginal incisional hernia repair^[59], transvaginal liver, diaphragm, ovaries, and peritoneum biopsies^[60], and transvaginal appendectomy^[61]. This last one is especially important, as two of the three cases presented had an umbilical port inserted in order to complete appendectomy. As seen before in cholecystectomy, the use of a transabdominal port is essential to make natural orifice approaches feasible or at least easier at this time. Hybrid NOTES procedures are seen as a safe way to accomplish pure NOTES in the future. For this aim, hybrid procedures are developing in humans and achieving new goals like transvaginal nephrectomy^[62], transrectal rectosigmoidectomy^[63], sleeve gastrectomy^[64], transvaginal liver resection^[65], transvaginal splenectomy^[66], transgastric cholecystectomy^[67], transanal rectal cancer resection^[43], intragastric stapled cystogastrostomy of a pancreatic pseudocyst^[68], and adjustable gastric banding^[69].

In 2009, de Sousa *et al*^[70] published the first series of pure NOTES transvaginal cholecystectomies. The authors performed four cholecystectomies using two

endoscopes introduced simultaneously in the abdominal cavity through a transvaginal incision. Dissection was accomplished with conventional endoscopic instruments. Ligation of the cystic duct and artery was performed using endoscopic clips. Vaginal closure was achieved using the direct-vision suture technique. More recently, Bessler *et al*^[71] described a different technique for pure NOTES cholecystectomy in a 35-year-old-woman. Instead of using two endoscopes, the authors used an extra-long 5-mm articulating retractor placed into the abdomen *via* a separate colpotomy made under direct vision using the flexible endoscope in a retroflexed position. This method overcame the retracting limitations that obliged the use of a transabdominal port.

Despite all the enthusiasm around NOTES, other clinical advantages besides the absence of skin incision remain to be fully proven. Although most studies claim that greater operative time would be compensated by shorter hospital stays, prospective control studies are lacking^[72]. Hensel *et al*^[73] reported a retrospective case-control study where hybrid transvaginal cholecystectomy group showed a lower need for analgesics, faster mobilization, more comfortable recovery and a shorter hospital stay than the conventional laparoscopy group.

Finally, patients' perspectives and expectations about NOTES are not yet fully understood. An interesting questionnaire-based study was derived to identify their preferences between different available surgical options upon a hypothetical scenario of an acute appendicitis^[74]. Single port surgery (SPS) was the most popular method followed by conventional laparoscopy. Open surgery and NOTES were the least preferred. Choosing between SPS and NOTES only, 80.6% opted for SPS, 11.8% NOTES, and 5.6% declined surgery. The most popular route of access for NOTES was oral (37.7%). Another study asked women about their concerns and opinions regarding transvaginal surgery^[75]. The majority of women (68%) indicated that they would want a transvaginal procedure in the future because of decreased risk of hernia and decreased operative pain (90% and 93%, respectively), while only 39% were concerned with the improved cosmesis of NOTES surgery. Of the women polled, nulliparous women and those under age 45 years were significantly more often concerned with how transvaginal surgery may affect healthy sexual life and fertility issues. Of the women who would not prefer transvaginal surgery, a significant number indicated concerns over infectious issues.

THE FUTURE OF NOTES

NOTES promises a new and innovative era of minimal access surgery based on traditional laparoscopic and endoscopic techniques. Researchers all over the world are investigating ways to improve NOTES procedures in order to make it easier and safer. With careful development of new equipment and techniques, NOTES may be a reasonable option to conventional laparoscopic procedures. It may even become the method of choice for selected surgical procedures in the future.

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S-editor Tian L L-editor Rutherford A E-editor Li JY

Rebamipide promotes healing of colonic ulceration through enhanced epithelial restitution

Tomohisa Takagi, Yuji Naito, Kazuhiko Uchiyama, Toshimitsu Okuda, Katsura Mizushima, Takahiro Suzuki, Osamu Handa, Takeshi Ishikawa, Nobuaki Yagi, Satoshi Kokura, Hiroshi Ichikawa, Toshikazu Yoshikawa

Tomohisa Takagi, Yuji Naito, Kazuhiko Uchiyama, Toshimitsu Okuda, Katsura Mizushima, Takahiro Suzuki, Osamu Handa, Takeshi Ishikawa, Nobuaki Yagi, Satoshi Kokura, Hiroshi Ichikawa, Toshikazu Yoshikawa, Department of Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 602-8566 Kyoto, Japan

Author contributions: Takagi T and Naito Y conceived the experiments; Okuda T, Suzuki T and Ishikawa T performed the majority of *in vivo* experiments; Uchiyama K and Hand O performed the majority of *in vitro* experiments; Mizushima K, Yagi N and Kokura S performed molecular biological analysis; Takagi T, Ichikawa H and Yoshikawa T analyzed the data and were also involved in editing the manuscript. All authors discussed the results and commented on the manuscript.

Supported by A Grant-in-Aid for Scientific Research (B) to Toshikazu Yoshikawa (Grant No. 21390184) and Challenging Exploratory Research to Yuji Naito (No. 08101559) from the Japan Society for the Promotion of Science; A City Area Program to Toshikazu Yoshikawa and Yuji Naito from Ministry of Education, Culture, Sports, Science and Technology, Japan; An Adaptable and Seamless Technology Transfer Program through target-driven R&D to Yuji Naito from Japan Science and Technology Agency

Correspondence to: Yuji Naito, MD, PhD, Department of Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi-Hirokoji, Kamigyō-ku, 602-8566 Kyoto, Japan. ynaito@koto.kpu-m.ac.jp

Telephone: +81-75-2515508 Fax: +81-75-2510710

Received: December 2, 2010 Revised: January 18, 2011

Accepted: January 25, 2011

Published online: September 7, 2011

Abstract

AIM: To investigate the efficacy of rebamipide in a rat model of colitis and restitution of intestinal epithelial cells *in vitro*.

METHODS: Acute colitis was induced with trinitro-

benzene sulfonic acid (TNBS) in male Wistar rats. Rats received intrarectal rebamipide treatment daily starting on day 7 and were sacrificed on day 14 after TNBS administration. The distal colon was removed to evaluate the various parameters of inflammation. Moreover, wound healing assays were used to determine the enhanced restitution of rat intestinal epithelial (RIE) cells treated with rebamipide.

RESULTS: Intracolonic administration of rebamipide accelerated TNBS-induced ulcer healing. Increases in the wet weight of the colon after TNBS administration were significantly inhibited by rebamipide. The wound assay revealed that rebamipide enhanced the migration of RIE cells through phosphorylation of extracellular signal-regulated kinase (ERK) and activation of Rho kinase.

CONCLUSION: Rebamipide enema healed intestinal injury by enhancing restitution of RIE cells, *via* ERK activation. Rebamipide might be a novel therapeutic approach for inflammatory bowel disease.

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Key words: Rebamipide; Experimental colitis; Intestinal epithelial cells; Extracellular signal-regulated kinase; Rho kinase

Peer reviewer: Ibrahim A Al Mofleh, Professor, Department of Medicine, College of Medicine, King Saud University, PO Box 2925, 11461 Riyadh, Saudi Arabia

Takagi T, Naito Y, Uchiyama K, Okuda T, Mizushima K, Suzuki T, Hand O, Ishikawa T, Yagi N, Kokura S, Ichikawa H, Yoshikawa T. Rebamipide promotes healing of colonic ulceration through enhanced epithelial restitution. *World J Gastroenterol* 2011; 17(33): 3802-3809 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i33/3802.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3802>

INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic and recurrent intestinal inflammatory disorder whose precise pathogenesis remains unknown^[1]. In patients with IBD, a variety of cells and soluble factors mediate extensive mucosal damage, and epithelial cell damage is frequently observed^[2]. Generally, after sustaining mucosal injury, the intestinal epithelium rapidly reestablishes its integrity *via* restitution, proliferation, and differentiation of epithelial cells^[3]. Among these three steps, restitution is thought to be most critical for intestinal mucosal healing^[4]. Therefore, the promotion of restitution remains an important therapeutic target. Along these lines, various molecules involved in regenerating the intestinal epithelium are currently under consideration for clinical use^[5].

Rebamipide is an amino acid derivative of 2(1H)-quinolinone, and is a gastric mucosal protective and ulcer-healing agent that has been widely used for treatment of acute and chronic gastritis and gastric ulcer. It is already known that rebamipide has anti-inflammatory properties including scavenging of free radicals, suppression of pro-inflammatory cytokine production, inhibition of inflammatory cell migration and adherence, and promotion of prostaglandin and mucus production^[6]. Recently, rebamipide has been used as a gastric protective agent and for treatment of UC^[7]. First, Makiyama *et al.*^[8] have reported that rebamipide enema had an anti-inflammatory effect in a patient with proctitis-type UC. They also have reported the efficacy of rebamipide enemas in active distal UC and proctitis in a prospective study^[9]. Furthermore, it has been demonstrated that rebamipide enema is safe and useful in corticosteroid-refractory or -dependent patients with the active distal type of UC^[10]. In addition, Matsumoto *et al.*^[11] have reported that rebamipide enema ameliorates disease activity in patients with left-side ischemic colitis. Thus, these clinical data suggest that rebamipide shows promise in terms of its potential for repairing intestinal injury. However, the detailed molecular mechanism of action of rebamipide against intestinal inflammation remains unclear.

Therefore, in the present study, we aimed to assess the effect of rebamipide in intestinal inflammation by using the trinitrobenzene sulfonic acid (TNBS)-induced colitis model, a well-accepted IBD model. Furthermore, we analyzed the possible mechanisms involved in rebamipide-mediated mucosal restitution by using a well-established model of intestinal epithelial wound healing *in vitro*^[12,13].

MATERIALS AND METHODS

Reagents

All chemicals were prepared immediately before use. Rebamipide {2-(4-chlorobenzoylamino)-3[2-(1H)-quinolinon-4-yl] propionic acid} was a kind gift from Otsuka Pharmaceutical Co. Ltd. (Tokyo, Japan). TNBS and 3, 3', 5, 5'-tetramethylbenzidine were obtained from

Wako Pure Chemicals (Osaka, Japan). We used MEK1/2 inhibitor as an extracellular signal-regulated kinase (ERK) inhibitor (U0126; BIOMOL International LP, Plymouth Meeting, PA, United States) and Y27632 [(1)-(R)-*trans*-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride] as the Rho kinase inhibitor (Biaffin GmbH and Co KG, Kassel, Germany). All other chemicals were of the highest quality commercially available.

In vivo study animals

Male Wistar rats weighing 180-200 g were obtained from Shimizu Laboratory Supplies Co. Ltd. (Kyoto, Japan). The animals were housed at 22 °C in a controlled environment with 12 h of artificial light per day, and were allowed access to rat chow and water *ad libitum*. The animals were maintained and all experimental procedures were carried out in accordance with the National Institutes of Health (NIH) guidelines for the use of experimental animals. All experimental protocols were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine (Kyoto, Japan).

Induction of colitis

TNBS-induced colitis was established using the method of Morris *et al.*^[14]. The rats were lightly anesthetized with pentobarbital following a 48-h fast, and then a rubber catheter (outer diameter, 2 mm) was inserted *via* the anus, such that the tip was 8 cm from the anus. TNBS dissolved in 50% ethanol (120 mg/mL) was instilled into the lumen of the colon *via* the catheter (volume, 0.25 mL). Following the instillation of TNBS at 30 mg per rat, the anus was occluded with a clip for 1 h.

Treatment protocol

All animals were randomized into groups that received rebamipide or physiological saline vehicle. We focused on the effects of rebamipide during healing after colonic mucosal injury. One percent rebamipide was intrarectally administered (2 mL/kg) twice daily starting on day 7 after induction of colitis, until day 14.

Evaluation of colonic damage

The rats were sacrificed on day 14 and the distal colon was removed and opened by longitudinal incision. The wet colon weight was measured immediately thereafter. As indices of inflammation, damage was estimated macroscopically as the sum of the mucosal score. The mucosal score was rated on a six-point scale (0-5) according to the criteria established by Morris *et al.*^[14] (Table 1). The degree of colitis was evaluated by an independent observer who did not have previous knowledge of the treatment. For the histological examination, formalin-fixed tissue was stained with hematoxylin and eosin. The colon histological score was evaluated using the histopathological grading system of Ameho *et al.*^[15] by an observer blinded to the treatment. This grading, which takes into account the degree of infiltration, the presence of erosion, ulceration, or necrosis, and the depth and

Table 1 Criteria for scoring gross morphological damage of the colon^[12]

Mucosal score	Gross morphology
0	No damage
1	Localized hyperemia, but no ulcers
2	Liner ulcers with no significant inflammation
3	Liner ulcers with inflammation at one site
4	Two or more sites of ulceration and/or inflammation
5	Two or more major sites of inflammation and ulceration extending > 1 cm along the length of the colon

surface of the lesion, is scaled from 0 to 6.

***In vitro* study of intestinal epithelial cell line**

Non-transformed rat intestinal epithelial (RIE) cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin. The cells were incubated at 37 °C in a humidified atmosphere supplemented with 5% CO₂. RIE cells were trypsinized and seeded into 60-mm culture dishes. Experiments were performed when the cells reached confluency.

Wound assay

Wound assays were performed using a previously described method with minor modifications^[16]. Confluent monolayers of RIE cells in 60-mm culture dishes were washed with phosphate-buffered saline, and cells were cultured for an additional 24 h in serum-free medium. Subsequently, cell monolayers were disrupted using an extra long 10-µL pipette tip (Pelican Life Sciences, San Diego, CA, United States), followed by a cycle of washing in serum-free medium to yield a cell-free zone in the culture dishes. The process of migration was monitored using an inverted phase-contrast microscope at 0, 6 and 12 h after induction of the artificial wound. Changes in the cell-free zone were analyzed with the ImageJ software (Wayne Rashband; NIH, Bethesda, MD, United States), and this analysis was performed by the same individual under blind conditions to prevent observer bias. To investigate the effects of rebamipide on RIE cell migration, the cells were co-incubated with rebamipide (2 mmol/L) after wound induction. Furthermore, to investigate involvement of the ERK signaling pathway and the Rho kinase pathway, cells were co-treated with U0123 (10 µmol/L) or Y27632 (1 µmol/L) after wounding.

Western blotting analysis

To determine whether rebamipide was involved in the ERK signaling pathway, proteins were obtained from RIE cells at 0, 5, 15 and 20 min after stimulation with 2 mmol/L rebamipide. The total proteins were mixed with SDS sample buffer. The samples were then subjected to 10% SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (Atto Corporation, Tokyo, Japan). The membrane was blocked with 2% bovine serum albumin in Tris-

buffered saline that contained 0.1% Tween (TBS-T) at room temperature for 30 min. Western blotting analysis was carried out using rabbit polyclonal anti-p44/42, phospho-p44/42 (1:1000), and actin antibody (1:1000) as an internal control at room temperature for 1 h. After three washes with TBS-T, the membrane was incubated with anti-rabbit IgG-horseradish peroxidase (1:3000; GE Healthcare UK Ltd., Little Chalfont, Bucks, United Kingdom) at room temperature for 45 min. The signals were visualized using an ECL kit (GE Healthcare) according to the manufacturer's instructions.

Statistical analysis

Results are presented as the mean ± SE. Overall differences between groups were determined by one-way ANOVA. Whenever one-way ANOVA was significant, differences between individual groups were analyzed by Bonferroni's multiple comparisons test. Differences of $P < 0.05$ were considered significant. All analyses were performed using the GraphPad Prism 4 program (San Diego, CA, United States) for a Macintosh computer.

RESULTS

Therapeutic effect of rebamipide on TNBS colitis

In rats exposed to TNBS, macroscopic findings in the colon demonstrated severe colitis with hyperemia, edema, thickening, ulceration, and necrosis. It has already been demonstrated that the lesion area reaches its maximum on day 2 or 3 after TNBS treatment, after which it decreases in a time-dependent manner^[13,17]. In order to focus on the effects of mucosal healing by rebamipide enema, all rats were administered either placebo or rebamipide solution starting on day 7 after induction of TNBS, until day 14. On day 14, severe colitis with thickening of the mucosa and ulceration were still observed in the placebo group (Figure 1A). In contrast, rats treated with 1% rebamipide showed smaller erosions and mild edema in the colon (Figure 1A). Thus, the colonic mucosal damage score on day 14 had significantly increased due to TNBS administration in the sham-treated group. Increases in the mucosal damage score were significantly inhibited by treatment with 1% rebamipide (Figure 1B). Furthermore, the colonic wet weight was significantly increased in the TNBS colitis group. This increase was significantly decreased by treatment with 1% rebamipide (Figure 1C).

The therapeutic effects of 1% rebamipide enema were also confirmed by histological examination. Figure 2A shows the representative histological features of a normal colon (day 0) and those of the control group (day 14) and the rebamipide-treated group (day 14). TNBS administration induced marked thickening of the colonic wall, with transmural infiltration and aggregation of numerous inflammatory cells (Figure 2A), which is in contrast to the features of the normal colon, which does not show transmural infiltration or aggregation of inflammatory cells (Figure 2A). On the contrary, in rats treated with 1%

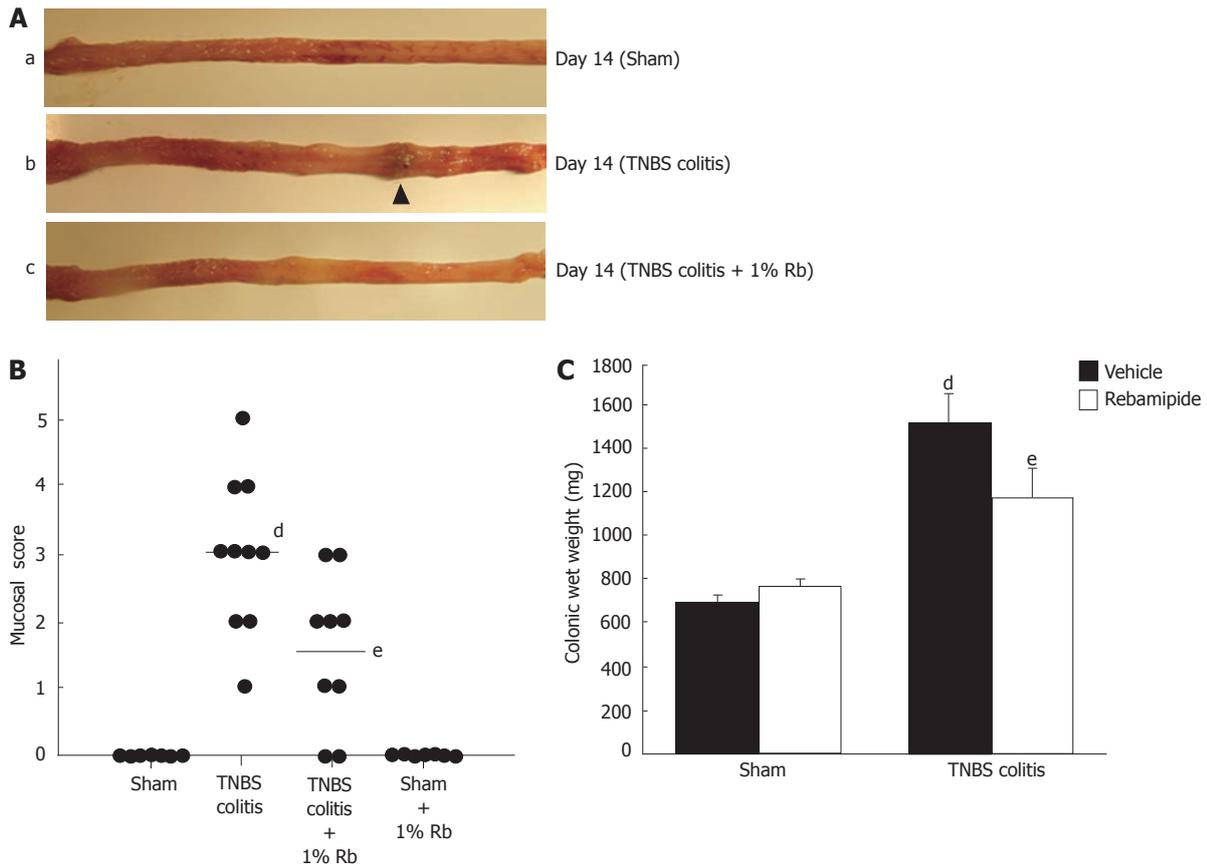


Figure 1 Effects of 1% rebamipide on macroscopic findings, mucosal damage score, and wet colon weight on day 14 after trinitrobenzene sulfonic acid-induced injury. A: Severe colitis was induced with hyperemia, edema, thickening, ulceration, and necrosis in trinitrobenzene sulfonic acid (TNBS)-colitis rats (b) compared to sham-operated rats (a). These changes were reduced in rats treated with 1% rebamipide (TNBS-colitis rats treated with 1% rebamipide) (c). B: A 1% rebamipide enema was administered twice daily starting on day 7 after induction of colitis, until day 14. Rats were sacrificed on day 14, and the mucosal damage score was evaluated. Data are expressed as a scatter plot. ^a*P* < 0.01 vs sham-treated rats. ^b*P* < 0.05 vs TNBS-induced colitis rats receiving the vehicle. C: Rats were sacrificed on day 14 and the distal colon was removed, after which, the wet colon weight was immediately measured. Data represent the mean ± SE of seven rats. ^d*P* < 0.01 vs sham-treated rats receiving the vehicle. ^e*P* < 0.01 vs TNBS-induced colitis rats receiving the vehicle.

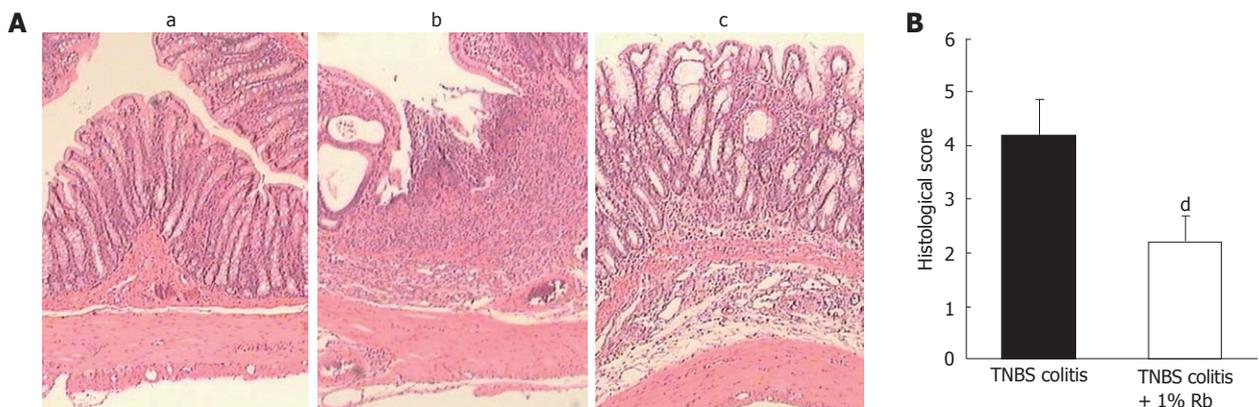


Figure 2 Effects of 1% rebamipide on histological findings in the colon on day 14 after trinitrobenzene sulfonic acid-induced injury. A: Histological appearance of colonic tissue in sham-operated rats (a), trinitrobenzene sulfonic acid (TNBS)-colitis rats (b), and TNBS-colitis rats treated with 1% rebamipide (c). Histological examination revealed that TNBS administration induced marked thickening of the colonic wall, which was associated with transmural infiltration of inflammatory cells. In contrast, both mural wall thickening and infiltration of inflammatory cells were inhibited in rats treated with 1% rebamipide. Hematoxylin and eosin staining (× 40). B: Histological score was evaluated. Data represent the mean ± SE of six rats. ^a*P* < 0.01 vs TNBS-colitis rats receiving the vehicle.

rebamipide, inhibition of both mural wall thickening and inflammatory cell infiltration was observed (Figure 2A). More importantly, rebamipide enema promoted restit-

tion of the colonic epithelium in the ulcerative area. The histological score was increased in the TNBS colitis group, and this increase was significantly inhibited by treatment

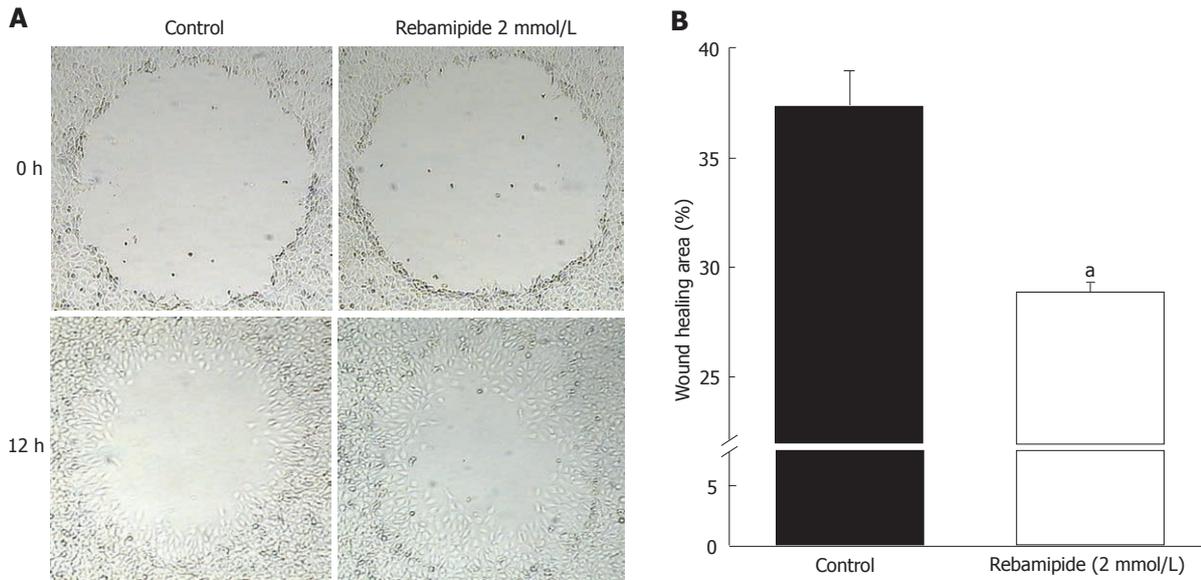


Figure 3 Restitution of rat intestinal epithelial cells around an artificially created wound in control and rebamipide-treated groups. A: Restitution of rat intestinal epithelial (RIE) cells was evaluated using a wound assay. The denuded area of RIE cells recovered in a time-dependent manner after wound induction. Restitution of the denuded area was promoted by rebamipide at 12 h after wound induction. B: To investigate the effects of rebamipide on RIE cell migration, cells were co-cultured with 2 mmol/L rebamipide after wound induction. Wound repair in 2 mmol/L rebamipide-treated cells occurred significantly earlier than it did in the controls. Datas represent the mean \pm SE of four experiments. ^a $P < 0.05$ vs controls.

with 1% rebamipide (Figure 2B).

Effects of rebamipide treatment as determined by wound assay using RIE cells

To investigate the effect of rebamipide on restitution of the intestinal epithelium, we performed a wound assay using RIE cells. The cell-free area at the wound site gradually decreased in a time-dependent manner, and complete recovery was observed 15 h after wound induction (data not shown). Restitution was significantly enhanced by rebamipide treatment (Figure 3A). Treatment with 2 mmol/L rebamipide accelerated wound healing compared to the control group (Figure 3B).

To investigate whether the promotion of restitution by rebamipide was involved in ERK signaling, RIE cells were stimulated with 2 mmol/L rebamipide by using a specific ERK inhibitor (U01263). As shown in Figure 4A, the ERK inhibitor blocked the promotion of wound healing by rebamipide. Moreover, to confirm the involvement of the ERK signaling pathway in the enhanced restitution associated with rebamipide treatment, we performed western blot analysis using phosphorylation-status-dependent and -independent antibodies against ERK1/2 (44 and 42 kDa) at 0, 5, 15 and 20 min after treatment with 2 mmol/L rebamipide. The western blots revealed ERK phosphorylation in RIE cells 5 min after rebamipide treatment (Figure 4B). Furthermore, to examine the role of Rho kinase in rebamipide-enhanced restitution, RIE cells were stimulated with rebamipide by using Y27632, a Rho kinase inhibitor (1 μ mol/L). The inhibition of Rho kinase canceled the promotion of wound healing by rebamipide treatment.

DISCUSSION

In the present study, we demonstrated that rebamipide enema promoted wound healing in rats with TNBS-induced colonic ulceration. In this model, the area of colonic ulceration peaked on day 2 or 3 after TNBS treatment, and subsequent amelioration was observed in a time-dependent manner. In this study, rats were treated with 1% rebamipide enema starting on day 7 after TNBS injury induction, until day 14. Rebamipide clearly accelerated colonic wound healing under these conditions. These findings are consistent with previous results showing the beneficial effects of rebamipide enema in patients with active UC^[7-10], as well as with the results of a study using another experimental colitis model treated with rebamipide enema^[18-20].

Rebamipide is a gastric protective and ulcer healing agent that was developed in Japan. It is used clinically in Japan in combination with acid suppressive agents for gastric mucosal protection, acute and chronic gastritis treatment, and gastroduodenal ulcer healing. More interestingly, accumulating evidence suggests that rebamipide exerts protective and healing effects on other tissues. In fact, rebamipide has been shown to be effective in the treatment of patients with UC. In addition, the therapeutic effect of rebamipide may not be limited to the colon alone: indeed, this agent has been demonstrated by both clinical and basic research as effective for the treatment of stomatitis^[21] and pulmonary^[22], renal^[23] and liver damage^[24-26], and it also provides corneal protection^[27-29].

The mechanisms responsible for amelioration of colitis by rebamipide have not been fully elucidated but may involve inhibition of reactive oxygen species produc-

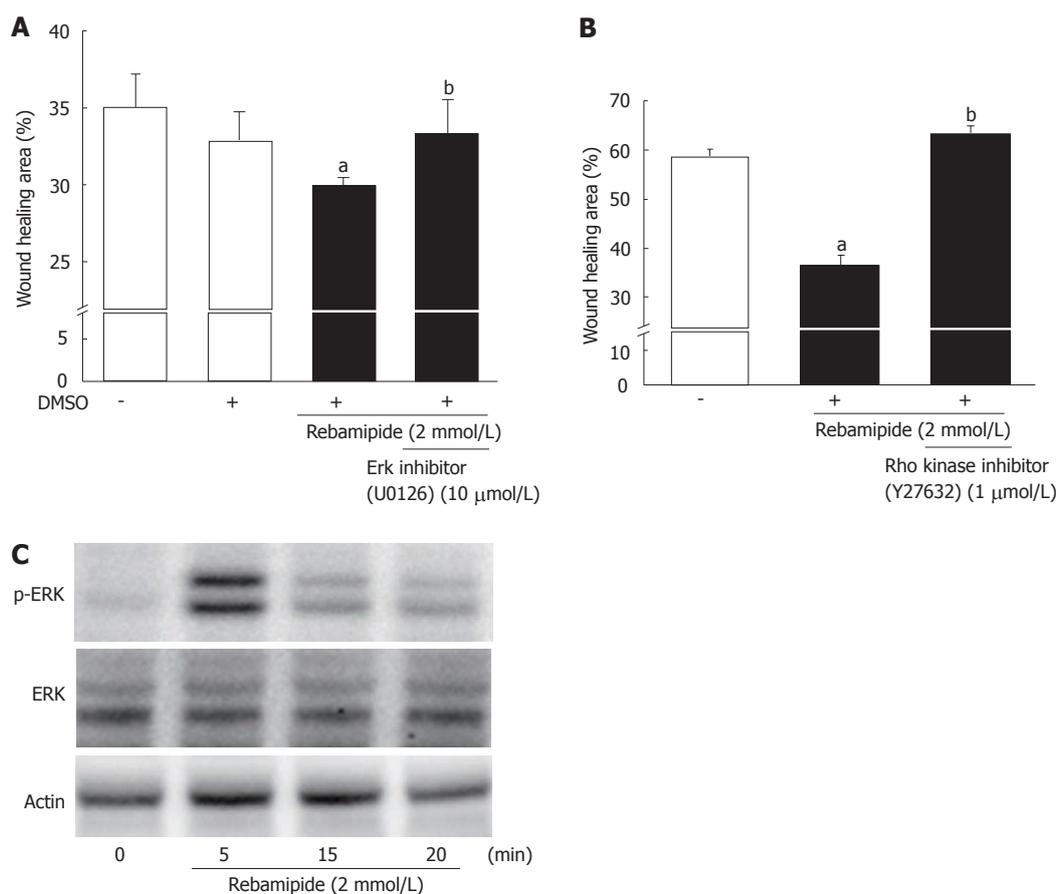


Figure 4 Involvement of extracellular signal-regulated kinase and Rho in rebamipide-treated rat intestinal epithelial cells. **A:** Rat intestinal epithelial (RIE) cells were treated with rebamipide (2 mmol/L) with or without an extracellular signal-regulated kinase inhibitor (U01263, 10 μmol/L) after wound induction. The wound healing area (12 h later) was then monitored. Data represent the mean ± SE of four experiments. ^a $P < 0.05$ vs controls. ^b $P < 0.05$ vs the 2 mmol/L rebamipide-treated group. **B:** Expression of p44/42 and phospho-p44/42 in RIE cells incubated with rebamipide (2 mmol/L) was measured using western blot analysis. Actin antibody was used as an internal control. Representative data from three observations is shown. **C:** RIE cells were treated with rebamipide (2 mmol/L) with or without Rho kinase inhibitor (Y27632, 1 μmol/L) after wound induction. The wound healing area (6 h later) was then monitored. Data represent the mean ± SE of four experiments. ^a $P < 0.05$ vs controls. ^b $P < 0.05$ vs the 2 mmol/L rebamipide-treated group. DMSO: Dimethyl sulfoxide.

tion^[19,20,30], suppression of neutrophil accumulation^[31,32], increases in trans-epithelial electrical resistance^[33], and induction of hepatocyte growth factor expression^[34]. However, it remains unclear whether rebamipide enhances restitution of RIE cells. It is already known that rebamipide enhances gastric epithelial restitution, but the same has yet to be proven in intestinal epithelial restitution. In cases of intestinal mucosal injury, restitution is an important step in re-establishing mucosal integrity, and restitution is the most rapid post-injury response; in effect, restitution restores the continuity of the intestinal epithelial layer, primarily by redistribution of epithelial cells. This process is completed by the migration of local epithelial cells along the underlying matrix, which does not require epithelial cell proliferation^[35,36]. In this study, to assess the effects of rebamipide on intestinal epithelial restitution, the round wound assay was evaluated using RIE cells. Our results showed that 2 mmol/L rebamipide treatment promoted the restitution of RIE cells.

With regard to the role of the ERK signaling pathway in epithelial restitution, several studies have been reported in which activation of the ERK signaling path-

way played an important role in epithelial wound closure. In the present study, rebamipide promoted ERK phosphorylation. This result is in agreement with the results of Gazel *et al.*^[37] and Wang *et al.*^[38]. Moreover, rebamipide-promoted restitution was suppressed by U01263, a specific ERK inhibitor. These data indicate that accelerated restitution by rebamipide is at least partly mediated by the ERK pathway. Tanigawa *et al.*^[39] have demonstrated that rebamipide induces ERK phosphorylation in gastric cancer cells and inhibits cell growth through Smad signaling. Although they used gastric cancer cells, they also found that rebamipide induced phosphorylation of ERK.

Furthermore, we investigated whether rebamipide-promoted restitution was related to Rho kinase activation. Rho kinase has been identified as one of the effectors of the small GTP-binding protein Rho. Accumulating evidence has demonstrated that the Rho/Rho kinase pathway plays an important role in various cellular functions, including cell contraction, cell proliferation, gene expression, and especially cell migration^[40,41]. With regard to restitution of IE cells, Santos *et al.*^[42] have found that

Rho protein is one of the essential elements of a mechanism by which growth factors induce cell migration to restore mucosal integrity, and Rao *et al.*^[43] have demonstrated that activation of Rho kinase results in increased phosphorylation of the myosin light chain, which leads to cell migration. In this investigation, we used Y27632, which has been widely used as a specific inhibitor of the Rho-associated coiled-coil forming protein serine/threonine kinase family of protein kinases^[44]. Co-treatment with Y27632 cancelled the effect of rebamipide on the restitution of RIE cells. These data indicate that rebamipide enhanced the restitution of RIE cells *via* ERK phosphorylation and Rho kinase activation. However, the detailed mechanism of rebamipide-induced ERK and Rho kinase activation remains unknown. Further investigations are needed to elucidate this mechanism.

In summary, the present study indicates that treatment with rebamipide can promote the healing of TNBS-induced intestinal injury, which is associated with acceleration of intestinal epithelial restitution. The present results suggest that rebamipide has great potential as a new therapeutic agent for the treatment of inflammation-associated intestinal injury.

COMMENTS

Background

Ulcerative colitis (UC) is a chronic and recurrent disorder of the colon and rectum. While the precise pathogenesis of UC remains unknown, medical management of patients with acute exacerbation of UC symptoms focuses on achieving remission by inhibiting intestinal inflammation and repairing mucosal injury. However, some patients with inflammatory bowel disease do not respond, or respond incompletely, to the existing treatments. Therefore, it is important to investigate new anti-inflammatory strategies.

Research frontiers

Rebamipide is a gastric mucosal protective and ulcer-healing agent, and has been used for treatment of UC. However, the detailed mechanism of action of rebamipide against intestinal inflammation such as UC remains unclear. In this study, the authors investigated the therapeutic efficacy of rebamipide in an experimental rat model of colitis and evaluated the restitution of intestinal epithelial cells treated with rebamipide *in vitro*.

Innovations and breakthroughs

The present study indicated that treatment with rebamipide could promote the healing of trinitrobenzene sulfonic acid-induced intestinal injury, which has been associated with acceleration of intestinal epithelial restitution through extracellular signal-regulated kinase and Rho kinase activation. The present results suggest that rebamipide has great potential as a new therapeutic agent for the treatment of inflammation-associated intestinal injury.

Applications

By understanding how rebamipide inhibits intestinal inflammation and promotes healing of the intestinal injury, rebamipide may represent a future therapeutic agent for treatment of patients with UC.

Terminology

Rho kinase has been identified as one of the effectors of the small GTP-binding protein Rho. Accumulating evidence has demonstrated that the Rho/Rho kinase pathway plays an important role in various cellular functions, including cell contraction, cell proliferation, gene expression, and especially, cell migration.

Peer review

This is an interesting, well-designed study with good documentation of results.

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S- Editor Tian L L- Editor Kerr C E- Editor Li JY

Dickkopf3 overexpression inhibits pancreatic cancer cell growth *in vitro*

Yu-Mei Gu, Yi-Hui Ma, Wu-Gan Zhao, Jie Chen

Yu-Mei Gu, Yi-Hui Ma, Wu-Gan Zhao, Jie Chen, Department of Pathology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China

Author contributions: Chen J and Gu YM designed the research; Gu YM, Ma YH and Zhao WG performed the research and analyzed the data; Gu YM and Chen J wrote the paper.

Supported by National Natural Science Foundation of China, No. 30471970; National Science and Technology Support Project (the 11th Five-Year Plan) of China, No. 2006BAI02A14; Scientific Research Special Projects of Health Ministry of China, No. 200802011 and National Data Sharing Project in Human Health, No. 2005DKA32403

Correspondence to: Jie Chen, Professor, Department of Pathology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China. xhblk@163.com

Telephone: +86-10-65295490 Fax: +86-10-65295490

Received: February 17, 2011 Revised: April 13, 2011

Accepted: April 20, 2011

Published online: September 7, 2011

Abstract

AIM: To elucidate the role of dickkopf3 (Dkk3) in human pancreatic cancer cell growth.

METHODS: Dkk3 mRNA and protein expression in human pancreatic cancer cell lines were detected by real-time reverse transcription polymerase chain reaction (real-time RT-PCR), Western blotting and immunofluorescence. Methylation of the Dkk3 promoter sequence was examined by methylation-specific polymerase chain reaction (MSP) and Dkk3 mRNA expression was determined by real-time RT-PCR after 5-aza-2'-deoxycytidine (5-aza-dC) treatment. The effects of Dkk3 on cancer cell proliferation and *in vitro* sensitivity to gemcitabine were investigated by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) after transfecting the Dkk3 expression plasmid into human pancreatic cancer cells. The expression of β -catenin, phosphorylated extracellular signal-regulated protein kinases (pERK) and

extracellular signal-regulated protein kinases (ERK) was also examined by real-time RT-PCR and Western blotting after upregulating Dkk3 expression in human pancreatic cancer cells.

RESULTS: The results show that the expression levels of both Dkk3 mRNA and protein were low in all pancreatic cancer cell lines tested. The Dkk3 promoter sequence was methylated in the MIA PaCa-2 and AsPC-1 cell lines, which showed reduced Dkk3 expression. These two cell lines, which initially had a methylated Dkk3 promoter, showed increased Dkk3 mRNA expression that was dependent upon the dosage and timing of the DNA demethylating agent, 5-aza-dC, treatment ($P < 0.05$ or $P < 0.01$). When Dkk3 expression was upregulated following the transfection of a Dkk3 expression plasmid into MIA PaCa-2 cells, the ability of cells to proliferate decreased ($P < 0.01$), and the expression of β -catenin and pERK was downregulated ($P < 0.01$). Sensitivity to gemcitabine was enhanced in Dkk3 expression plasmid-transfected cells.

CONCLUSION: Our findings, for the first time, implicate Dkk3 as a tumor suppressor in human pancreatic cancer, through the downregulation of β -catenin expression *via* the ERK-mediated pathway.

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Key words: Cell growth; Dickkopf3; *In vitro*; Overexpression; Pancreatic cancer

Peer reviewers: Kazuaki Takabe, MD, PhD, Assistant Professor of Surgery and Assistant Professor of Biochemistry and Molecular Biology, Surgical Oncology, VCU Massey Cancer Center, Virginia Commonwealth University/Medical College of Virginia, PO Box 980011, Richmond VA 23298-0011, United States; Catherine Greene, PhD, Senior Lecturer, Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin 9, Ireland

Gu YM, Ma YH, Zhao WG, Chen J. Dickkopf3 overexpres-

sion inhibits pancreatic cancer cell growth *in vitro*. *World J Gastroenterol* 2011; 17(33): 3810-3817 Available from: URL: <http://www.wjnet.com/1007-9327/full/v17/i33/3810.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3810>

INTRODUCTION

Pancreatic cancer is the sixth leading cause of cancer death in China^[1]. The overall five-year survival rate is approximately 1%-3%, and the median survival period after diagnosis is only 4 to 5 mo. Pancreatic cancer remains one of the most aggressive human cancers, with an exceedingly poor prognosis because of its late onset of symptoms^[2], rapid progression, frequent metastasis and insensitivity to chemotherapy and radiotherapy. Therefore, recognizing the factors associated with pancreatic cancer progression is critical for its treatment.

Dickkopf (Dkk) family proteins, including Dkk1/2/3/4, are secreted modulators of the canonical Wnt signaling pathway^[3]. Dkk1, Dkk2 and Dkk4, antagonists of Wnt signaling^[4,5], interact with Wnt coreceptors, low-density lipoprotein receptor-related protein 5/6 (LRP5/6) and Kremen^[6,7]. Dkk3 interacts with kremen1 and kremen2, but not with LRP5/6^[8], and has been proposed to act as a tumor suppressor. Dkk3 is downregulated in some tumors, and it inhibits tumor growth^[9-25]. For example, in cervical cancer and malignant glioma, Dkk3 regulates tumor cell growth and decreases β -catenin expression^[16,23]. Dkk3 can induce cancer cell apoptosis by c-Jun-NH2-kinase (JNK) activation in testicular and prostate cancer cells^[9,26]. The Dkk3 promoter sequence is methylated in several tumors, such as breast cancer, hepatoma, bladder cancer and malignant astrocytic gliomas^[27-32]. In lung adenocarcinomas, however, Dkk3 inhibits cancer cell apoptosis by decreasing the intracellular level of reactive oxygen species and functions as an oncogene^[33]. Dkk3 knock-out mice showed no enhanced tumor formation^[34]. Recently, other studies have demonstrated that Dkk3 plays distinct roles in different cells^[8].

To date, no study has investigated Dkk3 expression and its roles in human pancreatic cancer cell behavior. To better understand the role of Dkk3 in pancreatic cancer progression, we investigated Dkk3 expression and promoter sequence methylation in human pancreatic cancer cells. The effects of Dkk3 on cell proliferation and sensitivity to gemcitabine were simultaneously observed after expression was increased in MIA PaCa-2 cells, following transfection with the Dkk3 expression plasmid.

MATERIALS AND METHODS

Cell lines and cell culture

The human pancreatic cancer cell lines PANC-1, MIA PaCa-2, AsPC-1 and BxPC-3 were purchased from the American Type Culture Collection (Manassas, Virginia, United States). AsPC-1 and BxPC-3 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, MO, United States)

and PANC-1 and MIA PaCa-2 cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, MO, United States). All media were supplemented with 10% fetal calf serum (Tianjin Haoyang Biological Manufacture Co., LTD, China), 100 μ g/mL streptomycin and 100 U/mL penicillin, and the cultures were grown at 37 °C in a humidified atmosphere containing 5% CO₂.

Construction of and transient transfection with a plasmid expressing human Dkk3

Total RNA was extracted from PANC-1 cells using TRIzol reagent (Invitrogen, CA, United States), according to the manufacturer's protocol. The cDNAs were synthesized using the TaKaRa RNA polymerase chain reaction (PCR) Kit (TaKaRa, Japan). A full-length cDNA encoding human Dkk3 was cloned by PCR using 500 ng cDNA as a template and primers containing HindIII and BamHI restriction enzyme sites (Table 1). The PCR products were ligated into pcDNA3.1 (Invitrogen, CA, United States) to create the plasmid pcDNA3.1-Dkk3. MIA PaCa-2 cells were transfected with the pcDNA3.1 vector or pcDNA3.1-Dkk3 using FuGENE (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's protocol.

Reverse transcription polymerase chain reaction

Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, CA, United States) according to the manufacturer's protocol. The cDNAs were synthesized using the TaKaRa RNA PCR Kit (TaKaRa, Japan). The optimal PCR conditions were 94 °C for 5 min; 35 cycles at 94 °C for 40 s, 61 °C (Dkk3)/52 °C (β -actin) for 40 s, 72 °C for 40 s; and 72 °C for 10 min. PCR products (5 μ L) were separated by electrophoresis in a 2.0% agarose gel. Primer sequences for Dkk3 and β -actin are listed in Table 1.

RNA preparation and real-time reverse transcription polymerase chain reaction

Total RNA was isolated from the cells, with or without 5-aza-2'-deoxycytidine (5-aza-dC) treatment, using TRIzol reagent (Invitrogen, CA, United States) according to the manufacturer's protocol. First-strand cDNA was synthesized from 500 ng of total RNA using the TaKaRa RNA PCR Kit (TaKaRa, Japan). PCR was conducted on a 7500 Real Time PCR System (Applied Biosystems, United Kingdom) in combination with the SYBR green PCR master mix (Applied Biosystems, United Kingdom). Melting curve analyses following amplification were performed to ensure product specificity. The relative expression levels of Dkk3 mRNA and β -catenin mRNA were normalized to mRNA level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same cDNA sample. Δ Ct was calculated by subtracting the Ct of GAPDH mRNA from the Ct of the mRNA of interest. $\Delta\Delta$ Ct was then calculated by subtracting the Δ Ct of the control from the Δ Ct of the sample. The fold change in mRNA was calculated according to the equation $2^{-\Delta\Delta Ct}$. Primer sequences for Dkk3, β -catenin and GAPDH are listed in Table 1.

Table 1 Oligonucleotide primers used in the study

	Sequence (5' to 3')	T _A (°C)	Cycles
PCR			
Dkk3 (full-length)	Forward: CCCAAGCTTATGCAGCGGCTTGGGGC Reverse: CGCGGATCCCTAAATCTCTCCCTCCCAGCAGT	53	35
Real-time RT-PCR			
Dkk3	Forward: ACAGCCACAGCCTGGTGTA Reverse: CCTCCATGAAGCTGCCAAC	60	40
β-catenin	Forward: AAAATGGCAGTGCCTTTAG Reverse: TTGAAGGCAGTCTGTCTGTA	60	40
GAPDH	Forward: GCACCGTCAAGGCTGAGAAC Reverse: GCCTTCTCCATGGTGGTGAA	60	40
RT-PCR			
Dkk3	Forward: AAGGCAGAAGGAGCCACGAGTGC Reverse: GGCCATTTTGGTGCAGTGACCCCA	61	35
β-actin	Forward: AAATCGTGCCTGACATTAA Reverse: CTCGTCTACTCTCTGCTTG	52	35
MSP			
Dkk3 unmethylated	Forward: TTAGGGGTGGTGGTGGGGT ^[32] Reverse: CTACATCTCCACTTACACCCA ^[32]	59	34
Dkk3 methylated	Forward: GGGCGGGCGGGCGGGG ^[32] Reverse: ACATCTCCGCTTACGCCCG ^[32]	59	34

T_A: Annealing temperature; Real-time RT-PCR: Real-time reverse transcription polymerase chain reaction; Dkk3: Dickkopf3; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MSP: Methylation-specific polymerase chain reaction.

Bisulfite modification and methylation-specific polymerase chain reaction

Genomic DNA was isolated from pancreatic cancer cell lines using the TIANamp Genomic DNA kit (Tiangen Biotech Co., LTD, Beijing, China). One microgram of genomic DNA was bisulfite-modified using the Cp-Genome™ DNA Modification Kit (Chemicon, MA, United States) according to the manufacturer's protocol. Methylation-specific polymerase chain reaction (MSP) was performed at 95 °C for 5 min, followed by 34 cycles at 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s. The final extension was at 72 °C for 10 min. Each PCR reaction was performed using 0.5 units of HotStarTaq Plus DNA Polymerase (Qiagen GmbH, Hilden, Germany). The primers are listed in Table 1. The specificity of the MSP primers in detecting the Dkk3 methylation status was demonstrated using unmethylated and methylated DNA as a template (EpiTect Control DNA Set; Qiagen GmbH, Hilden, Germany).

5-aza-dC treatment

Cells were seeded at a density of 4×10^4 cells/well in a six-well plate. After overnight incubation, the cells were treated with 10 μmol/L and 20 μmol/L of the DNA demethylating agent 5-aza-dC (Sigma-Aldrich, Steinheim, Germany) for 48 h or 72 h. Control cells were incubated with dimethyl sulfoxide and fresh medium.

Immunofluorescence and confocal microscopy

Cells grown on coverslips were washed and fixed with 4% paraformaldehyde, followed by washing with 0.2% Triton X-100. Coverslips were incubated with nonimmune animal serum to reduce nonspecific binding. The coverslips

were subsequently incubated at 4 °C overnight with an anti-Dkk3 rabbit polyclonal antibody (1:100, Santa Cruz, CA, United States). Rhodamine-conjugated AffiniPure goat anti-rabbit IgG was used as the secondary antibody (1:200, Zhongshan Goldenbridge Biotechnology Co., LTD, Beijing, China). Counterstaining was performed using 1 μg/mL 4',6-diamidino-2-phenylindole. Expression and localization of Dkk3 were observed under a confocal microscope (Leica, Mannheim, Germany).

Western blotting

The cells in culture were washed twice with ice-cold PBS, and proteins were extracted with M-PER mammalian protein extraction reagent (Pierce Biotechnology, Rockford, United States). Samples were centrifuged at $14000 \times g$ for 10 min. Aliquots of cell lysates containing 40 μg protein were separated on a 12% SDS-polyacrylamide gel and transferred to PVDF membranes (Millipore, MA, United States). The membranes were blocked with 10% skim milk and incubated with Dkk3 antibody (1:1500, Santa Cruz, CA, United States), β-catenin antibody (1:1500, BD Transduction Laboratories, San Diego, United States), phosphorylated extracellular signal-regulated protein kinase antibody (pERK antibody, 1:2000, Cell Signaling, MA, United States), extracellular signal-regulated protein kinase antibody (ERK antibody, 1:2000, Cell Signaling, MA, United States) and β-actin antibody (1:2000, Santa Cruz, CA, United States) at 4 °C overnight, followed by their corresponding secondary antibodies (1:2000, Zhongshan Goldenbridge Biotechnology Co., LTD, Beijing, China) at room temperature for 2 h. The membrane-bound proteins were detected using the Pierce ECL Western blotting substrate (Pierce Biotechnology, Rockford, United States).

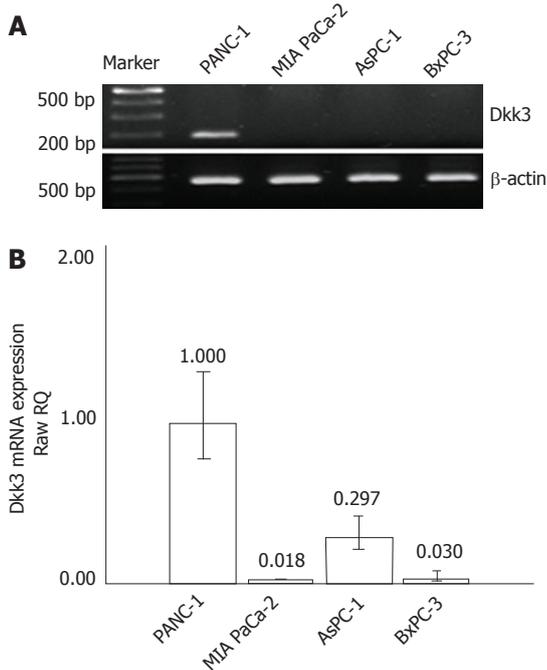


Figure 1 Dickkopf3 expression in human pancreatic cancer cell lines (PANC-1, MIA PaCa-2, AsPC-1 and BxPC-3). A, B: Dickkopf3 (Dkk3) mRNA expression was detected by reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR. Dkk3 mRNA expression was low in all cell lines examined. Dkk3: Dickkopf3; RQ: Relative quantitation.

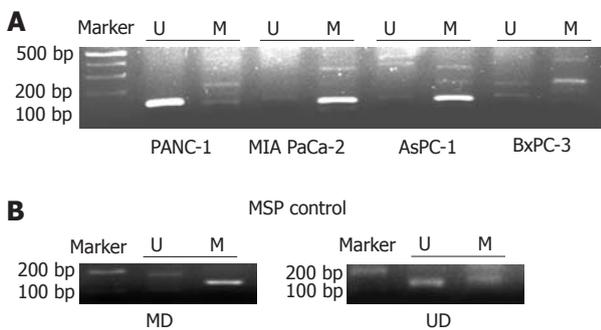


Figure 2 Dickkopf3 promoter methylation analysis in human pancreatic cancer cell lines. A: Methylation-specific PCR (MSP) was performed with bisulfite-treated DNA from pancreatic cancer cells. The Dickkopf3 (Dkk3) promoter was significantly methylated in MIA PaCa-2 and AsPC-1 cells; B: MSP controls demonstrate the specificity of the Dkk3 primers used. Methylated bisulfite-converted DNA exclusively yields amplification products with primers specific to methylated Dkk3 promoter sequences; unmethylated bisulfite-converted DNA yields exclusively amplification products with primers recognizing unmethylated Dkk3 promoter sequences. MD: Methylated bisulfite-converted DNA; UD: Unmethylated bisulfite-converted DNA; U: PCR products amplified with primers recognizing unmethylated Dkk3 promoter sequences; M: Amplification generated with methylation-specific primers.

Determination of dose-response curve

For determination of the dose-response curve, MIA PaCa-2 cells were transfected with pcDNA3.1-Dkk3 or pcDNA3.1. Six hours after transfection, cells were seeded in 96-cell plates in triplicate at a density of 3000 cells/well and were allowed to adhere. Gemcitabine (LILLY, France) was added to the medium 24 h after transfection.

Cell proliferation was determined 72 h after gemcitabine addition using the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (MTS, Promega, WI, United States), according to the manufacturer's protocol. The spectrophotometric absorbance of each sample was measured at 490 nm using the TECAN spectra (Thermo, Austria). Percent proliferation relative to the controls was calculated based on the MTS read-out; the IC₅₀ value was defined as the concentration of drug that produced a 50% reduction in absorbance relative to the control.

Cell growth assay

For the cell growth assay, MIA PaCa-2 cells were transfected with pcDNA3.1-Dkk3 or pcDNA3.1. At 6 h after transfection, cells were seeded in 96-well plates in triplicate at a density of 1000 cells/well and were allowed to adhere overnight. At 24 h, 48 h and 72 h, cell proliferation was determined using MTS (Promega, WI, United States) according to the manufacturer's protocol. The spectrophotometric absorbance of each sample was measured at 490 nm using the TECAN spectra (Thermo, Austria).

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. Unless otherwise indicated, the level of significance for differences between data sets was assessed using *t* test and one-way analysis of variance. Data are expressed as the mean \pm SD. *P* < 0.05 was considered statistically significant.

RESULTS

Dkk3 is downregulated in pancreatic cancer cell lines

Dkk3 expression was assessed in four human pancreatic cancer cell lines (PANC-1, MIA PaCa-2, AsPC-1, BxPC-3). A low level of Dkk3 mRNA was observed in all cell lines, although Dkk3 expression in PANC-1 cells was slightly higher than in the other three cell lines (Figure 1). Dkk3 protein expression was too low to detect by Western blotting or immunofluorescence (data not shown).

Methylation of the Dkk3 promoter in pancreatic cancer cell lines

Through the use of MSP, we found that the Dkk3 promoter sequence was significantly methylated in MIA PaCa-2 and AsPC-1 cells, which were the cell lines with reduced Dkk3 expression. Conversely, the Dkk3 promoter sequence was unmethylated in the PANC-1 cells, which had slightly higher Dkk3 expression (Figure 2).

Demethylation of the Dkk3 promoter

Because methylation of the Dkk3 promoter sequence was detected in MIA PaCa-2 and AsPC-1 cells, we chose to treat these two cell lines with 10 μ mol/L and 20 μ mol/L, respectively, of the DNA methyltransferase inhibitor 5-aza-dC. After treatment with 5-aza-dC, for 48 h or 72 h, the cells were harvested to determine Dkk3 mRNA expression by real-time reverse transcription PCR. The results showed that these two cell lines with methylated Dkk3 promoters showed increased Dkk3 mRNA expression,

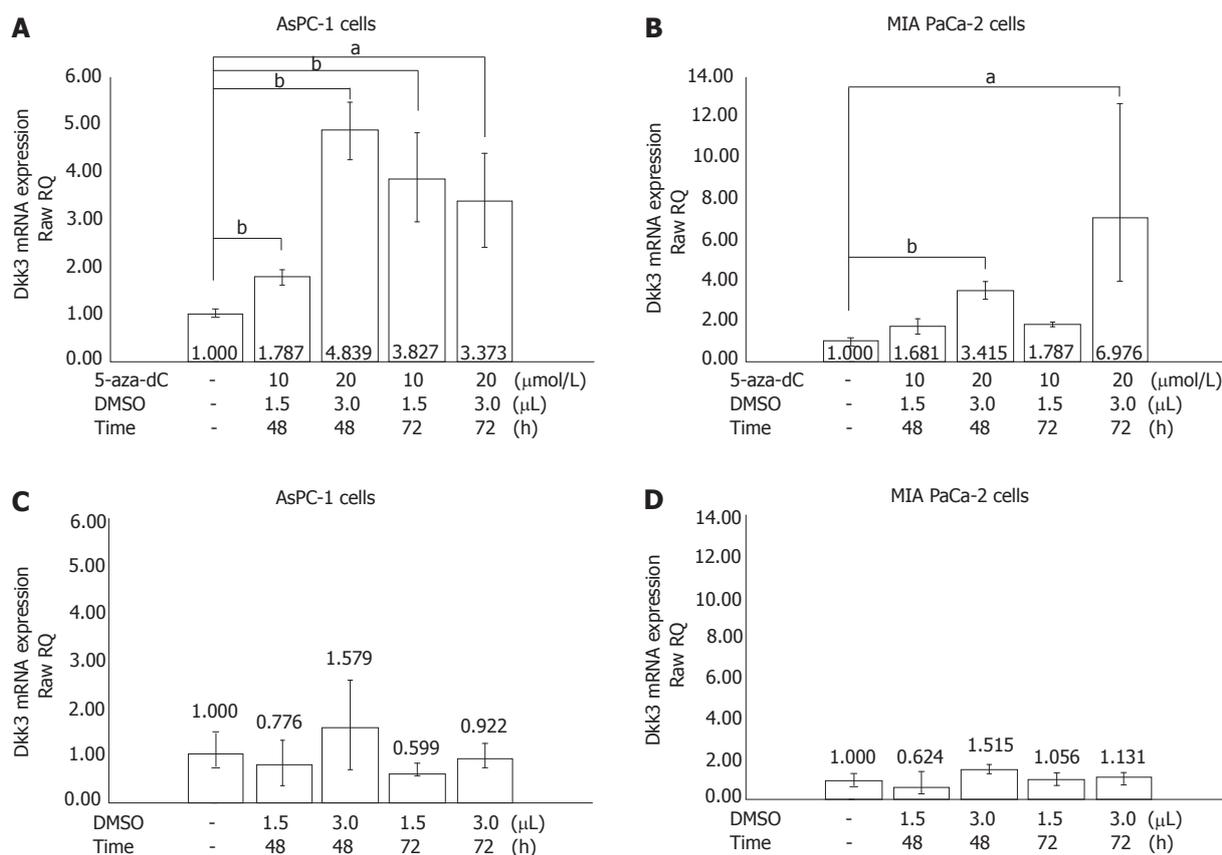


Figure 3 Dickkopf3 mRNA expression after demethylation *in vitro*. A, B: AsPC-1 and MIA PaCa-2 cells were treated with 10 μmol/L and 20 μmol/L of the DNA demethylating agent, 5-aza-dC, for 48 h or 72 h, respectively. The results show that these two cell lines, in which the Dickkopf3 (Dkk3) promoter was initially heavily methylated, had increased Dkk3 mRNA expression that was dependent on the dosage and timing of 5-aza-dC treatment (^a*P* < 0.05 vs untreated MIA PaCa-2 cells or untreated AsPC-1 cells, ^b*P* < 0.01 vs untreated MIA PaCa-2 cells or untreated AsPC-1 cells); C, D: Control cells were incubated with dimethyl sulfoxide and fresh medium. RQ: Relative quantitation; Dkk3: Dickkopf3; DMSO: Dimethyl sulfoxide.

which was dependent on the dosage and timing of 5-aza-dC treatment (*P* < 0.05 or *P* < 0.01) (Figure 3).

Overexpression of Dkk3 suppresses pancreatic cancer cell growth and β-catenin expression

To study the roles of Dkk3 in the progression of pancreatic cancer, MIA PaCa-2 cells were transfected with pcDNA3.1-Dkk3 or pcDNA3.1. After transfection, Dkk3 mRNA and protein levels significantly increased in the pcDNA3.1-Dkk3-transfected cells (*P* < 0.01), while no significant changes were observed in the pcDNA3.1-transfected cells (Figure 4A and B). At 48 h and 72 h after transfection, the β-catenin mRNA and protein expression levels were significantly decreased in the pcDNA3.1-Dkk3-transfected cells (*P* < 0.01) (Figure 4B and C). The protein expression of pERK was also decreased, but there was no significant change in total ERK expression (Figure 4B). The results of the MTS assay showed that in the pcDNA3.1-Dkk3-transfected cells, proliferation capacity was lower than in the pcDNA3.1-transfected cells (*P* < 0.01) (Figure 4D).

Sensitivity of Dkk3-overexpressing pancreatic cancer cells to gemcitabine

A dose-response curve was constructed, and the IC₅₀ val-

ues were compared to determine the influence of Dkk3 overexpression in pancreatic cancer cells on the effect of gemcitabine on cell growth. MIA PaCa-2 cells were transfected with pcDNA3.1-Dkk3 or pcDNA3.1. Seventy-two hours after gemcitabine addition, the IC₅₀ values for gemcitabine were 0.621 μmol/L for pcDNA3.1-Dkk3-transfected cells and 1.877 μmol/L for pcDNA3.1-transfected cells (Figure 4E). These results show that the IC₅₀ value of the Dkk3-overexpressing cells was significantly lower than that of the control cells.

DISCUSSION

Dkk3 is expressed in many normal human tissues^[35]. It was previously reported that Dkk3 expression is generally low in some tumors, such as sporadic epithelial ovarian cancer, cervical cancer, mammary tumors, malignant melanoma, hepatoma and kidney, pancreas, gastric and lung cancers^[12,15,16,18,19,31,32]. Additional studies also revealed the association between Dkk3 expression and cancer metastasis or prognosis in gastric cancer, renal cancer and head and neck squamous cell carcinoma^[36-38]. However, Dkk3 expression and its roles in pancreatic cancer remain unknown. In this study, we detected Dkk3 expression in human pancreatic cancer cells. We found that both Dkk3

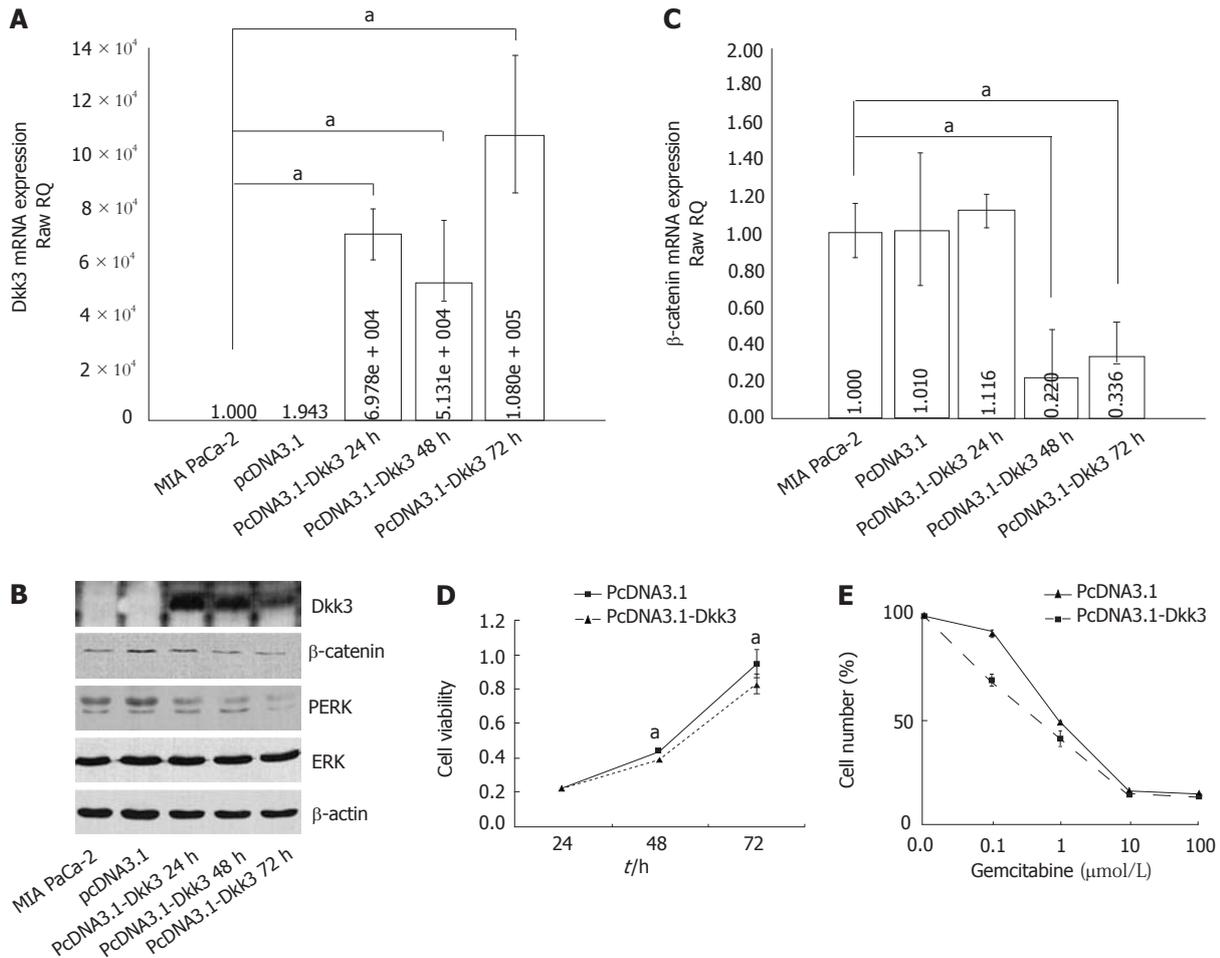


Figure 4 The effects of dickkopf3 overexpression on pancreatic cancer cells. MIA PaCa-2 cells were transfected with pcDNA3.1-dickkopf3 (Dkk3) or pcDNA3.1 vector. A, B: After transfection, Dkk3 mRNA and protein expression were examined by real-time reverse transcription polymerase chain reaction (real-time RT-PCR) and Western blotting. The results show that in the pcDNA3.1-Dkk3-transfected MIA PaCa-2 cells, Dkk3 expression was significantly upregulated ($P < 0.01$, $^aP < 0.01$ vs MIA PaCa-2 cells). B, C: β -catenin expression was examined by real-time RT-PCR and western blotting. β -catenin expression was downregulated 48 h and 72 h after transfecting pcDNA3.1-Dkk3 into MIA PaCa-2 cells ($P < 0.01$, $^aP < 0.01$ vs MIA PaCa-2 cells). B: The expression of extracellular signal-regulated protein kinases (ERK) and phosphorylated extracellular signal-regulated protein kinases (pERK) was examined by western blotting. The expression of pERK was simultaneously downregulated, without a significant change in total ERK expression. D: MTS assay results showed that the proliferative ability of pcDNA3.1-Dkk3-transfected cells was lower than that of pcDNA3.1-transfected cells ($P < 0.01$, $^aP < 0.01$). E: Dose-response analysis of pcDNA3.1- or pcDNA3.1-Dkk3-transfected MIA PaCa-2 cells with gemcitabine treatment. Seventy-two hours after gemcitabine addition, the IC50 values for gemcitabine were 0.621 $\mu\text{mol/L}$ for pcDNA3.1-Dkk3-transfected cells and 1.877 $\mu\text{mol/L}$ for pcDNA3.1-transfected cells. PERK: Phosphorylated extracellular signal-regulated protein kinases; ERK: Extracellular signal-regulated protein kinases; RQ: Relative quantitation; Dkk3: Dickkopf3; DMSO: Dimethyl sulfoxide.

protein and mRNA expression levels were low in all cell lines examined. Our results are partly in agreement with those of Takahashi N *et al*^[39].

Methylation of the Dkk3 promoter has been observed in hepatocellular carcinoma, breast cancer, malignant astrocytic glioma, acute myeloid and lymphoblastic leukemia and gastrointestinal and bladder cancers^[27-30,40-44]. Our MSP results showed that the Dkk3 promoter was methylated in MIA PaCa-2 and AsPC-1 cells, in which Dkk3 expression was low. After treatment with the DNA methyltransferase inhibitor 5-aza-dC, MIA PaCa-2 and AsPC-1 cells, which initially bore heavily methylated Dkk3 promoters, showed increased Dkk3 mRNA expression. In the present study, we demonstrated for the first time that decreased Dkk3 gene expression was associated with promoter methylation in two human pancreatic cancer cell lines (MIA PaCa-2 and AsPC-1). The inhibition

of DNA methyltransferase activity by 5-aza-dC led to a reversion of methylation and upregulated expression of the previously downregulated gene.

Additional studies have recently demonstrated that Dkk3 has distinct roles in regulating the malignant behavior of cancer cells, depending on which cells are examined. For example, Dkk3 can reduce malignancy in mouse prostate cancer RM9 cells *in vitro* and *in vivo*^[25]. Dkk3 can induce apoptosis or cell death in human bladder cancer, prostate cancer, breast cancer and lung cancer cells^[9,20,24,45]. Dkk3 can inhibit tumor growth and metastasis in an orthotopic prostate cancer model^[10]. While Jung *et al*^[33] found that Dkk3 acts as an antiapoptotic molecule in lung adenocarcinoma, our results show that Dkk3 overexpression inhibited pancreatic cancer cell growth. The results revealed that in the pcDNA3.1-Dkk3-transfected MIA PaCa-2 cells, β -catenin mRNA and protein expres-

sion levels were both downregulated. Phosphorylation of ERK was decreased. These data demonstrate that Dkk3 suppressed MIA PaCa-2 cell growth by inhibiting β -catenin expression. Our results were consistent with the findings of Yue *et al.*^[45] in lung cancer. We hypothesize that Dkk3 acts as a Wnt signal transduction inhibitor in human pancreatic cancer cells.

Gemcitabine is the most commonly used chemotherapy drug for pancreatic cancer. Notably, our results show that gemcitabine's IC₅₀ value for pcDNA3.1-Dkk3-transfected cells was significantly lower than that for the control cells. Dkk3 overexpression enhanced the sensitivity of pancreatic cancer cells to gemcitabine.

In summary, our results suggest that Dkk3 acts as a tumor suppressor in human pancreatic cancer cells by downregulating β -catenin expression *via* the ERK-mediated pathway. Dkk3 may be a valid adjunctive target of gemcitabine for the treatment of human pancreatic cancer.

COMMENTS

Background

Pancreatic cancer is the sixth leading cause of cancer death in China. The overall five-year survival rate is approximately 1%-3%. Pancreatic cancer remains one of the most aggressive human cancers. Recognizing the factors associated with pancreatic cancer progression is critical for its treatment.

Research frontiers

Dickkopf family proteins are secreted modulators of the canonical Wnt signaling pathway. Dickkopf 3 (Dkk3) is a member of the dickkopf family proteins. Dkk3 is downregulated in some tumors, and its overexpression inhibits tumor growth. The Dkk3 promoter sequence is methylated in several tumors. However, in lung adenocarcinomas, Dkk3 functions as an oncogene. Recently, other studies have demonstrated that Dkk3 plays distinct roles in different cells.

Innovations and breakthroughs

To date, no study has investigated Dkk3 expression and its roles in human pancreatic cancer cell behavior. In this study, the authors investigated Dkk3 expression and promoter sequence methylation in human pancreatic cancer cells. The effects of Dkk3 on cell proliferation and sensitivity to gemcitabine were simultaneously observed after expression was increased in MIA PaCa-2 cells, following transfection with the Dkk3 expression plasmid. According to the experimental results, the authors for the first time, confirmed that Dkk3 acts as a tumor suppressor in human pancreatic cancer cells by downregulating β -catenin expression *via* the ERK-mediated pathway. Dkk3 overexpression enhanced the sensitivity of pancreatic cancer cells to gemcitabine.

Applications

This study indicates that Dkk3 may be a valid adjunctive target of gemcitabine for the treatment of human pancreatic cancer.

Peer review

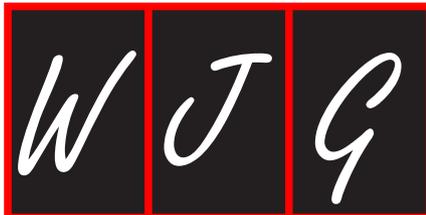
This is a paper that reports that Dkk3 is a tumor suppressor gene in pancreatic cancer. The findings are interesting, and overall writing is good.

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S- Editor Tian L L- Editor Webster JR E- Editor Xiong L



Balanced propofol sedation administered by nonanesthesiologists: The first Italian experience

Alessandro Repici, Nico Pagano, Cesare Hassan, Alessandra Carlino, Giacomo Rando, Giuseppe Strangio, Fabio Romeo, Angelo Zullo, Elisa Ferrara, Eva Vitetta, Daniel de Paula Pessoa Ferreira, Silvio Danese, Massimo Arosio, Alberto Malesci

Alessandro Repici, Nico Pagano, Cesare Hassan, Alessandra Carlino, Giacomo Rando, Giuseppe Strangio, Fabio Romeo, Angelo Zullo, Elisa Ferrara, Eva Vitetta, Daniel de Paula Pessoa Ferreira, Silvio Danese, Alberto Malesci, Department of Gastroenterology, IRCCS Istituto Clinico Humanitas, Via Manzoni 56, 20089 Rozzano, Milan, Italy

Massimo Arosio, Department of Anaesthesiology, IRCCS Istituto Clinico Humanitas, Via Manzoni 56, 20089 Rozzano, Milan, Italy

Author contributions: Repici A, Pagano N and Hassan C designed research; all the authors performed research; Repici A, Pagano N and Hassan C analyzed data; Repici A, Pagano N and Hassan C wrote the paper.

Correspondence to: Alessandro Repici, MD, Digestive Endoscopy Unit, IRCCS Istituto Clinico Humanitas, Via Manzoni 56, 20089 Rozzano, Milano, Italy. alessandro.repici@humanitas.it

Telephone: +39-02-82242579 Fax: +39-02-82244590
Received: October 10, 2010 Revised: October 29, 2010

Accepted: November 5, 2010

Published online: September 7, 2011

Abstract

AIM: To assess the efficacy and safety of a balanced approach using midazolam in combination with propofol, administered by non-anesthesiologists, in a large series of diagnostic colonoscopies.

METHODS: Consecutive patients undergoing diagnostic colonoscopy were sedated with a single dose of midazolam (0.05 mg/kg) and low-dose propofol (starter bolus of 0.5 mg/kg and repeated boluses of 10 to 20 mg). Induction time and deepest level of sedation, adverse and serious adverse events, as well as recovery times, were prospectively assessed. Cecal intubation and adenoma detection rates were also collected.

RESULTS: Overall, 1593 eligible patients were included.

The median dose of propofol administered was 70 mg (range: 40-120 mg), and the median dose of midazolam was 2.3 mg (range: 2-4 mg). Median induction time of sedation was 3 min (range: 1-4 min), and median recovery time was 23 min (range: 10-40 min). A moderate level of sedation was achieved in 1561 (98%) patients, whilst a deep sedation occurred in 32 (2%) cases. Transient oxygen desaturation requiring further oxygen supplementation occurred in 8 (0.46%; 95% CI: 0.2%-0.8%) patients. No serious adverse event was observed. Cecal intubation and adenoma detection rates were 93.5% and 23.4% (27.8% for male and 18.5% for female, subjects), respectively.

CONCLUSION: A balanced sedation protocol provided a minimalization of the dose of propofol needed to target a moderate sedation for colonoscopy, resulting in a high safety profile for non-anesthesiologist propofol sedation.

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Key words: Colonoscopy; Propofol; Sedation

Peer reviewers: Luis Bujanda, PhD, Professor, Department of Gastroenterology, CIBEREHD, University of Country Basque, Donostia Hospital, Paseo Dr. Beguiristain s/n, 20014 San Sebastián, Spain; Zvi Fireman, MD, Associate Professor of Medicine, Head, Gastroenterology Department, Hillel Yaffe Med Ctr, POB 169, 38100 Hadera, Israel

Repici A, Pagano N, Hassan C, Carlino A, Rando G, Strangio G, Romeo F, Zullo A, Ferrara E, Vitetta E, Ferreira DPP, Danese S, Arosio M, Malesci A. **Balanced propofol sedation administered by nonanesthesiologists: The first Italian experience.** *World J Gastroenterol* 2011; 17(33): 3818-3823 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i33/3818.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3818>

INTRODUCTION

Colorectal cancer (CRC) represents a major cause of morbidity and mortality in western countries^[1]. Despite the fact that it has been shown to be highly effective in preventing CRC incidence, colonoscopy is usually perceived as an invasive and potentially painful procedure, resulting in a low uptake rate when compared with less invasive options, such as fecal tests or flexible sigmoidoscopy^[2-4].

To improve acceptability and tolerability of colonoscopy, different protocols of sedation have been adopted^[5]. Such regimens have been mainly restricted to benzodiazepines alone or in combination with opioids, because of the relatively high safety of these substances. Although these drugs result in a substantial improvement of patients' and endoscopists's experiences, some drawbacks have been observed. In particular, due to a relatively long half-life, a slow induction of sedation and a delayed discharging time with significant cost of monitoring have been reported^[6]. Moreover, a significant proportion of patients are quite dissatisfied by the sub-optimal degree of sedation provided by this protocol, and morbidity and mortality as a result of respiratory depression have also been reported^[7,8].

Propofol represents a short-acting sedative agonist of gamma-aminobutyric acid receptor in the central nervous system, and it is mainly used for the induction and maintenance of deep sedation during surgical procedures^[9]. Because of its short half-life (2-4 min) and high lipid solubility, propofol has the distinct advantages of a rapid induction of sedation and a fast recovery. When applied to gastrointestinal (GI) endoscopy, patient satisfaction with propofol has been shown to be equivalent or superior to that of benzodiazepines and/or narcotics^[7]. Propofol, however, is a respiratory depressant with a narrow therapeutic range and without a reversal agent, resulting in a significant risk of inducing a too deep level of sedation, complicated by hypoventilation, apnea or cardiovascular depression. Moreover, it lacks a reversal agent. For this reason, propofol is largely administered by anesthesiologists or anesthesiologist nurses^[10]. When considering the very high number of colonoscopies performed worldwide - 14 million every year in the United States alone^[11] - anesthesiologist capacity is, however, likely to be insufficient to assure propofol sedation for this procedure.

It has been recently shown that propofol may be an effective and safe agent when used by non-anesthesiologists to target an adequate level of sedation^[12]. A recent systematic review of the literature, including 646 080 cases, provided adequate evidence to the American Gastroenterological Association for them to support propofol administration by non-anesthesiologists (NAP), because of the extraordinary rarity of life-threatening episodes^[13]. Most of these series were based on the use of propofol alone, during which NAP targeted a deep level of sedation. To further improve the safety profile, it has been suggested that a substantial reduction of the

propofol dose may be achieved by administering this drug in association with other sedative agents, such as midazolam or meperidine^[14,15]. This protocol has been defined as balanced propofol sedation (BPS), and, differently from NAP, it targets a moderate level of sedation.

No study has addressed the use of propofol by non-anesthesiologists for colonoscopy in Italy, and very few in Europe^[13]. This is largely related to the product label of the drug which allows its administration only by physicians trained in general anesthesia. Due to the lack of an adequate anesthesiologist capacity and the low fee of reimbursement for a colonoscopy in the public system, virtually all the procedures are performed without propofol.

Only a few colonoscopy series have addressed the efficacy and safety of BPS for colonoscopy, most of them including only a few hundred of patients^[14,15,17-23]. The purpose of this study was to prospectively assess the safety and the efficacy of endoscopist-administered BPS to target a moderate level of sedation for colonoscopy in a large series of consecutive patients.

MATERIALS AND METHODS

From February 2008 to December 2009, outpatients who presented to our unit for diagnostic colonoscopy were eligible for the study if they were between 18 and 75 years of age, American Society of Anesthesiology (ASA) class I or II, and capable of providing written informed consent for study participation. Exclusion criteria were inability to provide informed consent, history of allergic reactions or hypersensitivities to midazolam, propofol, eggs, or soybeans, high-risk head and neck anatomy (Mallampati score > 2) that could complicate airway rescue, sleep apnea syndrome, ASA class > II.

The use of propofol by non-anesthesiologists in Italy is, at the time being, prevented by the specification in the product label that the use of this drug is exclusively allowed for anesthesiologists or intensive care unit physicians. For this reason, the administration of propofol within the present study has been performed under a study protocol that was supported by our Institution (Istituto Clinico Humanitas) and approved by the institutional review board. Nine endoscopists participated in this protocol, being authorized to administer propofol.

Patients underwent BPS administered by an endoscopist who was not involved in the endoscopic procedure. The physicians administering sedation were certified in advanced cardiac life support and had also successfully completed an intensively structured training program in propofol administration and laryngeal mask use under an anesthesiologist tutorship. The same anesthesiologist was always on call during the procedure time. Baseline vital signs (heart rate, blood pressure, oxygen saturation) were obtained in all patients before induction of sedation. Endoscopy-dedicated nurses also attended the procedure.

BPS was structured as follows: after a single dose of midazolam (0.05 mg/kg; Hameln pharmaceuticals gmbh, Hameln, Germany), a starter bolus of 0.5 mg/kg

Table 1 Scale for assessing Alertness/Sedation

Responsiveness	Score
Responds readily to name spoken in normal tone	5
Lethargic response to name spoken in normal tone	4
Responds only after name is called loudly and/or repeatedly	3
Responds only after mild prodding or shaking	2
Responds only after painful trapezius squeeze	1
Does not respond to painful trapezius squeeze	0

of propofol (Diprivan, Astra-Zeneca, Stockholm, Sweden) was administered. Repeated boluses of 10 to 20 mg of propofol were then administered on-demand with a 30-60 s interval for the entire duration of the procedure. Propofol bolus frequency and dose were titrated to the patient response, including vital signs and manifestations of restlessness or discomfort. The maximum dose allowed to be administered was 200 mg. Throughout the procedure, all patients received oxygen 2 L/min by nasal cannula. Continuous pulse oximetry, heart rate, electrocardiography, and end-expiratory carbon dioxide were monitored, with blood pressure being assessed at 5-min intervals. Level of sedation was evaluated according to the Scale for assessing Alertness/Sedation (MOAA/S), as reported in Table 1. In detail, deep sedation was defined as MOAA/S 1, moderate as MOAA/S 2-4, and minimal as MOAA/S 5. The following parameters were recorded: patient demographics, procedure indication and duration, midazolam dose, propofol dose, induction time, recovery time, cecal intubation rate, and polyp detection rate. The baseline values and changes in vital signs or oxygen saturation (SpO₂) from the baseline were also recorded. Adverse events were defined as hypoxia (i.e., a reduction in oxygen saturation < 90% for more than 20 s) requiring supplemental oxygen (O₂) by nasal cannula (NC) in excess of 2 L/min; and transient hypotension (< 90 mmHg) or bradycardia (< 60 beats/min) not requiring any active medical treatment. Serious adverse events were defined as hypoxia requiring positive pressure ventilation or laryngeal mask use; hypotension (< 90 mmHg) or bradycardia (< 60 beats/min) requiring medical treatment (i.e., infusion of liquid) other than propofol titration; and any event requiring the administration of a benzodiazepine antagonist (flumazenil). After the procedure, the patients were transported to the recovery room where blood pressure, SpO₂ and heart rate were measured continuously until discharge. Discharge was possible when blood pressure was within 20% of the initial value, SpO₂ > 90%, and the patient was able to drink and walk autonomously. Recovery time was measured from the time the patient entered the recovery area until departure by the recovery room nurse.

RESULTS

During the study period, 1593 eligible patients were in-

cluded. Of these, 789 (49%) were male, the median age being 60 years (range: 22-75 years). Clinical indication for colonoscopy was evaluation of symptoms in 876 (55%) cases, screening or surveillance of a previous neoplastic lesion in 542 (34%), work-up of a positive fecal test in 96 (6%), and follow up of inflammatory bowel diseases in the remaining 79 (5%) cases.

Baseline mean heart rate and mean blood pressure were 71 ± 13 beats per min and 103 ± 16 mmHg, respectively. BPS was administered to all the patients. The median dose of midazolam was 2.3 mg (range: 2-4 mg), and the median dose of propofol administered was 70 mg (range: 40-120 mg). The median induction time of sedation (i.e., between the initiation of sedation and colonoscope insertion) was 3 min (range: 1-4 min). The deepest level of sedation was moderate in 1561 (98%) patients and deep in the remaining 32 (2%) cases. General anesthesia was not observed in any patient.

There was no serious adverse event related to any of the 1593 patients. The only adverse events observed with BPS were episodes of transient oxygen desaturation requiring O₂ supplementation by NC in excess of 2 L/min in 8 (0.46%; 95% CI: 0.2%-0.8%) patients. No patient required mask ventilation or endotracheal intubation. Although a transient decrease in blood pressure was common (446 patients, 28%), no episodes of sustained hypotension or bradycardia requiring active therapy were observed. No patient required administration of a benzodiazepine antagonist. Median recovery time was 23 min (range: 10-40 min).

The overall cecal intubation rate was 93.5%, corresponding to 1491 complete colonoscopies. Incomplete procedures were due to poor bowel cleaning in 72 (4.5%) patients and sigmoid strictures in 30 (2%) cases. The median procedural time was 11.3 min (range: 9-22 min), consisting of a median intubation time of 4 min (range: 3-9 min) and a median withdrawal time of 6.3 min (range: 4.2-11.9 min). Adenoma detection rate was 23.4% (27.8% for male, and 18.5% for female subjects). No major procedure-related complication occurred.

DISCUSSION

Our study showed that a BSP protocol, based on the co-administration of propofol with benzodiazepine, was a feasible, effective and safe approach for colonoscopy in a large series of consecutive patients. In particular, following a careful and rigid selection of the patients, BSP was successfully administered by non-anesthesiologist endoscopists without requiring anesthesiologist intervention in any of the cases. No BSP-related serious adverse event occurred in the study population, as outlined by the evidence that a midazolam-reversal agent was not needed in any patient. A transient oxygen desaturation was observed in only 0.5% of the study population, and it was treated conservatively in all cases.

The high safety profile of the BSP observed in our study appears to be strictly related to the very low dose

of propofol needed to target a moderate sedation, because of the additional effect of midazolam. Despite the fact that this was a non-randomized study in which a propofol-alone arm was not included, the median dose of propofol shown in the present study, corresponding to 70 mg per patient, appeared to be much lower than the 200-400 mg range described in previous propofol-alone series^[16]. A similarly low propofol dose was also reported in previous series in which BSP was adopted^[14,15,17-23]. When considering the potential legal implications related to NAP, the ability to minimize the dose of propofol needed appears as an attractive goal for the endoscopists. The very low rate of oxygen desaturation observed in our study may also be related to the systematic adoption of capnography to monitor our patients. It has been suggested that capnography may anticipate the diagnosis of propofol-induced hypoventilation as compared to the simple assessment of oxygen saturation^[24].

It could be argued, however, that co-administration of midazolam could reduce the propofol-related advantages. In particular, the slow metabolism of benzodiazepines could result in a prolonged recovery time, reducing the efficiency of an endoscopic turnover system. The median recovery time in our series was consistently lower than 30 min. This value favorably compares with previous accounts of midazolam alone, in which a recovery time as long as 70 min was reported^[25]. Such a difference in favor of the BSP regimen is presumably due to the relatively low dose of midazolam administered, the median being 2.1 mg per patient. Moreover, midazolam was administered only at the beginning of the procedure as a bolus, so that the drug started to be metabolized during the procedure itself, lasting on average 11 min.

Quality of colonoscopy procedures in our series appeared to reach the required standards, showing no interference of BSP in the diagnostic or operative procedures. In particular, the adjusted cecal intubation rate of 93.5% in a mixed setting with symptomatic and screening indications is remarkably superior to the 80.7% recently reported in an Italian survey, in which the use of propofol was not reported^[26]. Of note, in a similarly designed Italian study, it was observed that the intubation rate in sedation-assisted colonoscopies was superior to that of those performed without sedation^[27].

It could be argued that the results of our study were not unexpected; the safety of BSP having already been shown in previous studies. However, most of these series included only 100-200 patients^[14,15,17-23], so that a greater confirmation of BSP safety in over 1500 subjects was needed. Moreover, this is the first Italian study in which NAP was applied to colonoscopy, and, more generally, to adults. This would appear to be of major importance when considering that the use of propofol in Italy is prevented by an unequivocal recommendation in the product label stating that only anesthesiologists are allowed to administer such a drug. The safety profile of BSP in our study should call for dedicated studies aiming to ascertain whether such a recommendation is really a protection for the patients and whether it is consistent

with literature data or simply represents an obstacle preventing a safe propofol-assisted colonoscopy to most patients. Indeed, in Italy, due to the lack of anesthesiologist capacity, virtually all the colonoscopies are performed without propofol, using at best benzodiazepines and/or narcotics^[25].

There are limitations to the present analysis. Our main target was to evaluate BSP efficacy in targeting a moderate level of sedation when administered by non-anesthesiologists, whilst we did not assess the level of satisfaction of patients or endoscopists with our sedation protocol. However, there is enough evidence regarding a higher satisfaction level with propofol as compared to midazolam^[16]. Moreover, the short induction time clearly reflects a propofol type of sedation rather than the effect of midazolam. Secondly, we did not compare the propofol/midazolam BSP with other protocols, such as propofol alone or propofol with narcotics with or without midazolam. However, most of the propofol-related toxicity is associated with its narrow therapeutic window, so that it is unlikely that such a high safety profile would be achieved by protocols based on doses of propofol substantially larger than those reached in our experience. Thirdly, we did not blind the discharging nurse regarding the type of sedation, so that we cannot exclude a bias in the computation of the recovery time. Fourthly, we did not assess the alertness level after several hours from discharge, so that we cannot exclude a prolonged effect of midazolam bolus in our series. Fifthly, although our study included over 1500 subjects, we cannot exclude extremely rare events that have been associated with the use of propofol, such as neurologic injuries or even death. However, the lack of severe episodes of respiratory or cardiovascular depression reassures us about the safety of BSP. Moreover, no death has been reported up to now with the use of NAP in colonoscopy; all the cases having been associated with upper GI endoscopy or biliary maneuvers^[13]. According to the study protocol, we systematically used a non-anesthesiologist physician for monitoring propofol administration. It could be argued that this represents a waste of resources, requiring two endoscopists to perform one procedure. However, this simply reflects a prudent choice within the study protocol to prevent eventual litigation for an off-label use of the drug. It has already been shown that appropriately trained nurses may assist the endoscopist in propofol administration and sedation monitoring with a clear saving of resources. Finally, we did not use specific scales of recovery after the completion of the colonoscopy, considering discharge possible on the basis of blood pressure, SpO₂, and the patients' ability to drink and walk autonomously.

In conclusion, we report a large consecutive series showing the efficacy and safety of BSP for colonoscopy, when administered by non-anesthesiologists. When considering the controversy regarding NAP use for GI endoscopy, the very low dose of propofol allowed by the co-administration of midazolam appears to be a rational approach to maximize sedation efficacy and to minimize

propofol toxicity at the same time.

ACKNOWLEDGMENTS

We are indebted to Dr. Larry Cohen for his valuable support in reviewing and commenting on the present manuscript.

COMMENTS

Background

Non-anesthesiologists propofol administration (NAP) represents an effective and safe alternative to sedation with benzodiazepines/narcotics for colonoscopy. NAP generally involves the administration of propofol alone to target a deep level of sedation. By associating propofol with other sedative agents, such as midazolam, a moderate level of sedation may be targeted, resulting in a substantial reduction of the propofol dose.

Research frontiers

Despite being validated in small controlled trials, such a balanced propofol sedation has never been tested in a large cohort.

Innovations and breakthroughs

In a large prospective study involving 1593 patients, a balanced propofol sedation consisting of the co-administration of propofol and midazolam resulted in a moderate level of sedation in 98% of colonoscopies. Recovery time also appeared to be favorably short. Such a balanced protocol of sedation appeared to be highly safe, the only serious event being a transient oxygen desaturation requiring further oxygen supplementation in less than 1% of the patients. The median dose of propofol administered was 70 mg, being less than 120 mg in the entire series. The overall cecal intubation and adenoma detection rates were 93.5% and 23.4%, respectively. No major procedure-related complication occurred.

Applications

A balanced administration of propofol by non-anesthesiologists may be safely implemented in dedicated centers.

Peer review

The paper assessed the efficacy and safety of a balanced approach using midazolam in combination with propofol administered by non-anesthesiologists in a large series of diagnostic colonoscopies. It is very interesting.

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S- Editor Tian L L- Editor Logan S E- Editor Li JY

Is it better to use two elastographic methods for liver fibrosis assessment?

Ioan Sporea, Roxana Șirli, Alina Popescu, Simona Bota, Radu Badea, Monica Lupșor, Mircea Focșă, Mirela Dănilă

Ioan Sporea, Roxana Șirli, Alina Popescu, Simona Bota, Mirela Dănilă, Department of Gastroenterology and Hepatology, University of Medicine and Pharmacy Timișoara, 10 Iosif Bulbuca Bv., 300736 Timișoara, Romania

Radu Badea, Monica Lupșor, Department of Medical Imaging, 3rd Medical Clinic, University of Medicine and Pharmacy, Cluj-Napoca, 21-23 Croitorilor str., 400162 Cluj-Napoca, Romania

Mircea Focșă, Department of Biophysics and Medical Informatics, University of Medicine and Pharmacy Timișoara, 14 Tudor Vladimirescu str., 300173 Timișoara, Romania

Author contributions: Sporea I wrote the paper, and designed and supervised the study; Șirli R, Popescu A, Bota S, Badea R, Lupșor M and Dănilă M performed research; Focșă M, Bota S and Șirli R analyzed the data; Șirli R revised the manuscript.

Correspondence to: Dr. Ioan Sporea, Professor, Department of Gastroenterology and Hepatology, University of Medicine and Pharmacy Timișoara, 10 Iosif Bulbuca Bv., 300736 Timișoara, Romania. isporea@umft.ro

Telephone: +40-256-309455 Fax: +40-256-488003

Received: December 4, 2010 Revised: March 25, 2011

Accepted: April 1, 2011

Published online: September 7, 2011

Abstract

AIM: To find out if by combining 2 ultrasound based elastographic methods: acoustic radiation force impulse (ARFI) elastography and transient elastography (TE), we can improve the prediction of fibrosis in patients with chronic hepatitis C.

METHODS: Our study included 197 patients with chronic hepatitis C. In each patient, we performed, in the same session, liver stiffness (LS) measurements by means of TE and ARFI, respectively, and liver biopsy (LB), assessed according to the Metavir score. 10 LS measurements were performed both by TE and ARFI; median values were calculated and expressed in kilopascals (kPa) and meters/second (m/s), respectively. Only TE and ARFI measurements with IQR < 30% and

SR \geq 60% were considered reliable.

RESULTS: On LB 13 (6.6%) patients had F0, 32 (16.2%) had F1, 52 (26.4%) had F2, 47 (23.9%) had F3, and 53 (26.9%) had F4. A direct, strong correlation was found between TE measurements and fibrosis ($r = 0.741$), between ARFI and fibrosis ($r = 0.730$) and also between TE and ARFI ($r = 0.675$). For predicting significant fibrosis ($F \geq 2$), for a cut-off of 6.7 kPa, TE had 77.5% sensitivity (Se) and 86.5% specificity (Sp) [area under the receiver operating characteristic curve (AUROC) 0.87] and for a cut-off of 1.2 m/s, ARFI had 76.9% Se and 86.7% Sp (AUROC 0.84). For predicting cirrhosis ($F = 4$), for a cut-off of 12.2 kPa, TE had 96.2% Se and 89.6% Sp (AUROC 0.97) and for a cut-off of 1.8 m/s, ARFI had 90.4% Se and 85.6% Sp (AUROC 0.91). When both elastographic methods were taken into consideration, for predicting significant fibrosis ($F \geq 2$), (TE \geq 6.7 kPa and ARFI \geq 1.2 m/s) we obtained 60.5% Se, 93.3% Sp, 96.8% positive predictive value (PPV), 41.4% negative predictive value (NPV) and 68% accuracy, while for predicting cirrhosis (TE \geq 12.2 kPa and ARFI \geq 1.8 m/s) we obtained 84.9% Se, 94.4% Sp, 84.9% PPV, 94.4% NPV and 91.8% accuracy.

CONCLUSION: TE used in combination with ARFI is highly specific for predicting significant fibrosis; therefore when the two methods are concordant, liver biopsy can be avoided.

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Key words: Transient elastography; Acoustic radiation force impulse elastography; Liver stiffness; Combined methods

Peer reviewer: Ned Snyder, MD, FACP, AGAF, Professor of Medicine, Chief of Clinical Gastroenterology and Hepatology, Department of Internal Medicine, The University of Texas Medical Branch, 301 University Blvd., Galveston, Texas

77555-0764, United States

Sporea I, Şirli R, Popescu A, Bota S, Badea R, Lupşor M, Focşa M, Dănilă M. Is it better to use two elastographic methods for liver fibrosis assessment? *World J Gastroenterol* 2011; 17(33): 3824-3829 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i33/3824.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3824>

INTRODUCTION

Liver fibrosis evaluation in patients with hepatitis C virus (HCV) infection is essential for prognosis assessment and also for a decision regarding therapy. In many centers, liver biopsy (LB) is the “normal” means of fibrosis assessment. In the last few years, non-invasive methods for the evaluation of liver fibrosis have become more and more popular, especially in France and, subsequently, throughout the world.

Non-invasive methods for liver fibrosis assessment are: biological (serological) tests^[1-5], ultrasound based (elastographic) methods, such as transient elastography (TE)^[6-9], real time elastography^[10-12] and acoustic radiation force impulse (ARFI) elastography^[13-15] and magnetic resonance imaging (MRI) elastography^[16,17]. Each method has certain advantages: only a few milliliters of blood are required for the serological tests, a special “ultrasound” examination is required for the elastographic methods and finally, a MRI examination reveals information about many abdominal organs and at the same time evaluates the liver stiffness (LS). All these methods have some disadvantages, the major one being that they are not 100% sensitive or 100% specific compared to the LB which is still considered the “gold standard”.

Some authors have proposed to combine different noninvasive methods for liver fibrosis evaluation, hoping to increase the accuracy or maybe to decrease the number of LBs needed to solve unclear cases^[18]. Some years ago, Castera^[18] proposed to use only ALT for the evaluation of liver activity in patients with chronic hepatitis C, and for fibrosis to combine a FibroTest with a FibroScan. If these noninvasive tests are concordant, then LB can be avoided. In this study, when the FibroScan and FibroTest results agreed, significant fibrosis ($F \geq 2$) was confirmed by LB in 84% of the cases, severe fibrosis ($F \geq 3$) in 95% of cases, and cirrhosis ($F = 4$) in 94% of the cases.

The purpose of this study is to find out if, by combining 2 ultrasound based elastographic methods: ARFI elastography and TE, we can improve the prediction of fibrosis severity in patients with chronic HCV hepatitis.

MATERIALS AND METHODS

Patients

We performed a bicentric study in two university hospitals (Timisoara and Cluj-Napoca) that included 197 patients with chronic HCV hepatitis (anti HCV antibodies positive, with or without cytology for at least 6 mo, PCR HCV RNA



Figure 1 Acoustic radiation force impulse measurement in the liver.

positive). In all these patients, in the same session, LS was evaluated by means of TE (FibroScan[®]) and ARFI elastography, and LB was performed in order to assess the fibrosis stage. Patients with other causes of chronic hepatitis (HBV infection, chronic alcohol abuse, cholestatic chronic hepatitis, nonalcoholic steatohepatitis, autoimmune chronic hepatitis, haemochromatosis, Wilson’s disease) were excluded from our study. Informed consent was obtained from each patient included in the study and the study protocol was approved by the local ethical committee.

TE

TE was performed in all patients with a FibroScan[®] device (EchoSens[®] - Paris, France) by experienced physicians (more than 500 TE), blinded to the results of LB and ARFI measurements. In each patient, 10 valid measurements were performed, after which a median value of LS was obtained, measured in kilopascals (kPa). Only patients in which LS measurements by means of TE had a success rate of at least 60%, with an interquartile range (IQR) < 30%, were included in our study. The success rate was calculated as the ratio of the number of successful acquisitions over the total number of acquisitions. IQR is the difference between the 75th percentile and the 25th percentile, essentially the range of the middle 50% of the data.

ARFI elastography

ARFI elastography was performed in all the patients with a Siemens Acuson S2000TM ultrasound system. The ultrasound probe automatically produces an acoustic “push” pulse that generates shear-waves which propagate into the liver. Their speed, measured in meters/second (m/s), is displayed on the screen. The propagation speed increases with fibrosis. The operator can select the depth at which the liver elasticity is evaluated by placing a “measuring box” (10 mm long and 5 mm wide) in the desired place (Figure 1). The patients were examined in left lateral decubitus, with the right arm in maximum abduction. Scanning was performed between the ribs in the right liver lobe in order to avoid cardiac motion (approximately in the place where we usually perform LB), 1 cm under the capsule, with minimal scanning pressure applied by the operator, while the patients were asked to stop breathing for a mo-

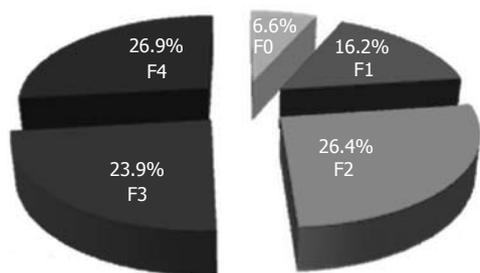


Figure 2 Severity of fibrosis in the studied group.

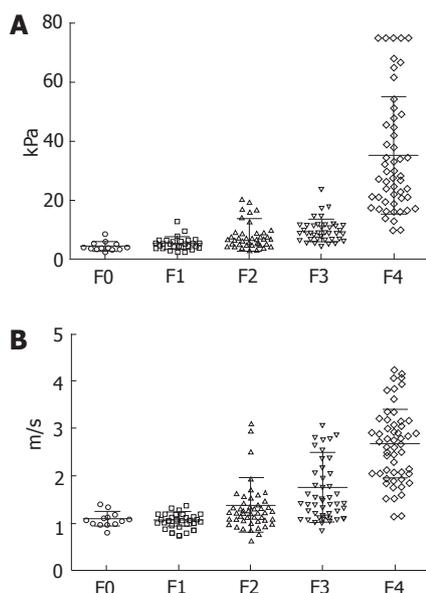


Figure 3 Liver stiffness measurements by means of transient elastography (A) and acoustic radiation force impulse elastography (B) for various stages of fibrosis.

ment, in order to minimize breathing motion.

We performed 10 measurements in every patient, and a median value was calculated, the result being measured in m/s. Only patients in which LS measurements by means of ARFI had a success rate of at least 60%, with an IQR < 30%, were included in our study. Operators were blinded to the results of LB and TE measurements.

LB

LB was performed in all the patients using echoguided TruCut technique, with a 1.8 mm (14 G) diameter automatic needle device-Biopty Gun (Bard GMBh), or echoassisted, using Menghini type modified needles, 1.4 and 1.6 mm in diameter. Only LB fragments including at least 6 portal tracts were considered adequate for pathological interpretation and included in our study. The LBs were assessed, according to the Metavir score, by a senior pathologist (one in each center) blinded to the results of TE and ARFI measurements. Fibrosis was staged on a 0-4 scale: F0-no fibrosis; F1-portal fibrosis without septa; F2-portal fibrosis and few septa extending into lobules;

F3-numerous septa extending to adjacent portal tracts or terminal hepatic venules and F4-cirrhosis.

Statistical analysis

The data we obtained from our patients were collected in a Microsoft Excel file, the statistical analysis being performed using MedCalc and GraphPad Prism programs. The predictors for the stage of fibrosis (ARFI and TE measurements) were numeric variables, so the mean and standard deviation were calculated.

Associations between assay results and fibrosis stage according to the Metavir scoring system (range: 0-4, ordinal scale), were described using the Spearman rank correlation coefficient (*r*).

The diagnostic performances of ARFI and TE were assessed by using receiver operating characteristics (ROC) curves. ROC curves were thus built for the detection of significant fibrosis ($F \geq 2$ Metavir) and cirrhosis ($F = 4$ Metavir). Optimal cut-off values were chosen to maximize the sum of sensitivity (Se) and specificity (Sp). Se and Sp were calculated according to standard methods. Exact confidence intervals of 95% were calculated for each predictive test.

RESULTS

Our study group included 197 patients, 119 women and 78 men, mean age 50 ± 9.8 years. On LB 13 (6.6%) patients had F0, 32 (16.2%) had F1, 52 (26.4%) had F2, 47 (23.9%) had F3 and 53 (26.9%) had F4 (Figure 2).

We obtained valid TE measurements in 187/197 patients (94.9%) and valid ARFI measurements in 191/197 patients (96.9%).

A direct, strong correlation was found between the values of liver stiffness evaluated by TE and fibrosis ($r = 0.741$) (Figure 3A), between the values of liver stiffness measured by ARFI and fibrosis ($r = 0.730$) (Figure 3B) and also between the values of liver stiffness evaluated by means both of TE and ARFI ($r = 0.675$).

The predictive values of TE and ARFI, alone, for $F \geq 2$ and F4, respectively, are presented in Table 1.

By combining the two elastographic methods (values both for TE and ARFI above the mentioned cut-offs) the specificity increased, statistically significant as compared to ARFI ($F \geq 2$: 93.3% vs 86.7%, $P = 0.04$; $F = 4$: 94.4% vs 85.6%, $P = 0.007$) but not as compared to TE ($F \geq 2$: 93.3% vs 86.7%, $P = 0.05$; $F = 4$: 94.4% vs 89.6%, $P = 0.12$), of course with lower sensitivity (Table 2), with very good positive predictive value (PPV) (96.3%) for significant fibrosis ($F \geq 2$ Metavir). By combining the two elastographic methods for F4, we obtained a very high negative predictive value (NPV), along with very good PPV and accuracy (Table 2).

The accuracy of the combined tests (TE + ARFI) was statistically significant better than ARFI alone for predicting cirrhosis (91.8% vs 83.4%, $P = 0.02$), but not as compared to TE alone (91.8% vs 91.4%, $P = 0.96$).

Table 1 Predictive value of transient elastography and acoustic radiation force impulse alone, for $F \geq 2$ and $F = 4$ (%)

	$F \geq 2$							$F = 4$						
	Cut-off	AUROC	Se	Sp	PPV	NPV	Accuracy	Cut-off	AUROC	Se	Sp	PPV	NPV	Accuracy
TE	6.7 kPa	0.87	77.5	86.7	94.8	54.9	79.6	12.2 kPa	0.97	96.2	89.6	78.1	98.3	91.4
ARFI	1.2 m/s	0.84	76.9	86.7	95.7	54.1	79.3	1.8 m/s	0.91	90.4	85.6	50.3	95.8	83.4

TE: Transient elastography; ARFI: Acoustic radiation force impulse; AUROC: Area under the receiver operating characteristic curve; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

Table 2 Predictive value of transient elastography and acoustic radiation force impulse in combination for $F \geq 2$ and $F = 4$ (%)

	$F \geq 2$							$F = 4$						
	Cut-off	Se	Sp	PPV	NPV	Accuracy	Cut-off	Se	Sp	PPV	NPV	Accuracy		
TE + ARFI	6.7 kPa and 1.2 m/s	60.5	93.3	96.8	41.1	68	12.2 kPa and 1.8 m/s	84.9	94.4	84.9	94.4	91.8		
TE or ARFI	6.7 kPa or 1.2 m/s	86.1	71.1	90.9	60.3	82.7	12.2 kPa or 1.8 m/s	96.2	83.3	68	98.3	86.8		

TE: Transient elastography; ARFI: Acoustic radiation force impulse; AUROC: Area under the receiver operating characteristic curve; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

DISCUSSION

Discussions regarding the replacement of LB in the evaluation of liver fibrosis with non-invasive methods are currently very active. Arguments in favor of maintaining LB are: it allows a correct evaluation of fibrosis as well as of the activity of the disease; it can provide arguments for the etiology (Mallory bodies, *etc*) or it can evaluate the presence and severity of hepatocytes' fatty infiltration. Arguments against this method are: it is an invasive one (there is a risk for complications, even if it is low); it is usually a stressful method for the patients; in some cases, good quality histological specimens are not obtained and, also, there are some questions regarding sampling variability.

In some countries, such as France, the non-invasive methods for fibrosis assessment have replaced the LB in a large number of cases. To become accepted worldwide, these non-invasive methods must be very accurate, in order to replace a well recognized method such as LB.

TE measures the liver stiffness of a fragment that is approximately a cylinder 1 cm in diameter and 4 cm long, 500 times bigger than the specimen obtained by LB. This examination is more or less blind, but the other method that we used for the elastographic evaluation of the liver, ARFI, is performed under clear ultrasonographic visualization of the area of interest (the operator being able to choose the area to be examined).

The criticism, especially of TE, is that it is not able to differentiate between contiguous stages of fibrosis (F0 vs F1, or F1 vs F2). On the other hand, from the point of view of the clinician, it is important to know if the patient has only mild fibrosis (probably with no need for treatment in HCV patients), or moderate or severe fibrosis. And for this purpose, TE is quite a good method (the following AUROCs were reported: 0.79 for $F \geq 2$, 0.91 for $F \geq 3$ and 0.97 for $F = 4$)^[7].

The other elastographic method, ARFI technology,

involves targeting an anatomic region to be interrogated for elastic properties, with the use of a region of-interest cursor, while performing real-time B-mode imaging. Tissue from the region of interest is mechanically excited by using short-duration (262 μ s) acoustic pulses with a fixed transmit frequency of 2.67 MHz to generate localized tissue displacements. The displacements result in shear-wave propagation away from the region of excitation and are tracked by using US correlation-based methods^[19]. The shear-wave propagation velocity is proportional to the square root of tissue elasticity. Results are expressed in meters per second (m/s). The technique is new and published data suggest that ARFI and TE have a similar predictive value for fibrosis assessment.

In a study performed by Friedrich-Rust *et al*^[9] in which ARFI was compared to LB and blood markers in 86 patients with chronic hepatitis (HBV or HCV), the Spearman correlation coefficients between the histological fibrosis stage and ARFI, TE, FibroTest and APRI score were statistically significant: 0.71, 0.73, 0.66 and 0.45 respectively ($P < 0.001$).

In the study performed by Lupşor *et al*^[14], 112 patients with chronic hepatitis were evaluated. All the patients underwent LB (fibrosis stage assessed according to the Metavir scoring system), ARFI and FibroScan. The mean ARFI values for different stages of fibrosis were: 1.079 ± 0.150 m/s (F0-F1), 1.504 ± 0.895 m/s (F2), 1.520 ± 0.575 m/s (F3) and 2.552 ± 0.782 m/s (F4). The mean values were statistically significant different only between F3 and F4. The following cut-off values were proposed for various stages of fibrosis: $F \geq 1: 1.19$ m/s; $F \geq 2: 1.34$ m/s; $F \geq 3: 1.61$ m/s; and $F \geq 4: 2$ m/s.

Since both types of elastographic evaluation are available in our Department, we tried to see if by combining them we can improve their predictive value for fibrosis assessment. Firstly, we evaluated their predictive value alone for significant fibrosis ($F \geq 2$ Metavir) and cir-

rhosis ($F = 4$), after which we evaluated their predictive value in combination. If both methods were concordant ($TE \geq 6.7$ kPa and $ARFI \geq 1.2$ m/s), we obtained a high specificity (93.3%) for predicting significant fibrosis ($F \geq 2$), also with a very good positive predictive value (96.8%), so in those cases there was no need to perform LB before initiating treatment. In our study group, 152/197 patients had significant fibrosis ($F \geq 2$ Metavir) on LB. TE and ARFI were concordant for significant fibrosis in 92 of 152 patients. Therefore, we could avoid 60.5% of the LBs in our group of patients.

Also, by combining the two elastographic methods for predicting cirrhosis (F4) ($TE \geq 12.2$ kPa and $ARFI \geq 1.8$ m/s), the results were very good, with 94.4% Sp, 94.4% NPV and 91.8% accuracy, so the combined methods are excellent for confirming, but also for excluding the presence of cirrhosis. In our study group, 53 patients had cirrhosis ($F = 4$ Metavir on LB). TE and ARFI were concordant for liver cirrhosis in 45 from the 53 patients with $F = 4$ on LB (84.9%).

Other published data tried to combine different non-invasive methods for a better evaluation of liver stiffness. In a study published in 2005 by Castera *et al.*¹⁸, 183 patients with chronic HCV hepatitis were evaluated by LB, TE, FibroTest and APRI. The best performance was obtained by combining FibroScan and FibroTest, with areas under the ROC curve of 0.88 for $F \geq 2$, 0.95 for $F \geq 3$, and 0.95 for $F = 4$. When FibroScan and FibroTest results agreed, significant fibrosis ($F \geq 2$) was confirmed by LB in 84% of the cases, severe fibrosis ($F \geq 3$) in 95% of cases, and cirrhosis ($F = 4$) in 94% of the cases.

In another study published in 2010, Castera *et al.*²⁰ evaluated two algorithms for liver fibrosis prediction: one combined TE and FibroTest (Castera) and the other APRI and FibroTest (SAFE biopsy). In all patients a LB was performed. Significant fibrosis ($F \geq 2$ Metavir) was present in 76% of patients and cirrhosis (F4) in 25%. TE failure was observed in eight cases (2.6%). For significant fibrosis, the Castera algorithm saved 23% more liver biopsies than SAFE biopsy (71.9% *vs* 48.3%, respectively, $P < 0.0001$), but its accuracy was significantly lower (87.7% *vs* 97.0%, respectively; $P < 0.0001$). Regarding cirrhosis, the accuracy of the Castera algorithm was significantly higher than that of SAFE biopsy (95.7% *vs* 88.7%, respectively; $P < 0.0001$). The number of saved liver biopsies did not differ between the two algorithms (78.8% *vs* 74.8%, $P = NS$).

Shahenn²¹ published a meta-analysis which compared the performances of TE and FibroTest for the prediction of liver fibrosis in patients with chronic HCV hepatitis. Thirteen studies were identified, 9 regarding FibroTest (1679 patients) and 4 regarding TE (546 patients). In heterogeneous analysis for significant fibrosis, the AUROC curves for FibroTest and TE were 0.81 and 0.83, respectively. At a threshold of approximately 0.60, the sensitivity and specificity of FibroTest were 47% (35%-59%) and 90% (87%-92%). For TE (threshold approximately 8 kPa), corresponding values were 64% (50%-76%) and 87% (80%-91%), respectively. However, the diagnostic ac-

curacy of both tests was associated with the prevalence of significant fibrosis and cirrhosis in the study populations. For cirrhosis, the summary AUROCs for FibroTest and FibroScan were 0.90 and 0.95 (0.87-0.99).

In a study published in 2010 by Cross *et al.*²², 187 patients with chronic HCV hepatitis were evaluated by means of LB, TE and the King score. Liver fibrosis was scored using the Ishak score; significant fibrosis was defined as Ishak fibrosis stage F3-F6, and cirrhosis defined as Ishak fibrosis F5-F6. The AUROCs for TE, the King score and TE + King score for the diagnosis of Ishak F3-F6 were 0.83, 0.82 and 0.85, respectively and 0.96, 0.89 and 0.93, respectively, for the diagnosis of cirrhosis ($F \geq 5$ Ishak). The negative predictive values for the diagnosis of cirrhosis, using the optimal cut-off results for TE (10.05 kPa), the King score (24.3) and the two combined (26.1), were 98%, 91% and 94%, respectively.

Our study tried to establish whether the combination of TE and ARFI could provide some advantages for the evaluation of significant fibrosis in patients with chronic hepatitis C in comparison with a single elastographic method. By combining the two elastographic methods (values both for TE and ARFI above the mentioned cut-offs), the specificity increased (of course with lower sensitivity), with very good PPV (96.3%) for significant fibrosis ($F \geq 2$ Metavir). In our study group, 152/197 patients had significant fibrosis ($F \geq 2$ Metavir) on LB. TE and ARFI were concordant for significant fibrosis in 92 of 152 patients. Therefore, we were able to avoid 60.5% of LB in our group of patients.

Also, by combining the two elastographic methods for predicting cirrhosis (F4) ($TE \geq 12.2$ kPa and $ARFI \geq 1.8$ m/s), the results were very good, with 94.4% Sp, 94.4% NPV and 91.8% accuracy, so the combined methods are not only able to confirm, but also to exclude the presence of cirrhosis.

In conclusion, LS measurements assessed by means of both TE and ARFI strongly correlate to histological fibrosis in HCV patients. TE used in combination with ARFI is highly specific (approximately 93%) for predicting significant fibrosis ($F \geq 2$ Metavir), so that in patients with higher LS measurements than the proposed cut-offs for both methods, liver biopsy could be avoided. Also, in patients suspected of having severe fibrosis, if both methods are concordant, they are very good for confirming and excluding the presence of cirrhosis (94.4% Sp, 94.4% NPV).

COMMENTS

Background

Non-invasive methods for fibrosis assessment in chronic hepatitis, such as transient elastography (TE), are accepted more and more, tending to replace the invasive methods, especially in hepatitis C virus (HCV) chronic hepatitis. In the last few years, studies were published regarding the use of acoustic radiation force impulse (ARFI) elastography for fibrosis assessment in chronic hepatitis.

Research frontiers

Studies were published regarding the benefits of combining non-invasive methods for fibrosis evaluation (serological tests with or without TE), but not regarding a combination of elastographic methods (TE and ARFI).

Innovations and breakthroughs

The aim of this study was to find out if by combining ARFI and TE the prediction of fibrosis in patients with chronic HCV hepatitis can be improved, and the authors concluded that TE used in combination with ARFI is highly specific for predicting significant fibrosis; therefore when the two methods are concordant liver biopsy can be avoided.

Applications

In this study that included 197 patients with chronic C hepatitis, LS measurement by means of both TE and ARFI strongly correlated to the histological fibrosis. TE used in combination with ARFI was highly specific (93.3%) for predicting significant fibrosis ($F \geq 2$ Metavir); therefore in patients with higher LS measurements than the proposed cut-offs for both methods, liver biopsy could be avoided (positive predictive value 96.8%).

Terminology

TE (FibroScan) is an ultrasound-based method that uses the transmission of low-frequency vibrations to create an elastic shear wave that propagates into the liver, followed by the detection wave propagation velocity, which is proportional to the tissue stiffness, with faster wave progression occurring through stiffer tissue. ARFI technology involves targeting an anatomic region to be interrogated for elastic properties, with the use of a region-of-interest cursor, while performing real-time B-mode imaging. Tissue from the region of interest is mechanically excited to generate localized tissue displacements. The displacements result in shear-wave propagation away from the region of excitation and are tracked by using US correlation-based methods. The shear-wave propagation velocity is proportional to the square root of tissue elasticity.

Peer review

This is a well written paper that looks at TE (a well studied technology for liver fibrosis) and acoustic radiation impulse force (a technology for which there is much less clinical data). The data is well displayed.

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S- Editor Tian L L- Editor Rutherford A E- Editor Xiong L

YKL-40 expression in CD14⁺ liver cells in acute and chronic injury

Oscar Pizano-Martínez, Irinea Yañez-Sánchez, Pilar Alatorre-Carranza, Alejandra Miranda-Díaz, Pablo C Ortiz-Lazareno, Trinidad García-Iglesias, Adrian Daneri-Navarro, Mónica Vázquez-Del Mercado, Mary Fafutis-Morris, Vidal Delgado-Rizo

Oscar Pizano-Martínez, Irinea Yañez-Sánchez, Pilar Alatorre-Carranza, Alejandra Miranda-Díaz, Trinidad García-Iglesias, Adrian Daneri-Navarro, Mónica Vázquez-Del Mercado, Mary Fafutis-Morris, Vidal Delgado-Rizo, Department of Physiology, CUCS, University of Guadalajara, Guadalajara, Jalisco 44340, México

Pablo C Ortiz-Lazareno, Division of Immunology, Centro de Investigación Biomédica de Occidente-IMSS. Guadalajara, Jalisco 44340, México

Author contributions: Pizano-Martínez O and Yañez-Sánchez I contributed equally to this work; Pizano-Martínez O, Yañez-Sánchez I, and Delgado-Rizo V designed the research; Pizano-Martínez O, Miranda-Díaz A, Ortiz-Lazareno PC and García-Iglesias T performed the research; Daneri-Navarro A, Vázquez-Del Mercado M and Fafutis-Morris M contributed new reagents and analytic equipment; Pizano-Martínez O, Yañez-Sánchez I and Delgado-Rizo V analyzed the data; and Pizano-Martínez O, Yañez-Sánchez I, Alatorre-Carranza P and Delgado-Rizo V wrote the paper.

Supported by Complementary support CONACyT 90361

Correspondence to: Dr. Vidal Delgado-Rizo, Department of Physiology, CUCS, University of Guadalajara, Sierra Mojada 950, Col. Independencia, Building P second level, Guadalajara, Jalisco 44340, México. vidalrizo@gmail.com

Telephone: +52-33-10585307 Fax: +52-33-10585307

Received: October 28, 2010 Revised: January 17, 2011

Accepted: January 24, 2011

Published online: September 7, 2011

Abstract

AIM: To demonstrate that CD14⁺ cells are an important source of the growth factor YKL-40 in acute and chronic liver damage.

METHODS: Rats were inoculated with one dose of CCl₄ to induce acute damage. Liver biopsies were obtained at 0, 6, 12, 24, 48 and 72 h. For chronic damage, CCl₄ was administered three days per week for 6 or 8 wk. Tissue samples were collected, and cellular

populations were isolated by liver digestion and purified by cell sorting. YKL-40 mRNA and protein expression were evaluated by real-time polymerase chain reaction and western blot.

RESULTS: Acute liver damage induced a rapid increase of YKL-40 mRNA beginning at 12 h. Expression peaked at 24 h, with a 26-fold increase over basal levels. By 72 h however, YKL-40 expression levels had nearly returned to control levels. On the other hand, chronic damage induced a sustained increase in YKL-40 expression, with 7- and 9-fold higher levels at 6 and 8 wk, respectively. The pattern of YKL-40 expression in different subpopulations showed that CD14⁺ cells, which include Kupffer cells, are a source of YKL-40 after acute damage at 72 h [0.09 relative expression units (REU)] as well as after chronic injury at 6 wk (0.11 REU). Hepatocytes, in turn, accounted for 0.06 and 0.01 REU after 72 h (acute) or 6 wk (chronic), respectively. The rest of the CD14⁺ cells (including T lymphocytes, B lymphocytes, natural killer and natural killer T cells) yielded 0.07 and 0.15 REU at 72 h and 6 wk, respectively. YKL-40 protein expression in liver was detected at 72 h as well as 6 and 8 wk, with the highest expression relative to controls (11-fold; $P \leq 0.05$) seen at 6 wk. Macrophages were stimulated by lipopolysaccharide. We demonstrate that under these conditions, these cells showed maximum expression of YKL-40 at 12 h, with $P < 0.05$ compared with controls.

CONCLUSION: Hepatic CD14⁺ cells are an YKL-40 mRNA and protein source in acute and chronic liver injury, with expression patterns similar to growth factors implicated in inflammation-fibrogenesis.

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Key words: YKL-40; Kupffer cells; Liver cirrhosis; CD14⁺ cells

Peer reviewer: Maria-Angeles Aller, MD, PhD, Professor, Cátedra de Cirugía, Facultad de Medicina, Universidad Complutense de Madrid, Pza. de Ramón y Cajal s.n., Madrid 28040, Spain

Pizano-Martínez O, Yañez-Sánchez I, Alatorre-Carranza P, Miranda-Díaz A, Ortiz-Lazareno PC, García-Iglesias T, Daneri-Navarro A, Vázquez-Del Mercado M, Fafutis-Morris M, Delgado-Rizo V. YKL-40 expression in CD14⁺ liver cells in acute and chronic injury. *World J Gastroenterol* 2011; 17(33): 3830-3835 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i33/3830.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3830>

INTRODUCTION

YKL-40 glycoprotein (Chi3L1) is a growth factor named for its N-terminal amino acid sequence and molecular weight. It is related to 18-glycosylhydrolases, but lacks enzymatic activity^[1,2]. Under physiological conditions, human YKL-40 is secreted in small quantities by synovocytes and chondrocytes^[3,4]. However, *in situ* experiments demonstrated increased tissue mRNA and protein levels in osteoarthritis and rheumatism as well as in patients with inflammatory joint disease, where activated monocytes and macrophages are the main source.

The biological function of YKL-40 is unclear because no receptors have been identified to date. However, it has been reported that YKL-40 binds to stabilin type 1 and heparin sulfate *in vivo*. Furthermore, YKL-40 stimulates the proliferation and migration of connective tissue cells and fibroblasts through activation of the mitogen-activated protein kinase signaling pathway, suggesting that YKL-40 modulates fibrogenesis and tissue remodeling^[5,6].

Several studies have found elevated YKL-40 concentrations in sera of patients with liver diseases, such as hepatic fibrosis by hepatitis C virus^[7]. Serum concentrations of YKL-40 correlated with extracellular matrix (ECM) products secreted by hepatic stellate cells (HSCs) and fibroblasts (e.g., P11NP, hyaluronan, MMP-2, and TIMP-1). It has been suggested that YKL-40 concentrations reflect the degree of liver fibrosis. However, extensive clinical evaluation is still required, and other inflammatory diseases have to be excluded as potential causes of YKL-40 elevations.

In hepatic tissue, immunohistochemical analyses show strong YKL-40 staining around fibrotic areas^[3]; however, in this study it was not possible to discriminate the cells that produce YKL-40. Notably, HSCs from fibrotic liver tissue by *S. japonica* showed an increase of YKL-40 mRNA^[8].

Currently, the kinetics and source of YKL-40 in the liver under damage conditions are unknown. This study addressed these issues as part of a broader effort to elucidate the role of this molecule in hepatic inflammation and tissue repair.

MATERIALS AND METHODS

Animals

Rats were treated according to the guidelines for reproduction, care and use of laboratory animals stated by the Norma Oficial Mexicana (NOM-066-ZOO-1999). Twenty-seven male Wistar rats weighing 250 g each were selected for treatment with CCl₄ and divided into nine groups of three animals each. One group was untreated and used as controls. Six groups were inoculated with a single intragastric dose of 0.5 mL/100 g of CCl₄ (Sigma, 319961, United States) mixed 1:1 with mineral oil (Sigma, M5409, United States) to establish acute injury. These animals were sacrificed at time 0, 6, 12, 24, 48 and 72 h. To establish chronic liver injury, two groups were inoculated three times per week with an i.p. injection of 0.1 mL/100 g CCl₄: mineral oil at ratios of 1:6, 1:5, 1:4 (one week for each), then 1:3 until the animals were sacrificed at week 6 or 8.

Tissue samples

Procedures were performed under ether anesthesia. Livers were washed with PBS (Gibco, 70013-032, United Kingdom) at 4 °C, sectioned into small 100 mg pieces and stored in tubes at -70 °C. Samples for RNA extraction were stored in 500 µL of TRIzol (Invitrogen[®] BRL 15596-026, United States) at -70 °C.

RNA extraction

Liver tissue and cells were homogenized in 500 µL of TRIzol (Invitrogen[®] BRL United States). Chloroform (Sigma, C2432, United States) was added, and the samples were centrifuged for 15 min at 10 000 g and 4 °C. Total RNA was precipitated with isopropanol (Sigma, 19516, United States). The RNA pellet was washed with 75% ethanol and dissolved in RNase-free water. The final concentrations and quality of the RNA were determined by spectrophotometry.

Reverse-transcriptase polymerase chain reaction

Approximately 1 µg of total RNA was reverse-transcribed to cDNA in a 20 µL reaction using murine leukemia virus reverse transcriptase M-MLV (Invitrogen[®] BRL, 28025-013, United States). The mixture was prepared with 1 µg of RNA, 125 ng/µL of random primers (Invitrogen[®] BRL, 48190011, United States), 1 µL of 10 mmol/L dNTP mix and 12 µL of sterile distilled water. The mixture was heated for 5 min at 65 °C and quick chilled on ice. Four µL of 5X First Strand Buffer, 2 µL of 0.1 mol/L DTT and 1 µL of RNaseOUT (Invitrogen[®] BRL, 10777019, United States) were added, and the reaction was incubated at 37 °C for 2 min. Finally, 200 U/µL of M-MLV were added, and the total reaction was incubated at 25 °C for 10 min and then at 37 °C for 50 min. The reaction was inactivated by heating at 70 °C for 15 min^[9].

Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was performed

with an ABI Prism 7300 thermocycler (Applied Biosystems, United States). Each 20 μ L reaction contained 2 μ L of cDNA and TaqMan Universal PCR master mix (Applied Biosystems, 4364338, United States). The primers and probe set sequences were specific for 18S rRNA (constitutive gene; Applied Biosystems, FG18S RNA, United States) and YKL-40 (inducible gene; Applied Biosystems, Rn0149065, United States). All reactions were run in duplicate at universal thermocycler conditions for TaqMan[®] Gene Expression Assays (2 min at 50 °C, 10 min at 95 °C and 40 cycles at 95 °C for 15 s and 60 °C for 1 min). Results were analyzed with ABI Prism software^[9].

YKL40 expression in cell subpopulations

Male Wistar rats were anesthetized, and livers were perfused *via* the portal vein with calcium-free Gey's solution (Sigma, 69779, United States) supplemented with 100 U/mL of heparin. A second perfusion was done with calcium-free Gey's solution containing 100 U/mL of heparin, 0.06% collagenase (Fluka, 27678, United States) and 0.01% DNase (Roche, 13035000, Sweden). Liver tissues were extracted, homogenized and placed in a bottle containing Gey's solution, 0.005% collagenase and 0.001% DNase, and stirred gently for 30 min at 37 °C. The resultant suspension was filtered in 106 Nylon mesh, and 4 mL of MEM (Invitrogen[®] BRL, 11095, United States) supplemented with 10% fetal bovine serum (Invitrogen[®] BRL, 1082-139, United States) were added. The homogenates were centrifuged at 50 G for 2 min at 4 °C to precipitate hepatocytes. The supernatant was recovered and centrifuged at 400 G for 7 min at 4 °C. The pellet was resuspended with Gey's solution plus 25% albumin (Sigma, A7906, United States) and centrifuged at 350 G for 10 min at 4 °C to obtain non-parenchymal cells (NPCs). NPCs were washed, resuspended with Gey's solution, placed on a lymphoprep (Axis-Shield, LYS3773, Oslo, Denmark) gradient and centrifuged at 1800 *g* for 25 min. The white ring of mononuclear cells was recovered. Cells were washed and then divided into aliquots of 1×10^6 per tube for sorting. Next, cells were incubated with a CD14 primary antibody (Santa Cruz Biotechnology, M305, United States, 1:25) for 30 min in the dark at 4 °C. Cells were then washed and incubated with a FITC secondary antibody (Jackson ImmunoResearch, 71813, United Kingdom, 1:2000) for 30 min at 4 °C in the dark. Marked cells were washed and sorted (BD FACSAria) by CD14 protein surface marker expression.

Western blotting

Protein extraction from whole liver tissue and cellular populations was performed with the NE-PER (Pierce, 78833, United States) extraction reagent according to the manufacturer's instructions. Protein concentrations were quantified with the BioRad protein assay (BioRad, 500-13, 14, 15; United States). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was done at 12%, and the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane at 30 V overnight at 8 °C. The PVDF membrane was washed twice in tris buffered

saline (TBS) and blocked with 5% of non-fat milk used for 1 h at room temperature with constant stirring. The membrane was then incubated for 1 h at room temperature with primary antibodies against YKL-40 (Santa Cruz Biotechnology, sc-31722, United States, 1:200) or [-Actin (Santa Cruz Biotechnology, sc-47778, United States, 1:500)]. Membranes were then washed twice with tris buffered saline Tween-20 (TBST) and incubated for 30 min with an HRP-conjugated donkey anti-goat secondary antibody (Santa Cruz Biotechnology, sc-2020, United States, 1:4000) to detect YKL-40 or goat anti-mouse for [-actin (Roche, 11520709001, Sweden, 1:500)]. Membranes were then washed twice in TBST and TBS. The signal was detected with the BM chemiluminescence Western Blotting kit (Roche, 1520709, Sweden) according to the manufacturer's instructions. Relative expression units (REU) were calculated from densitometric values of YKL-40 and β -actin (Kodak MI SE 4.5 software, Kodak, United States).

In vitro overexpression of YKL-40

Alveolar macrophage cells (ATCC, NR8383) were cultured in RPMI 1640 medium (Invitrogen[®] BRL, 21870084, United States) supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic (Invitrogen[®] BRL, 15240062, United States). Upon reaching 70% confluence, cells were stimulated with LPS (Sigma, L4005, United States, 100 ng/L) for 3 h or 12 h; the medium was then discarded, and the cells were lysed in Trizol/TRIZOL reagent for mRNA extraction and evaluation of YKL-40 mRNA expression by real-time PCR.

Statistical analysis

The statistical analysis was performed with SPSS 10.0 software (SPSS Inc., United States), and significance was calculated by ANOVA. Data were expressed as mean \pm SD. We considered $P \leq 0.05$ to be significant.

RESULTS

YKL-40 mRNA expression

Real-time PCR was performed on liver tissue samples after acute or chronic injury to evaluate changes in YKL-40 mRNA expression. In all experiments, healthy animals served as the control group. At 6 and 12 h after CCl₄ administration, YKL-40 expression was increased 2- and 10-fold, respectively, compared to controls ($P \leq 0.05$). Maximum expression (26-fold) relative to healthy tissue samples occurred at 24 h. At 72 h, YKL-40 mRNA expression levels declined to a level close to that of the 0-h and healthy groups (Figure 1).

In the chronic liver injury model, YKL-40 mRNA expression was significantly increased relative to controls at both 6 and 8 wk (8 and 10 REU, respectively, $P < 0.05$) (Figure 1).

YKL-40 protein expression

We next investigated the presence of YKL-40 protein in whole liver tissue from healthy, acute and chronic

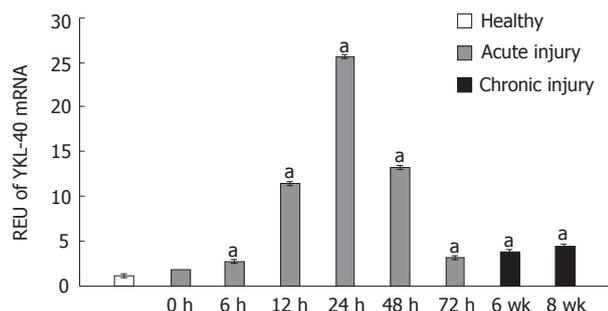


Figure 1 YKL-40 mRNA expression in whole liver tissue samples with acute and chronic injuries induced by CCl₄. After acute injury, YKL-40 mRNA increased in a gradual and constant pattern and then fell to control levels. The maximum 26-fold increase was reached at 24 h in the acute damage model. For the chronic injury model, at 6 and 8 wk, YKL-40 mRNA levels were increased 7- and 9-fold compared to controls, respectively. ^a*P* ≤ 0.05. REU: Relative expression units.

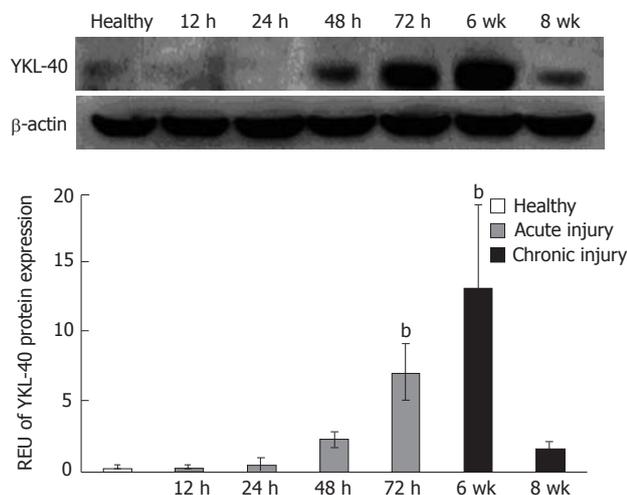


Figure 2 YKL-40 protein levels show a gradual increase. The maximum peak was at 72 h in the acute damage model and 6 wk in the chronic injury model, with 2.01 and 6.35-fold increases, respectively. ^b*P* ≤ 0.05 respect to the control group. REU: Relative expression units.

injury samples. In livers with acute injury, we observed that YKL-40 protein levels peaked at 72 h, with an increase of 2.0-fold compared to controls. At 12 and 24 h, YKL-40 levels were similar to those of healthy livers. In contrast, in the chronic injury model the maximum peak was observed at 6 wk with a significant decline apparent by 8 wk (*P* < 0.05) (Figure 2).

CD14⁺ cells are a source of YKL-40 protein

Based on their high levels of YKL-40 protein expression in whole liver samples, the 72-h group from the acute model and the 6-wk group from the chronic model were chosen for evaluation of protein expression in isolated cells. It has been reported that CD14⁺ cells population, which includes Kupffer macrophages, synthesize the largest amount of cytokines and growth factors. Additionally, they are found in abundance in the liver, accounting for about 20%-25% of NPCs^[10] and 80%-90% of macrophages in the whole body. Therefore, we evalu-

ated YKL-40 protein expression in this subpopulation.

Hepatocytes, CD14⁺ cells and CD14⁻ cells isolated from healthy animals did not show significant YKL-40 protein expression, with no statistical significance between groups (Figure 3A). However, when these cells types were isolated from CCl₄-treated animals, YKL-40 protein expression was elevated up to 4-fold compared with healthy animals. The highest level (0.09 REU) was detected in CD14⁺ cells in the acute damage model (Figure 3B). In the chronic injury model, both CD14⁺ and CD14⁻ cells were significant sources of YKL-40, with 0.15 and 0.11 REU, respectively (*P* < 0.05 compared to control cells) (Figure 3C).

Lipopolysaccharide induces YKL-40 mRNA expression in rat alveolar macrophages

Previous studies reported that CCl₄ induces damage to the intestinal tissue architecture^[11]. CCl₄ promotes translocation of bacteria and associated compounds like Lipopolysaccharide (LPS) towards the liver by portal blood flow. Here, LPS is taken up by Kupffer cells, which then synthesize cytokines and growth factors. To demonstrate this effect, rat alveolar macrophage cells were stimulated with LPS for 3 or 12 h. Our results showed that YKL-40 mRNA levels were increased 9- and 11-fold, respectively, compared with control cells without LPS stimulation (*P* ≤ 0.05). However, no significant difference was detected between the 3- and 12-h groups (Figure 4).

DISCUSSION

Several studies have found a correlation between serum YKL-40 levels and liver fibrosis stages^[10,12,13]. However, other studies have reported contradictory findings^[14-18]. Nevertheless, in the first set of studies, procollagen III peptide and hyaluronic acid showed better correlations with fibrosis than YKL-40, likely because these molecules are scar components. YKL-40 is presumably a growth factor that indirectly contributes to fibrosis by stimulating proliferation of the cells that produce ECM proteins. For this reason, we considered it important to study the expression kinetics and source of YKL-40 in models of acute and chronic liver injury.

Although liver fibrosis and cirrhosis are characterized by inflammatory infiltration, a process in which a great number of cells participate, Johansen *et al.*^[3] showed that the liver was a possible source of YKL-40. This study noted that strong YKL-40 immunostaining could be detected around fibrotic areas in liver tissue samples where fibrosis had been induced by alcohol and viral hepatitis, but it was impossible to distinguish the cellular source^[3].

We used the CCl₄ damage model because it resembles alcohol damage^[14] and because the kinetics of damage is well characterized. Our results show that YKL-40 mRNA levels began to increase at 12 h, with a maximum peak at 24 h (Figure 1). In the CCl₄ fibrosis model, increased mRNA levels of growth factors such as TGF-β and PDGF at 48 and 72 h after intoxication suggest their participation in the fibrogenic process through their bio-

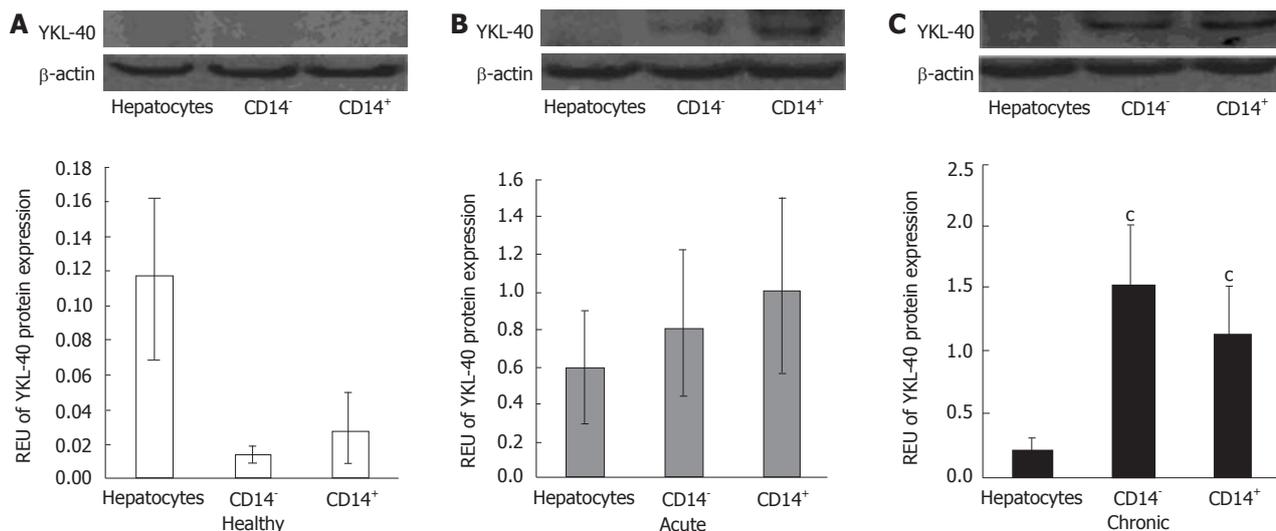


Figure 3 YKL-40 protein expression in cellular populations isolated from liver tissue. A: In healthy livers, expression of YKL-40 protein was lower in CD14⁺ and CD14⁻ subpopulations. B: In cells from livers exposed to acute damage, YKL-40 protein levels were higher in CD14⁺ cells. C: In the chronic injury model, hepatocytes showed low levels of YKL-40, with significant difference between CD14⁺ and CD14⁻ cells against hepatocytes (^c*P* < 0.05). REU: Relative expression units.

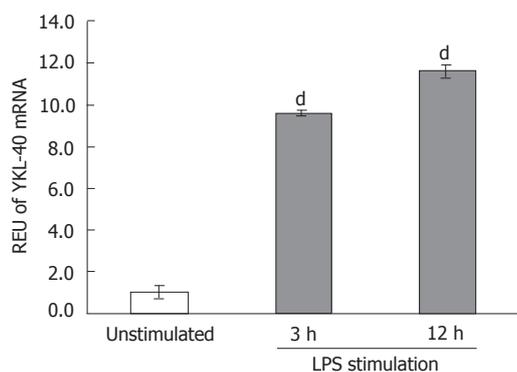


Figure 4 YKL-40 mRNA expression was induced by lipopolysaccharide in cell culture. After 3 and 12 h of stimulation with lipopolysaccharide, YKL-40 mRNA levels were increased by 9- and 11-fold, respectively, in a rat alveolar macrophage cell line (^d*P* < 0.05). Unstimulated cells were used as controls. REU: Relative expression units. LPS: Lipopolysaccharide.

logical activities of ECM synthesis and HSC-fibroblast proliferation respectively^[15,19]. Like PDGFβ, YKL-40 might activate the proliferating cell signaling pathway PI3K-AKT^[20].

Kupffer cells were the natural candidate source of YKL-40 because previous studies of joint, lung, kidney and skin inflammatory diseases^[2,5] identified macrophages as the main producer of this protein. In our study, CD14⁺ cells were a source of YKL-40, exhibiting 0.9 and 0.11 REU in the acute and chronic damage models, respectively. However, it is important to note that CD14⁻ cells also produced YKL-40 protein, with 0.7 and 0.15 REU in the acute and chronic injury models, respectively. This population includes immune cells such as natural killer (NK) and natural killer T (NKT) cells, which represent about 37% and 26% of non-parenchymal cells respectively. These cells are the first line of defense in liver infections and consequently have immunoregulatory properties: they can synthesize cytokines

(e.g., IFN-γ, TNF-α, IL-4, IL-10 and IL-13) (NK cells) or induce direct cellular destruction by TLR or CD1D molecules (NKT cells)^[11].

LPS might also be a trigger of YKL-40 gene expression. Carbon tetrachloride and alcohol injure the upper gastrointestinal tract, forming lesions in gastric and duodenal mucosa. This promotes increased intestinal permeability to endotoxins, notably LPS, peptidoglycan, flagellin and zymosan, which are found in liver blood influx as well. These antigens are taken up by monocytes and Kupffer cells^[12,13], which express TLR 1-8. LPS is known to be a ligand for TLR-4, a toll-like receptor widely expressed in monocytes and macrophages. This ligand-receptor interaction induces larger quantities of TNF-α, TGF-β and IL-10 cytokines^[12,13]. Considering that the type of intestinal and liver damage induced by CCl₄ is similar to that of ethanol consumption, we stimulated alveolar macrophages *in vitro* with 100 ng/L and found increases in YKL-40 mRNA levels of about 9- and 12-fold at 3 and 12 h, respectively. These results indicate that LPS could act as a direct stimulus to induce YKL-40 in macrophages during the establishment of damage.

COMMENTS

Background

Hepatic diseases are a major cause of death in the world; some have, as a singular characteristic, the presence of fibrosis. Hepatic fibrosis is difficult to diagnose because a hepatic biopsy is needed, and the condition of patients frequently makes it impossible to perform a biopsy. For several years, researchers have been looking for fibrosis markers in the blood in order to avoid having to perform a hepatic biopsy. One of these is YKL-40, a protein produced by several tissues under conditions causing stress and damage, such as alcohol consumption. YKL-40 has been used as a fibrosis marker with controversial results, particularly since it is not known precisely which cells produce this protein.

Research frontiers

The focus of this article is to elucidate which cells in the liver are producing YKL-4 and to try to understand the role of YKL-40 in the process of repairing

fibrotic hepatic tissue and the maintenance of a healthy liver.

Innovations and breakthroughs

Until the present moment, there has not been a study that describes in which cells and at what time YKL-40 is produced. Several research protocols in humans have been made: in 2003 Nojgaard detected YKL-40 and successfully related this protein with hepatic fibrosis, however in recent years other research has shown contradictory results. Johansen in 2000 published that, in liver biopsies, cells were producing YKL-40; however, with the methodology used it was impossible to distinguish which cells types were actively producing YKL-40. This work was initiated because the authors thought that it was necessary to know more about YKL-40 production and to focus on the hepatic cellular source.

Applications

The hepatic cells that produce YKL-40 are CD14⁺ and CD14⁻ at different times. This helps the authors to better understand how serum YKL-40 could be increased at different stages of disease, and attempt to better understand YKL-40 as a blood fibrosis marker and how this molecule participates in the process of hepatic health and illness.

Terminology

YKL-40: Protein produced by cells that helps to augment cell growth and to stimulate proteins that form the extracellular environment. CD14: Molecules in the cell membrane useful to classify and distinguish cells.

Peer review

A research study about a model of hepatic injury in the rat that is relevant since nowadays liver cirrhosis/fibrosis is a health problem worldwide. Many treatment modalities have been suggested to prevent the development of fibrosis but, unfortunately, none of them have uniformly yielded promising results. Therefore, a better knowledge of the pathogenic mechanisms involved in the inflammatory response developed during acute and chronic liver diseases makes possible the use of more effective therapeutic approaches in these patients.

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S- Editor Sun H L- Editor Rutherford A E- Editor Li JY

Ghrelin attenuates gastrointestinal epithelial damage induced by doxorubicin

Mohamed A Fahim, Hazem Kataya, Rkia El-Kharrag, Dena AM Amer, Basel al-Ramadi, Sherif M Karam

Mohamed A Fahim, Department of Physiology, Faculty of Medicine and Health Sciences, UAE University, Al Ain, PO Box 17666, United Arab Emirates

Hazem Kataya, Department of Biology, Faculty of Science, UAE University, Al Ain, PO Box 17666, United Arab Emirates
Rkia El-Kharrag, Sherif M Karam, Department of Anatomy, Faculty of Medicine and Health Sciences, UAE University, Al Ain, PO Box 17666, United Arab Emirates

Rkia El-Kharrag, Dena AM Amer, Basel al-Ramadi, Department of Microbiology and Immunology, Faculty of Medicine and Health Sciences, UAE University, Al Ain, PO Box 17666, United Arab Emirates

Author contributions: Kataya H, Fahim MA, al-Ramadi B and Karam SM designed this study; El-Kharrag R and Amer DAM performed the research; al-Ramadi B and Karam SM contributed analytical tools; El-Kharrag R, Amer DAM, al-Ramadi B and Karam SM analyzed the data; Fahim MA, al-Ramadi B and Karam SM wrote the paper.

Correspondence to: Sherif M Karam, Professor, Department of Anatomy, Faculty of Medicine and Health Sciences, UAE University, Al-Ain, PO Box 17666, United Arab Emirates. skaram@uaeu.ac.ae

Telephone: +971-3-7137493 Fax: +971-3-7672033

Received: December 13, 2010 Revised: March 5, 2011

Accepted: March 12, 2011

Published online: September 7, 2011

Abstract

AIM: To examine the influence of ghrelin on the regenerative potential of gastrointestinal (GI) epithelium.

METHODS: Damage to GI epithelium was induced in mice by two intravenous injections of doxorubicin (10 and 6 mg/kg). Some of the doxorubicin-treated mice received a continuous subcutaneous infusion of ghrelin (1.25 µg/h) for 10 d *via* implanted mini-osmotic pumps. To label dividing stem cells in the S-phase of the cell cycle, all mice received a single intraperitoneal injection of 5'-bromo-2'-deoxyuridine (BrdU) one hour before sacrifice. The stomach along with the duodenum were then removed and processed for histological examination and immunohistochemistry using anti-BrdU antibody.

RESULTS: The results showed dramatic damage to the GI epithelium 3 d after administration of chemotherapy which began to recover by day 10. In ghrelin-treated mice, attenuation of GI mucosal damage was evident in the tissues examined post-chemotherapy. Immunohistochemical analysis showed an increase in the number of BrdU-labeled cells and an alteration in their distribution along the epithelial lining in response to damage by doxorubicin. In mice treated with both doxorubicin and ghrelin, the number of BrdU-labeled cells was reduced when compared with mice treated with doxorubicin alone.

CONCLUSION: The present study suggests that ghrelin enhances the regenerative potential of the GI epithelium in doxorubicin-treated mice, at least in part, by modulating cell proliferation.

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Key words: Gastrointestinal cell proliferation; Gastrointestinal mucosal damage; Ghrelin

Peer reviewers: Dr. Jianyuan Chai, PhD, MS, BS, Assistant Professor, Research (09-151), VA Long Beach Healthcare System, 5901 E. 7th St, Long Beach, CA 90822, United States; Julio Mayol, MD, PhD, Department of Digestive Surgery, Hospital Clinico San Carlos, MARTIN-LAGOS S/n, Madrid, 28040, Spain

Fahim MA, Kataya H, El-Kharrag R, Amer DAM, al-Ramadi B, Karam SM. Ghrelin attenuates gastrointestinal epithelial damage induced by doxorubicin. *World J Gastroenterol* 2011; 17(33): 3836-3841 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i33/3836.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3836>

INTRODUCTION

Ghrelin is a novel 28-amino acid peptide which was simultaneously discovered by two independent research

groups more than 10 years ago^[1,2]. It was initially found to be a secretory product of a subset of enteroendocrine cells predominantly present in the stomach with a decreasing gradient toward the small intestine and the colon^[1,2]. It was also found to be produced in small amounts by several other organs such as the brain, pancreas, pituitary, kidney, lung and placenta^[3].

Functional studies revealed that ghrelin is involved in a wide variety of biological activities including growth hormone release^[4], stimulation of food intake and body weight gain^[5,6], gastrointestinal (GI) motility^[7], and modulation of cardiovascular function^[8]. Furthermore, it has been shown that ghrelin may also control proliferation and differentiation programs of neuronal and mesenchymal stem cells^[9] and modulate cell proliferation of some tissue progenitors^[10,11] and cell lines^[12-14].

In the stomach, while ghrelin has been shown to modulate the secretory activity of parietal cells^[15,16], it is not known whether this hormone is involved in the regulation of epithelial cell proliferation. It was shown earlier that parietal cells are the source of some instructive signals and their ablation in mice modulates the proliferation and differentiation program of gastric epithelial stem/progenitor cells^[17] and with age cause gastric carcinoma^[18]. Therefore, modulation of the secretory activity of these cells might also affect the proliferation/differentiation program of the epithelial progenitors. Surprisingly, when ghrelin knockout mice were examined, no abnormalities were reported in the GI mucosa^[19]. Recently, there has been increasing evidence to suggest a role for ghrelin in protection against gastric mucosal damage^[20,21]. The mechanism of mucosal protection was mainly attributed to the release of nitric oxide^[22].

The aims of this study were to determine whether ghrelin can be used to protect against GI mucosal damage induced by doxorubicin, and to test whether ghrelin protection, if any, is associated with modulation of cell proliferation in the progenitor cell zone of the GI epithelium.

MATERIALS AND METHODS

Animals

In this study, female BALB/c mice (2-3 mo old) were evaluated after being housed in sterile microisolator cages with sterile bedding, food, and water ad libitum. The animals were kept under a 12-h light/dark cycle and at room temperature (22-24 °C). The protocols described in this study were approved by the Animal Research Ethics Committee of the Faculty of Medicine, UAE University.

Chemotherapeutic treatment

To establish the experimental protocol of this study, we initially injected age-matched mice ($n = 40$) with a single dose of 5-fluorouracil (100 mg/kg body weight) or doxorubicin (10 mg/kg). Mice were then sacrificed at different time periods varying from 1 to 16 d. Gastro-duodenal tissues were collected to identify any changes

in mucosal integrity.

Experimental protocol

Mice ($n = 9$) were divided into three equal groups. Mice in the first group received ghrelin (Sigma, St. Louis, MO, United States) through Alzet micro-osmotic pumps (Durect Co, Cupertino, CA, United States) implanted subcutaneously which released ghrelin at a rate of 1.25 mg/h for 14 d. The pumps were prepared for implantation according to manufacturer's instructions and our previously published procedure^[23]. On the 8th and 9th day of ghrelin perfusion, mice received two intravenous injections of doxorubicin (10 and 6 mg/kg, respectively). In the second group, mice were subcutaneously infused with saline instead of ghrelin and then received two intravenous injections of doxorubicin on two consecutive days as in the first group. The third group of mice served as controls and, instead of ghrelin and doxorubicin, received only saline by infusion pump and by intravenous injections, respectively. To label dividing cells in the S-phase of the cell cycle, mice in all 3 groups received a single intraperitoneal injection of 5'-bromo-2'-deoxyuridine (BrdU, 120 mg/kg) one hour before sacrifice. At day 4 post-doxorubicin (or saline) second injection, the stomach along with the duodenum were removed under ether anesthesia and processed for morphological and immunohistochemical analysis.

Morphological analysis

To examine the histopathological changes that occurred in the wall of the GI tract, the stomach and proximal part of the duodenum were dissected from all mice under anesthesia and immediately processed for conventional histological examination^[23]. The tissues were fixed immediately in Bouin solution, dehydrated in ethanol, and infiltrated/embedded in paraffin. Five-micron-thick sections were mounted on slides and stained with periodic acid schiff and hematoxylin.

BrdU immunolabeling

To examine cell proliferation and estimate the number of cells in the S-phase of the cell cycle, paraffin tissue sections from all mice were processed to determine the localization of cells which incorporated BrdU^[23]. Briefly, tissue sections were first deparaffinized, hydrated, and incubated with 3% hydrogen peroxide to block endogenous peroxidase. The anti-BrdU antibody used was goat polyclonal^[24]. Biotinylated anti-goat immunoglobulin G was used as a secondary antibody which was identified by avidin-peroxidase and then di-aminobenzidine as a coloring agent. Immunoprobed tissue sections were scanned using the Olympus microscope to quantify BrdU-labeled cells. In each mouse, the numbers of BrdU-labeled cells per gastric gland or crypt-villus unit were averaged.

Statistical analysis

Data are presented as mean \pm SD. Differences between groups were evaluated using the Student *t* test. $P < 0.05$ was taken as statistically significant.

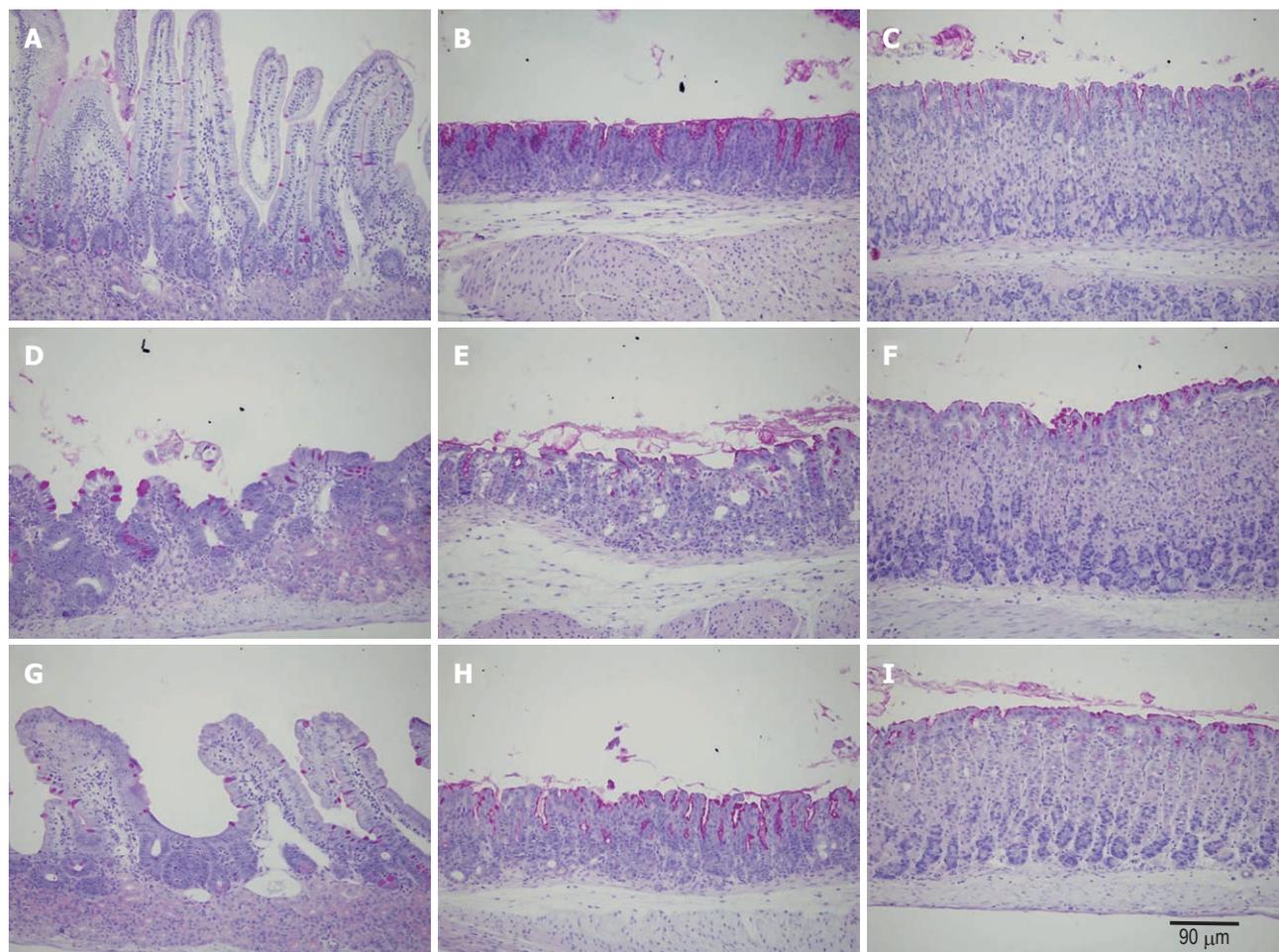


Figure 1 Light micrographs showing tissue sections obtained from the duodenum (A, D, G), pyloric antrum (B, E, H) and gastric corpus (C, F, I) of control (A-C), doxorubicin-treated (D-F) and ghrelin-plus-doxorubicin-treated (G-I) mice. All tissue sections were stained with periodic acid schiff and hematoxylin. Note that while there are apparent mucosal changes in D and E due to doxorubicin treatment, the tissues in G and H are more or less similar to control.

RESULTS

A single injection with chemotherapeutic agent induces mild effects in the GI mucosa

The damaging effect of the chemotherapeutic agents on GI mucosa was followed by microscopic examination of tissue sections. The dose regimen used for 5-fluorouracil showed inconsistent damaging effects in the mucosal tissues in the form of occasional vacuolation of the lining GI epithelial cells. Doxorubicin treatment induced a slightly more pronounced and consistent effect. Therefore, it was decided to use two intravenous injections of doxorubicin for the experimental protocol.

Protection of GI mucosa by ghrelin against the damaging effects of doxorubicin

Microscopic examination of gastroduodenal mucosa of control mice revealed the expected histological features of intact long oxyntic glands of the corpus region, short mucous glands of the pyloric antrum, and the very long crypt-villus units of the duodenum (Figure 1) as previously described^[25]. In the second group of doxorubicin-treated mice, while little changes were observed in the oxyntic glands in the form of a few scattered cells with vacuolated

cytoplasm, the antral glands showed more aggressive changes which induced dilations of the glandular lumen. Massive mucosal changes in the duodenum were observed (Figure 1D-F). The villi appeared blunt or much shorter and broader than those of control mice. In addition, the integrity of the villus epithelium was not intact. Signs of vacuolation and cell damage were evident throughout. In the ghrelin-plus-doxorubicin-treated mice, the glands in the corpus and antral regions appeared similar to those of control mice. Even the duodenal villi appeared intact, long and populated mainly by absorptive and goblet cells as in control mice (Figure 1G-I).

Modulation of BrdU-labeling in doxorubicin- and ghrelin-treated mice

To correlate the morphological changes with cell proliferation, we used the BrdU labeling method. BrdU was made available to cells in the S-phase of the cell cycle one hour before sacrifice. Gastroduodenal tissue sections were then immunolabeled using anti-BrdU antibody (Figure 2). In control mice, BrdU-labeled cells in the corpus and antral mucosae were located at the pit-gland junction close to the luminal surface (corpus) or the gland bottom (antrum). Counts revealed the presence of

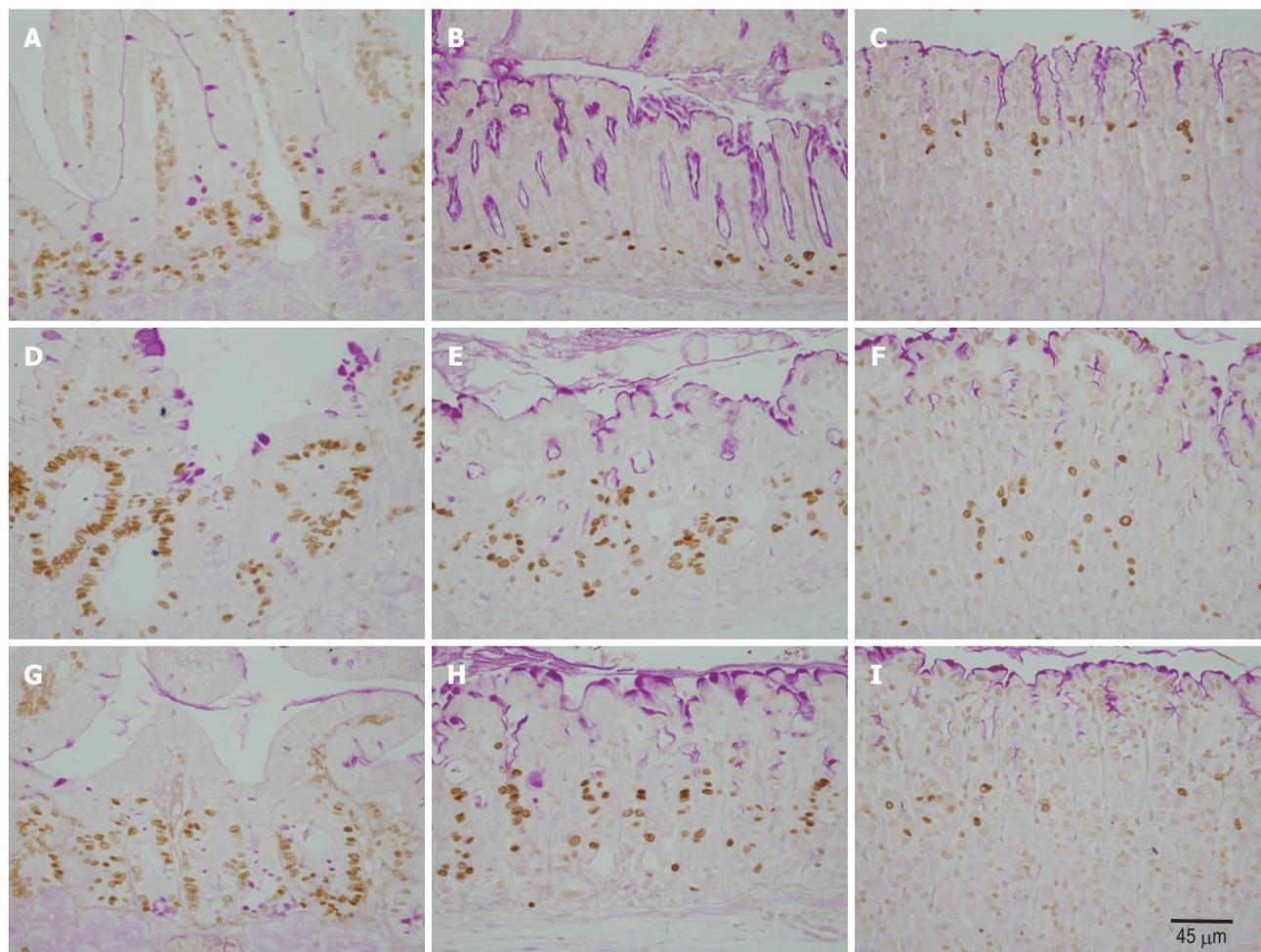


Figure 2 Immunohistochemical analysis of S-phase cells using anti-5'-bromo-2'-deoxyuridine antibody and tissue sections obtained from the duodenum (A, D, G), pyloric antrum (B, E, H) and gastric corpus (C, F, I) of control (A-C), doxorubicin-treated (D-F) and ghrelin/doxorubicin-treated (G-I) mice. Note that BrdU-labeled cells (brown nuclei) of doxorubicin-treated tissues in D, E and F appear more expanded when compared with control tissues in A, B and C. BrdU-labeled cells in G and H are still more expanded than in the control, but less prominent than in D and E.

3.5 ± 0.18 and 5.5 ± 0.18 cells per gland in the corpus and antrum, respectively (Figure 3). In the duodenum, dividing BrdU-labeled cells were in the lower portion of the crypts and averaged 10.7 ± 0.85 (Figure 3).

In doxorubicin-treated mice, the labeling pattern of BrdU-immunoreactive cells was altered in the corpus, antrum and duodenum. There was a general increase in the number of BrdU-labeled cells in the gastroduodenal mucosa. In addition, the distribution of BrdU-labeled cells was expanded and tended to be more scattered rather than localized to the gastric isthmus (Figure 2E, F). Counts revealed an increase in the number of BrdU-labeled cells up to 31.2 ± 4.07 , 12.1 ± 0.59 , 4.3 ± 0.18 in the duodenum, antrum and corpus, respectively. Each of these values was significantly higher than its corresponding value in control mice ($P > 0.01$).

Ghrelin-treated mice which also received doxorubicin showed an usual pattern in the distribution of dividing cells, however, the number of BrdU-labeled cells in the antrum and duodenum remained at a higher level than the control (Figures 2G, H, 3). These findings were confirmed when all mice were examined and BrdU-labeled

cells were quantified. When compared with control, the data showed a significant increase in the number of BrdU-labeled cells in the pyloric antrum and duodenum (9.6 ± 0.38 and 27.6 ± 2.75 , respectively). However, when these values were compared with those obtained from the second group of mice (treated only with doxorubicin), there was a decrease in S-phase labeled cells, but this was only significant in the antrum ($P > 0.02$).

DISCUSSION

Despite the tremendous effort in modern drug discovery and development, dyspepsia and GI mucosal damage remain frequent complications affecting life quality in cancer patients receiving chemotherapy. The available data demonstrate that ghrelin could be a potential protective agent against these complications. In rats treated with cisplatin, it was demonstrated that ghrelin can be used to prevent delayed gastric emptying, early satiety, anorexia, nausea and vomiting, all characteristic of cancer-associated dyspepsia syndrome^[26]. In the present study we induced GI mucosal damage using doxorubicin and

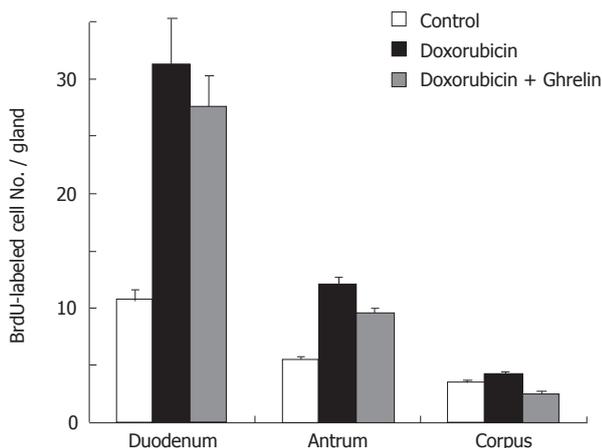


Figure 3 Analysis of 5'-bromo-2'-deoxyuridine-labeled cell counts in the duodenum, antrum and corpus of control, doxorubicin-treated and ghrelin/doxorubicin-treated mice.

demonstrated that ghrelin administration can also prevent the damaging effects of this chemotherapeutic agent.

Ghrelin appears to be a potent GI mucosal protective agent. Its effect on maintaining GI mucosal integrity was achieved using different experimental animal models. A recent series of studies showed that administration of ghrelin to rodents attenuated gastric mucosal lesions induced by ethanol^[20], stress^[21], ischemia-reperfusion^[27,28] and even HCl administration^[29]. The ghrelin effects on ethanol and stress models were mediated by release of nitric oxide as they were prevented by blocking nitric oxide synthase activity^[22]. The effects of ghrelin on the HCl-model was mediated not only by growth hormone secretagogue receptor, but also histamine H3 receptor, suggesting the involvement of histamine release in ghrelin-induced protection^[29].

It has been suggested that ghrelin is involved in several cell biological processes *via* different modes of actions: endocrine, paracrine and autocrine. We speculate that ghrelin exerted its GI mucosal protection *via* a paracrine effect. Examination of the GI mucosae of mice treated with two intravenous injections of doxorubicin revealed degenerative changes in their epithelial cell lining. However, mice which received ghrelin by continuous subcutaneous infusion showed minimal effects and appeared more or less similar to control mice when treated with the same dosage regimen of doxorubicin. The available data from the present study may suggest a mechanism which involves modulation of the cell cycle and induction of cell differentiation to substitute for the damaging effect of doxorubicin.

Several lines of evidence suggest that ghrelin modulates the proliferation of various cell types. It has been demonstrated that ghrelin stimulates proliferation of osteoprogenitor cells in bone tissue^[30] and neuronal progenitor cells in the spinal cord^[31]. Since cell proliferation and epithelial renewal are regarded as one of the protective mechanisms against GI mucosal damage, the question arises whether ghrelin also modulates prolifera-

tion of GI epithelial cells and hence could protect their integrity against noxious agents (such as chemotherapy). To answer this question we injected all mice used in our experiments with BrdU to label dividing cells during the S-phase of the cell cycle using immunohistochemistry. As expected, control mice showed BrdU-labeled cells in the isthmus regions of gastric glands and at the bottom of intestinal crypts. Doxorubicin-treated mice showed many more BrdU-labeled cells in both the gastric glands and intestinal crypts, probably to compensate for the damaged cells. However, in the presence of excess ghrelin, the damaging effects of doxorubicin were minimal and the number of BrdU-labeled cells was reduced, perhaps due to enhancement of cell differentiation. The other possibility is that ghrelin enhances cell differentiation and the increased proliferating cells observed in doxorubicin-treated mice were instructed by ghrelin to differentiate and migrate to restore the normal organization of the epithelium.

The protective effect of ghrelin against tissue damage is not restricted to the GI mucosa. Recent studies suggested that ghrelin promotes neuroprotective effects *via* stimulation of the regenerative potential of hippocampal neuroprogenitor cells to form new neurons. Incidentally, the expression of GHS-R was also demonstrated in the hippocampus^[10,11].

In conclusion, this study demonstrates the protective effect of ghrelin against GI mucosal damage induced by doxorubicin and provides an addition justification for its potential use during chemotherapy in cancer patients to improve their quality of life.

COMMENTS

Background

Normal gastrointestinal (GI) mucosa is characterized by its regenerative potential following damage. However, cancer patients receiving chemotherapy develop severe GI complications due to mucosal damage. Ghrelin has been suggested to play a protective role against these mucosal damaging effects.

Research frontiers

It is important to define the factors involved in GI mucosal protection and regulation of stem cell proliferation and differentiation. Here the authors provide evidence in support of the role of ghrelin in GI mucosal protection.

Innovations and breakthroughs

This study provides evidence that ghrelin protects against GI mucosal damage caused by doxorubicin.

Applications

The findings of this article could help in designing new modalities for GI mucosal protection and regeneration in cancer patients undergoing chemotherapy.

Peer review

This is an interesting and elegant experimental paper examining the effect of ghrelin on the intestinal tract of doxorubicin-treated mice. The investigators showed that ghrelin protects against doxorubicin-induced epithelial damage. In addition, they showed it modulates epithelial proliferation.

ACKNOWLEDGEMENT

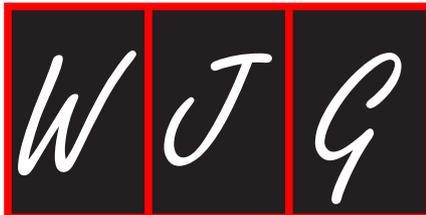
This paper is dedicated to our colleague Dr. H Kataya who initiated this collaborative study and suddenly passed away before its completion. The authors acknowledge the

Research Affairs section of the United Arab Emirates University which funded this interdisciplinary project.

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S- Editor Tian L L- Editor Webster JR E- Editor Li JY



Management of acquired bronchobiliary fistula: A systematic literature review of 68 cases published in 30 years

Guan-Qun Liao, Hao Wang, Guang-Yong Zhu, Kai-Bin Zhu, Fu-Xin Lv, Sheng Tai

Guan-Qun Liao, Hao Wang, Guang-Yong Zhu, Kai-Bin Zhu, Fu-Xin Lv, Sheng Tai, Department of General Surgery, the Second Affiliated Hospital of Harbin Medical University, Harbin 150086, Heilongjiang Province, China

Author contributions: Liao GQ and Tai S conceived the study, Liao GQ, Wang H, Zhu GY, Zhu KB, and Lv FX acquired and interpreted the data, Liao GQ, Wang H, and Tai S drafted the manuscript. All authors approved the final version of the paper.

Correspondence to: Sheng Tai, MD, PhD, Professor, Department of General Surgery, The Second Affiliated Hospital of Harbin Medical University, Harbin 150086, Heilongjiang Province, China. taishengster@gmail.com

Telephone: +86-451-86605719 Fax: +86-451-86605356

Received: November 28, 2010 Revised: January 22, 2011

Accepted: January 29, 2011

Published online: September 7, 2011

Abstract

AIM: To outline the appropriate diagnostic methods and therapeutic options for acquired bronchobiliary fistula (BBF).

METHODS: Literature searches were performed in Medline, EMBASE, PHMC and LWW (January 1980-August 2010) using the following keywords: biliobronchial fistula, bronchobiliary fistula, broncho-biliary fistula, biliary-bronchial fistula, tracheobiliary fistula, hepatobronchial fistula, bronchopleural fistula, and biliptysis. Further articles were identified through cross-referencing.

RESULTS: Sixty-eight cases were collected and reviewed. BBF secondary to tumors (32.3%, 22/68), including primary tumors (19.1%, 13/68) and hepatic metastases (13.2%, 9/68), shared the largest proportion of all cases. Biliptysis was found in all patients, and other symptoms were respiratory symptoms, such as irritating cough, fever (36/68) and jaundice (20/68). Half of the patients were treated by less-invasive methods such as endoscopic retrograde biliary drainage. Invasive approaches like surgery were used less frequently (41.7%, 28/67). The outcome was good at the end of the follow-

up period in 28 cases (range, 2 wk to 72 mo), and the recovery rate was 87.7% (57/65).

CONCLUSION: The clinical diagnosis of BBF can be established by sputum analysis. Careful assessment of this condition is needed before therapeutic procedure. Invasive approaches should be considered only when non-invasive methods failed.

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Key words: Bronchobiliary fistula; Digestive endoscopy; Endoscopic retrograde cholangio-pancreatography; Magnetic resonance cholangio; Percutaneous transhepatic cholangio; Iatrogenic damage; Congenital diaphragma defects; Hepatobiliary imino-diacetic acid scan

Peer reviewer: Beata Jolanta Jabłońska, MD, PhD, Department of Digestive Tract Surgery, University Hospital of Medical University of Silesia, Medyków 14 St. 40-752 Katowice, Poland

Liao GQ, Wang H, Zhu GY, Zhu KB, Lv FX, Tai S. Management of acquired bronchobiliary fistula: A systematic literature review of 68 cases published in 30 years. *World J Gastroenterol* 2011; 17(33): 3842-3849 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i33/3842.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3842>

INTRODUCTION

Bronchobiliary fistula (BBF) is a rare disorder, first reported by Peacock in 1850^[1]. It consists of abnormal interconnection between the biliary tract and bronchial trees. Unlike congenital bronchobiliary fistula, acquired BBF is usually regarded as a consequence of local infection, such as hydatid or amebic disease of the liver, hepatic abscess, trauma, obstruction of biliary tract and neoplasm^[1-58]. However, management of this condition can be very difficult and is often associated with a high rate of morbidity and mortality. Up to now, there has

been no widely accepted management strategy in this field. We searched the literature to outline the appropriate diagnostic methods and therapeutic options for acquired BBF.

MATERIALS AND METHODS

Data searching strategy

Literature searches were performed in Medline, EMBASE, PHMC and LWW (January 1980–August 2010), using the following keywords: biliobronchial fistula, bronchobiliary fistula, biliary-bronchial fistula, tracheobiliary fistula, hepatobronchial fistula, bronchopleural fistula and biliptysis. Two reviewers searched the literature independently in order to identify articles appropriate for inclusion in this review. Further articles were identified through cross-referencing. As a result, 68 cases were collected and reviewed.

Study criteria

Case reports and case series associated with acquired BBF and full texts of case reports in English were included. Cases did not provide basic information such as age, gender, primary diseases and etiological, clinical manifestation, therapeutic method, outcome and follow-up duration were excluded.

The clinical outcome was defined as cured when clinical symptoms such as biliptysis, fever and jaundice disappeared without use of drainage tube or with closure of the fistula. In addition, an adequate follow-up period (at least 2 mo) was necessary in patients without tumors. Treatment failure was defined as persistence of symptoms, and death due to bronchobiliary fistula or complications from the treatment.

RESULTS

General data

A summary of published case reports in recent 30 years concerning acquired BBF is shown in Table 1^[1–58]. Sixty-eight cases were retrieved. Information regarding the demographics, clinical data and other variables were not reported in a few cases, thus the denominator varies in the following proportion of cases. Forty-two (61%) of the patients were men. The median age of onset was 48.3 years (range, 14–87 years) (Table 1). Fistula involving both lungs has not been reported so far. Only one case reported by Weis^[2] showed that the BBF orifice was opened in the left lung (Figure 1).

Pre-existing conditions

According to the data, BBF secondary to tumors (32.3%, 22/68) including primary tumors (19.1%, 13/68) and hepatic metastases (13.2%, 9/68) shared the largest proportion. Bile duct obstruction (30.8%, 21/68), including biliary stenosis (17.6%, 12/68) and cholangiolithiasis (13.2%, 9/68) took the second position. Although liver tumor or chronic pancreatitis is associated with different degrees of biliary obstruction, it is considered as an independent

etiology. Hepatic hydatidosis had been regarded as the most common primary disease in developing countries for a long time, but only 8 (11.7%, 8/68) cases have been reported in recent 30 years. Other causes were trauma (10.2%, 7/68) and chronic pancreatitis (2.9%, 2/68). One case of hepatic abscess, subphrenic abscess, syphilis gummosa and acute cholecystitis each in a suprahepatic gall bladder as other single primary diseases has been respectively reported. Four cases were complicated with multiple primary diseases in this review (Figure 2).

Symptoms

Biliptysis was presented in all the 68 patients (Figure 3). The volume of scant bile-staining of the sputum and the expectoration of copious volumes of bile ranged from 200 mL to 600 mL and reached a maximum of 1.2 L daily. Respiratory symptoms, such as irritating cough, fever and jaundice, were other clinical features of BBF. More than half of the patients (36/68) presented with fever. The highest body temperature exceeded 39.0 °C. Abdominal pain was present in 14 patients and most in the right upper quadrant, but chest pain occurred in only eight patients. Respiratory disorders occurred in eight cases, including six cases of dyspneic disease. Symptoms like hepatic decompensation, portal hypertension, anorexia, anemia, nausea, vomiting and diabetes were sporadic. Pneumonia as the most common comorbidity was diagnosed in 10 patients, but bacteriologic results were only provided in two cases.

Therapeutic options

Less-invasive procedures had a tendency to be employed in treatment of BBF. Half of the patients underwent therapeutic endoscopy (49.2%, 33/67), including endoscopic retrograde biliary drainage (ERBD) in 24 cases, sphincterotomy in 16 cases, endoscopic nasobiliary drainage (ENBD) in 3 cases and endoscopic stone extraction in three cases. Percutaneous drainage was conducted in 5 cases. Percutaneous transhepatic cholangial drainage (PTCD) was used as a main treatment only in two patients.

Surgical procedures are invasive, and usually used as a final choice. In this review, 28 patients received open operations (41.7%, 28/67), including pulmonary lobectomy, resection and kprosthesis of fistulous tract in diaphragm, heplobectomy, hepaticocenterostomy and abscess drainage alone or in combination.

With the advance of modern medical techniques, histoacryl embolization under bronchoscopic guidance and n-Butyl cyanoacrylate *via* a bronchial approach brought new insights into this field. However, only one case each by the two techniques was reported by Kim JH and Goldman SY, respectively, and both patients died in the following four months because of hepatic failure or cancer^[6,14].

Clinical outcome and follow-up

The outcome at the end of follow-up period (range from 2 wk to 72 mo) was good in most of the 28 cases,

Table 1 Basic data collected from case reports in the literature published over past 30 years

Sex and age	Fistulae orifice in side of lung	Primary diseases	Clinical manifestation				Therapeutic approaches	Outcome	Follow-up duration (mo)
			Cough and bilipyrosis	Fever	Jaundice	Other manifestation and/or comorbidity			
F 79	Left	Biliary stenosis	Yes	No	No	Pneumonia, diabetes, paroxysmal atrial fibrillation, coronary artery disease	Surgery	Cure	NA
M 19	Right	Hepatic hydatidosis	Yes	Yes	No	NA	ERBD	Cure	15
M 35	Right	Cholangiolithiasis	Yes	NA	NA	Abdominal distension and pain	ENBD	Cure	11
M 47	Right	Hepatic hydatidosis	Yes	Yes	NA	Abdominal pain	ERBD	Cure	7
F 18	Right	Trauma	Yes	NA	NA	Diabetes and asthma	ERBD	Cure	12
F 43	Right	Hepatocellular carcinoma	Yes	Yes	NA	Abdominal pain	Percutaneous drainage	Cure	NA
F 56	Right	Liver abscess and biliary stenosis	Yes	Yes	Yes	Pneumonia	Histoacryl embolization under bronchoscopic guidance	Cure but died 3 mo later	3
M 29	Right	Biliary stenosis	Yes	Yes	NA	Pneumonia	Surgery	Cure	NA
M 22	Right	Trauma	Yes	No	NA	NA	ERBD	Cure	5
M 57	Right	Cholangiolithiasis and chronic pancreatitis	Yes	Yes	Yes	Chest pain, pleuritic, alcoholic cirrhosis, diabetes and COPD	ERBD and bronchoalveolar lavage	Cure	NA
M 55	Right	Biliary stenosis	Yes	NA	NA	NA	Surgery	Cure	7
M 41	Right	Hepatic hydatidosis	Yes	Yes	Yes	NA	ERBD	Cure	30
M 35	Right	Hepatic hydatidosis	Yes	Yes	Yes	Dyspnea	Surgery	Cure	12
M 20	Right	Trauma	Yes	NA	NA	NA	Surgery	Cure	NA
M 38	Right	Hepatic hydatidosis	Yes	Yes	Yes	NA	Surgery	Cure	NA
M 66	Right	Hepatocellular carcinoma	Yes	Yes	NA	Pneumonia	Surgery	Cure	NA
F 49	Right	Metastatic liver tumors	Yes	NA	NA	Pneumonia	N-Butyl cyanoacrylate <i>via</i> a bronchial approach	Cure	3
M 30	Right	Liver abscess	Yes	NA	NA	NA	Surgery	Cure	NA
F 64	Right	Biliary stenosis	Yes	Yes	Yes	Hepatomegaly	Percutaneous drainage	Failure	NA
M 65	Right	Metastatic liver tumors	Yes	No	No	Shortness of breath, pleuritic and chest pain	ERBD	Cure but died 3 mo later	3
M 12	Right	Undifferentiated sarcoma	Yes	NA	NA	NA	Surgery	Cure	NA
M 76	Right	Hepatocellular carcinoma	Yes	Yes	Yes	Pneumonia	Surgery	Failure	NA
F 68	Right	Biliary stenosis	Yes	No	No	Pneumonia	ERBD	Cure	24
M 71	Right	Cholangiocarcinoma	yes	Yes	NA	Pneumonia	ERBD	Cure	9
F 63	Right	Biliary stenosis	Yes	NA	NA	NA	Surgery	Cure	NA
M 52	Right	Metastatic liver tumors	Yes	NA	NA	Pneumonia and abdominal pain	Percutaneous drainage	Cure	3
F 55	Right	Hepatocellular carcinoma	Yes	Yes	NA	Dyspnea	ENBD	Cure	3
F 40	Right	Biliary stenosis	Yes	NA	NA	NA	ERBD	Cure	3
M 69	NA	Hepatocellular carcinoma	Yes	NA	Yes	Dyspnea and cirrhosis	ERBD	Cure but died 5 mo later	5
M 44	Right	Hepatocellular carcinoma	Yes	Yes	Yes	Abdominal pain	Surgery	Failure	NA
F 71	Right	Cholangiolithiasis	Yes	Yes	No	Chest pain	ERBD	Cure	NA
F 56	Right	Metastatic liver tumors	Yes	NA	NA	NA	ERBD	Failure	NA
M 65	Right	Metastatic liver tumors and cholangiolithiasis	Yes	NA	NA	Pneumonia	ERBD and Surgery	Cure	7
M 34	Right	Hepatocellular carcinoma	Yes	NA	NA	NA	Surgery	Cure	NA
M 67	Right	Cholangiolithiasis	Yes	Yes	NA	Nausea, vomiting	Percutaneous drainage and ERBD	Cure	NA
M 38	Right	Trauma	Yes	Yes	No	Tachypnoea	ERBD	Cure	NA
M 20	Right	Trauma	Yes	Yes	Yes	Shortness of breath	Surgery	Cure	NA
F 70	Right	Hepatocellular carcinoma	Yes	NA	NA	NA	ENBD	Cure	NA
M 40	Right	Biliary stenosis	Yes	NA	NA	NA	Histoacryl injection through the micro-catheter and ERBD	Cure	9

F 47	Right	Cholangiolithiasis	Yes	Yes	NA	Chest pain and abdominal pain	ERBD	Cure	10
M 61	Right	Carcinosarcoma	Yes	Yes	NA	Chest pain	Surgery	Cure	7
F 46	Right	Subphrenic abscess	Yes	Yes	NA	NA	Surgery	Cure	24
F 46	Right	Metastatic liver tumors	Yes	NA	Yes	NA	ERBD	Cure but died 9 mo later	9
M 56	Right	Metastatic liver tumors	Yes	Yes	Yes	Chest pain	ERBD	Cure	NA
M 61	Right	Biliary stenosis	Yes	NA	Yes	NA	ERBD	Cure but died 5 mo later	NA
F 64	Right	Biliary stenosis	Yes	Yes	Yes	Abdominal pain	ERBD	Cure	2
F 53	Right	Hepatic hydatidosis	Yes	Yes	Yes	Abdominal pain, ascites and respiratory distress	Endoscopic sphincterotomy alone	NA	NA
M 26	Right	Hepatic hydatidosis	Yes	Yes	Yes	Ascites and anemia	ERBD	Cure	NA
M 44	Right	Hepatic hydatidosis	Yes	NA	NA	NA	Surgery	Cure	NA
F 73	Right	Hepatocellular carcinoma	Yes	NA	NA	NA	ERBD	Cure but died 5 mo later	5
F 57	Right	Syphilis gummosa	Yes	Yes	NA	NA	Surgery	Failure	NA
M 54	Right	Chronic pancreatitis	Yes	Yes	NA	Chest pain and diabetes	Surgery	Cure	NA
F 58	Right	Uterine leiomyosarcoma with hepatic metastases	Yes	Yes	Yes	Dyspnea	PTCD	Cure	11
M 61	Right	Cholangiolithiasis	Yes	Yes	Yes	Abdominal pain, dyspnea	Endoscopic stone extraction and sphincterotomy	Cure	NA
F 71	NA	Metastatic liver tumors	Yes	NA	No	Abdominal pain	Surgery	Failure	NA
F 56	NA	Cholangiolithiasis	Yes	NA	NA	NA	Endoscopic sphincterotomy and stone extraction	Cure	NA
F 38	Right	Mucinous adenocarcinoma	Yes	NA	NA	NA	PTCD	Cure	NA
F 87	Right	Cholangiolithiasis	Yes	Yes	NA	NA	Endoscopic stone extraction and sphincterotomy	Cure	NA
M 18	Right	Biliary stenosis	Yes	NA	NA	NA	PTCD and balloon dilation cholangioplasty	Cure	NA
M 47	Right	Hepatic abscess and chronic pancreatitis	Yes	Yes	NA	Abdominal pain	Surgery	Cure	NA
M 63	Right	Acute cholecystitis in a suprahepatic gallbladder	Yes	NA	Yes	Abdominal pain, portosystemic encephalopathy and ascites	Surgery	Failure	NA
M 58	Right	Metastatic liver tumors	Yes	Yes	Yes	Hemoptysis	Surgery	Failure	NA
M 44	Right	Chronic pancreatitis	Yes	NA	NA	NA	Surgery	Cure	NA
M 15	Right	Trauma	Yes	Yes	NA	NA	Surgery	Cure	NA
M 14	Right	Cholangiolithiasis	Yes	Yes	Yes	Chest pain	Surgery	Cure	72
M 45	Right	Cholangiolithiasis	Yes	Yes	NA	Abdominal pain	Surgery	NA	72
F 26	NA	Biliary stenosis	Yes	NA	NA	NA	NA	NA	NA
M 21	Right	Trauma	Yes	NA	NA	Anorexia	Percutaneous drainage	Cure	4

M: Male; F: Female; ERBD: Endoscopic retrograde biliary drainage; ENBD: Endoscopic nasobiliary drainage; PTCD: Percutaneous transhepatic cholangial drainage; NA: Not applicable/available. COPD: Chronic obstructive pulmonary diseases.

and the recovery rate was 87.7% (57/65). Therapeutic endoscopy was safer than surgery, as 96.8% (30/31) *vs* 76.9% (20/26). Intraoperative complications as the cause of death occurred in one patient and recurrent BBF was found in four cases. Among the recurrent cases, one was cured by open surgery, one by percutaneous drainage and the other two cases by ERBD.

DISCUSSION

Local infection has been considered as a classic cause of BBF since Peacock's report in 1850^[1,59]. According to this review, tumor is the major cause of BBF. It should

be emphasized that the disease spectrum is changing. It may be correlated with the development of radical surgery and its complications. Primary tumors were all liver cancers in BBF patients as noticed in this review. Among the seven cases with information of tumor location, only two cases had the tumor near diaphragm surface and one case had diaphragm invasion. Metastatic tumors derived from gastrointestinal tract were reported in nine cases of BBF. The pathogenesis of BBF is various. It may involve the iatrogenic damage, diaphragm invasion, intrahepatic or extrahepatic biliary obstruction and tumor cachexia. Although biliary obstruction is the most common pathogeny in literature^[57], its nature is not easy to

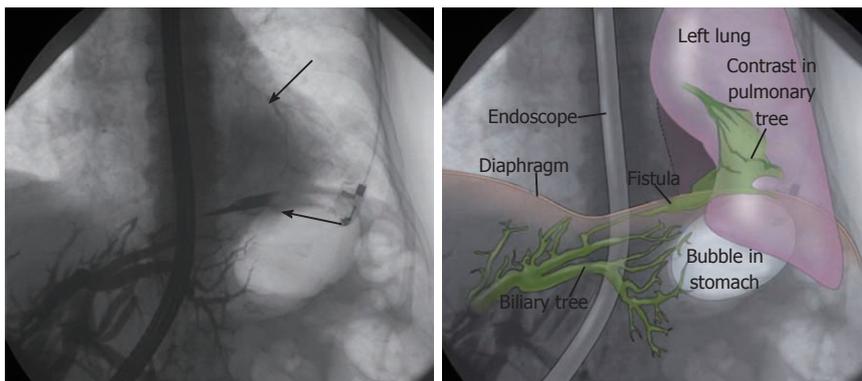


Figure 1 Picture reprint from the article of Weis^[2]. The arrows show the bronchobiliary fistula involving the left lung.

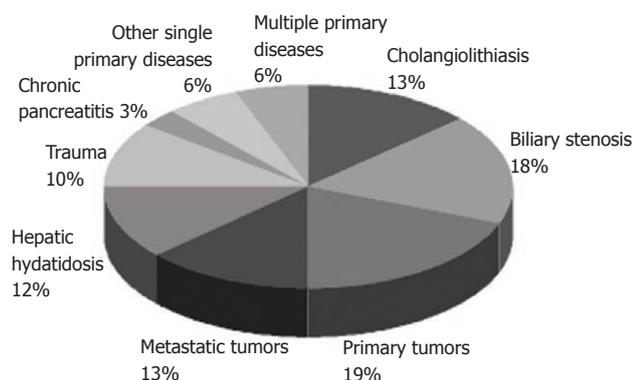


Figure 2 Primary diseases of bronchobiliary fistula. Multiple primary diseases include liver abscess and biliary stenosis, cholangiolithiasis and chronic pancreatitis, metastatic liver tumors and cholangiolithiasis, hepatic abscess and chronic pancreatitis.

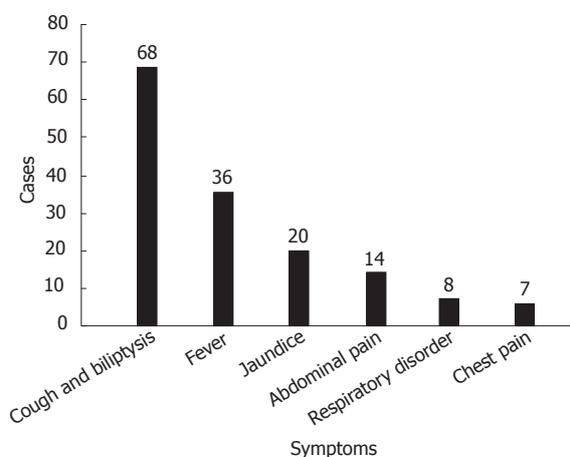


Figure 3 Clinical manifestations of bronchobiliary fistula.

define, and in most cases, it resulted from the presence of severe inflammation close to the primary lesion or in the hilus of the liver. The obstruction of the bile duct is often due to lithiasis, tumor, hydatid cyst, or postoperative stricture.

It should be emphasized that the recent surgery or invasive therapies before bilioptysis appearance was observed in 51 cases (75%). And in seven cases, the operation induced the stenosis of bile duct. It is considered that the stress, especially the iatrogenic damage is correlated with bronchobiliary fistula. We also found a congenital case^[60]. Therefore, we suspect that congenital fragile structure in diaphragma may be the basic pathogenesis in some cases.

Clinical diagnosis is usually made by bilioptysis. In some cases, it is inappropriately diagnosed as acute pneumonitis or chronic irritable cough producing greenish sputum^[59]. Patients having a long history of biliary tract disease can be diagnosed quite easily. However, some patients exhibited minimal signs of this disease. Sutherland *et al*^[61] described false bile ptyalism in patients with sickle cell diseases and hemolytic crisis. In the patients without these conditions, the presence of bile in the sputum is defined as pathognomonic bronchobiliary fistula. Conventionally, endoscopic retrograde cholangio-pancreatography

(ERCP) or percutaneous transhepatic cholangio (PTC) provided direct photographic evidence. This has been the most preferred choice for BBF. But, contrast-enhanced magnetic resonance cholangio (MRC) and hepatobiliary imino-diacetic acid (HIDA) scan not only ensures a definite diagnosis, but also has distinct advantage over other conventional non-invasional techniques^[62,63].

In terms of medical treatment, somatostatin and its analogues were often used for treating BBF, because it reduced its secretion in the gastrointestinal tract. But, up till now, not a single case has been completely cured only with medical treatment^[28]. Many patients complained that posture had an adverse impact on the volume of bilioptysis. But only few doctors considered this factor while treating these patients. Therefore, we suggest that doctors should instruct BBF patients, especially those without biliary obstruction, to refrain from bile postural drainage. In other words, patients should be advised to take orthostatic position and avoid supine position. To alleviate the symptoms and accelerate concrescence of the fistula, bile reflux should be decreased. In addition, electrolyte disturbances and digestive system disturbances occurred when a significant amount of bile is lost. Therefore, supporting therapy should be administered with appropriate prophylactic attention.

Recently, when resolution of a distal biliary obstruction was accomplished, non-surgical interventions *via* ERCP or PTC were successfully conducted. ERBD became much more prevalent. It was considered in more than 60% (24/39) of cases for non-surgical treatment in this review. Based on this review, its single application recovery rate is also much higher than traditional surgery (95.8%:76.9%). Although PTC and ENBD may lead to severe electrolyte disturbances and disturbances of the digestive system triggered by bile loss, it is convenient to recheck the healing of fistula using the radiographic technique with contrast media injection *via* drainage tube. This is practically more advantageous for managing the disease. In the past few years, histoacryl embolization under bronchoscopic guidance or the n-Butyl Cyanoacrylate *via* a bronchial approach were reported as new therapeutic methods^[6,14]. However, these methods should be proved by more clinical cases.

The open surgery should be the first choice when interventional techniques have failed or BBF secondary to tumors, biliary obstruction and trauma occurred. The type of operation depends on the primary tumor type, BBF location and involvement. The following surgical procedures were performed in BBF: drainage of right subphrenic or hepatic abscess, closure of fistula, resection of hydatid cysts or tumor, biliary drainage using T-tube, and bilioenteric anastomoses (e.g., Roux-en-Y hepaticojejunostomy). In case of diaphragmatic, pleural, bronchial, or pulmonary damages, closure of the diaphragm, pleural drainage, decortication or different pulmonary resections have been used. Gugenheim *et al.*^[59] recommended a two-stage approach for treating BBF: (1) external biliary drainage by percutaneous or surgical drainage of subphrenic abscess and/or direct percutaneous drainage of the intrahepatic biliary tract; and (2) treatment of the underlying cause. In the patients with biliary obstruction, the priority management was to treat the biliary disease.

Early diagnosis and treatment can alleviate the patient's sufferings. But there has been no evidence as to whether they can improve the prognosis of these patients. We have not obtained any information about the prevention of this disease.

In conclusion, we reviewed limited information of BBF published in the past 30 years. We could not completely understand its etiology and pathogenesis. The published experience suggests that ERCP or PTC should be a priority. But new diagnostic techniques such as contrast-enhanced MRC and HIDA scan have greater advantages. While considering the medical treatment, quite a few doctors have advocated non-operative therapy as the preferred choice. Individualized and multidisciplinary treatment should be emphasized in patients with primary diseases as the condition of each individual patient is quite different and complex.

COMMENTS

Background

Bronchobiliary fistula (BBF) is an uncommon disorder involving biliary channels

and the bronchial tree. Acquired BBF without proper management can induce death. With the improvement of non-invasive approaches, more satisfying outcome can be expected.

Research frontiers

Increasing non-invasive and less-invasive approaches to the management of acquired BBF can offer promising benefits to the patients with this condition. In this literature review, the authors point out that the definite procedure such as surgery should be considered only when the non-invasive methods failed.

Innovations and breakthroughs

Invasive procedure such as surgical excision of the fistula has been used widely, while less-invasive methods like external and internal stenting which can reduce biliary obstruction are of more value, since they are much safer and easier. The authors of this review searched the literature, provided more diagnostic and therapeutic options in management of acquired BBF.

Applications

BBF is rare and easily misdiagnosed as respiratory disease. Patients with this disease usually had poor outcome. However, there has been no guideline in BBF treatment up to date. This study reviewed the management strategy of BBF and summarized the experience from literatures in the past 30 years. It may contribute to the clinical treatment of BBF.

Terminology

Hepatobiliary imino-diacetic acid scan is a nuclear imaging procedure to evaluate the status of the gallbladder. N-Butyl cyanoacrylate is a tissue adhesive that applied as a monomer to moist tissue and polymerizes to form a bond. It is biodegradable slowly and used in all kinds of surgeries.

Peer review

This paper is a review of the world literature regarding BBF. Authors presented a spectrum of case reports concerning BBF. Idea of the summary about the knowledge of BBF is interesting.

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S- Editor Tian L L- Editor Ma JY E- Editor Li JY

Enhanced CT and CT virtual endoscopy in diagnosis of heterotopic pancreas

Dan Wang, Xiao-Er Wei, Lei Yan, Yu-Zhen Zhang, Wen-Bin Li

Dan Wang, Xiao-Er Wei, Lei Yan, Yu-Zhen Zhang, Wen-Bin Li, Institute of Diagnostic and Interventional Radiology, The Sixth Affiliated People's Hospital, Shanghai Jiao Tong University, Shanghai 200233, China

Author contributions: Wang D, Yan L and Zhang YZ collected the cases; Li WB analyzed the data; Wang D and Wei XE performed the post-processing software work and wrote the paper. Supported by Science and Technology Commission of Shanghai Municipality, Grant No. 08411951200

Correspondence to: Dr. Wen-Bin Li, MD, PhD, Professor, Vice Director of the Shanghai Jiaotong University Medical Imaging Institute, Institute of Diagnostic and Interventional Radiology, The Sixth Affiliated People's Hospital, Shanghai Jiao Tong University, No. 600, Yishan Road, Shanghai 200233, China. liwenbin@sh163.net

Telephone: +86-21-64369181-8993 Fax: +86-21-64844183

Received: January 24, 2011 Revised: February 22, 2011

Accepted: February 28, 2011

Published online: September 7, 2011

Abstract

AIM: To improve the diagnosis of heterotopic pancreas by the use of contrast-enhanced computed tomography (CT) imaging and CT virtual endoscopy (CTVE).

METHODS: A total of six patients with heterotopic pancreas, as confirmed by clinical pathology and immunohistochemistry in the Sixth Affiliated People's Hospital of Shanghai Jiao Tong University, Shanghai, China, were included. Non-enhanced CT and enhanced CT scanning were performed, and the resulting images were reviewed and analyzed using three-dimensional post-processing software, including CTVE.

RESULTS: Four males and two females were enrolled. Several heterotopic pancreas sites were involved; three occurred in the stomach, including the gastric antrum ($n = 2$) and lesser curvature ($n = 1$), and two were in the duodenal bulb. Only one case of heterotopic pancreas lesion occurred in the mesentery. Four cases had a solid yet soft tissue density that had a homogeneous pat-

tern when viewed by enhanced CT. Additionally, their CT values were similar to that of the pancreas. The ducts of the heterotopic pancreas tissue, one of the characteristic CT features of heterotopic pancreas tissue, were detected in the CT images of two patients. CTVE images showed normal mucosa around the tissue, which is also an important indicator of a heterotopic pancreas. However, none of the CTVE images showed the typical signs of central dimpling or umbilication.

CONCLUSION: CT, enhanced CT and CTVE techniques provide useful information about the location, growth pattern, vascularity, and condition of the gastrointestinal wall around heterotopic pancreatic tissue.

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Key words: Heterotopic pancreas; Computed tomography; Contrast enhancement; Computed tomography virtual endoscopy

Peer reviewers: Edward L Bradley III, MD, Professor of Surgery, Department of Clinical Science, Florida State University College of Medicine, 1600 Baywood Way, Sarasota, FL 34231, United States; Sara Regnér, MD, PhD, Department of Surgery, Institution of Clinical Sciences Malmö, Malmö University Hospital, Ing 82B, Universitetssjukhuset MAS, Malmö, SE-205 02, Sweden; Clement W Imrie, Professor, BSc(Hons), MB, ChB, FRCS, Lister Dept of Surgery, Glasgow Royal Infirmary, 11 Penrith Avenue, G46 6LU, Glasgow, United Kingdom

Wang D, Wei XE, Yan L, Zhang YZ, Li WB. Enhanced CT and CT virtual endoscopy in diagnosis of heterotopic pancreas. *World J Gastroenterol* 2011; 17(33): 3850-3855 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i33/3850.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3850>

INTRODUCTION

Heterotopic pancreas is a condition in which pancreatic tissue is found outside the boundaries of the normal pan-

Table 1 Clinical and computed tomography scanning data for the six patients with heterotopic pancreas

Case	Gender	Age	Symptom	Location	Growth pattern and modality
1	M	60 yr	Carcinoid syndrome	Duodenal bulb	Exogenous, superficially lobulated, well-defined border, subserosal outer boundary was rough
2	F	59 yr	Epigastric pain (3 wk)	Gastric antrum	Circumscribed tissue, ill-defined border
3	M	68 yr	Epigastric pain (1 yr)	Gastric antrum	Circumscribed tissue, superficially lobulated, ill-defined border
4	F	3 mo	Identified during choledochal cystectomy	Mesentery	No obvious abnormality detected by computed tomography
5	M	60 yr	Abdominal distension (2 mo)	Lesser curvature aspect of gastric antrum	Superficially lobulated, well-defined border
6	M	38 yr	Cachexia	Duodenal bulb	Exogenous, well-defined border

creas; such tissue is regarded as aberrant pancreas or an accessory pancreatic lesion. This tissue has no anatomical, vascular or neuronal connection with the main pancreas. Such anomalies can become apparent at any age, but they are most commonly found in the fourth, fifth and sixth decades of life, with a slight male predominance. Heterotopic pancreas frequently occurs in association with the gastrointestinal tract (stomach, duodenum, jejunum, ileum) but is also found associated with Meckel's diverticulum, mesentery, omentum, spleen, and gallbladder^[1]. In mice, the formation of a heterotopic pancreas may be caused by inactivation of the gene *IPF-1* (also known as *IDX-1*, *STF-1* or *PDX*), which leads to errors in embryological development that can result in the total absence of the pancreas^[1]. Symptoms are dependent on the location of the ectopic tissue, although the most common symptoms are epigastric pain, pyloric obstruction, cholecystitis, and intussusception^[1,2]. Some cases are identified as a result of such symptoms, but others are identified incidentally, such as during an unrelated surgery or during autopsy^[1,3,4].

Cases of heterotopic pancreas have been reported in recent years following descriptions of the general characteristics of the condition observed by endoscopic ultrasonography (EUS). However, recent improvements in multi-slice computed tomography (CT) technology and the wide use of enhanced CT scanning have provided a new approach for identifying heterotopic pancreas. In this study, we present six cases of heterotopic pancreas and highlight the associated CT features^[5].

MATERIALS AND METHODS

Patients

We retrospectively analyzed our database of all patients (about 350 000 patients) who underwent CT scanning at the Shanghai 6th People's Hospital Affiliated with Shanghai Jiao Tong University from January 2000 to December 2010. Six cases (four males, two females) were selected who were definitively diagnosed with heterotopic pancreatic tissue postoperatively by pathological examination and immunohistochemistry. With the exception of one patient aged 3 mo and another aged 38 years, the subjects were all aged between 59 and 68 years (Table 1). Three

patients complained of epigastric pain or abdominal distension, and two others presented with cachexia or carcinoid syndrome. The other case was identified incidentally (Table 1). This study was reviewed and approved by the Shanghai 6th People's Hospital Affiliated with Shanghai Jiao Tong University.

CT scanning procedure

Multi-slice CT scanning was performed in all of the six cases using the LightSpeed VCT (GE) or Sensation CT (SIEMENS). No abnormality was detected in one of the female patients who therefore did not undergo contrast-enhanced CT scanning. The other five cases underwent enhanced CT scanning after standard CT scanning. With the exception of the 3-mo-old infant, the patients were asked to hold their breath during the scan in order to reduce artifacts. The thickness of the scanning slices was either 5 mm or 7 mm. The CT data of some patients was further refined using reconstruction software, which enabled thinner slice data to be obtained. The reconstruction software was all supplied by multi-slice CT; this was one function of the multi-slice CT. After the reconstruction the thinnest slice was 0.625 mm.

Each patient received the contrast agent iopromide (dose, 50-70 mL) through the median cubital vein. The arterial phase scan was produced approximately 30 s after the start of the injection. Coronal and sagittal images were obtained, and scanning data were analyzed on the GE workstation ADW 4.3. Lesion sizes were measured, and CT values were calculated (Table 1). Images were analyzed by application of three-dimensional post-processing software, including CT virtual endoscopy (CTVE).

RESULTS

Clinical findings

The six patients comprised four males (aged 38, 60, 60 and 69 years) and two females (aged 3 mo and 59 years). All five adult patients had experienced symptoms over a period of time ranging from weeks to years. Three of the patients presented with symptoms that were directly indicative of gastrointestinal disease, which made the lesions

Table 2 Detailed data from non-enhanced and enhanced computed tomography images of five of the six patients with heterotopic pancreas

Case	Lesion dimensions (cm ³)	CT scanning		Enhanced CT scanning		Properties	Enhancement pattern
		Lesion	Pancreas	Lesion ¹	Pancreas ¹		
1	1.8 × 1.2 × 2.1	38.2	44.3	93.2	97.4	Solid	Homogeneous, highly enhanced
2	1.2 × 1.0 × 2.1	28.2	44.7	80	106.9	Solid	Homogeneous, slightly enhanced
3	2.0 × 1.3 × 2.0	6.9	41.5	13.5	95.5	Solid	Heterogeneous, slightly enhanced
5	1.7 × 1.9 × 2.0	-47.8	47.6	-35.8	93.4	Cystic with density of fat	Heterogeneous, slightly enhanced
6	1.9 × 1.3 × 1.0	43.3	47.1	29.1	106.6	Solid	Homogeneous, highly enhanced

Hu is the unit value for computed tomography (CT). ¹The CT values of both the lesion and the pancreas were measured during the arterial phase.

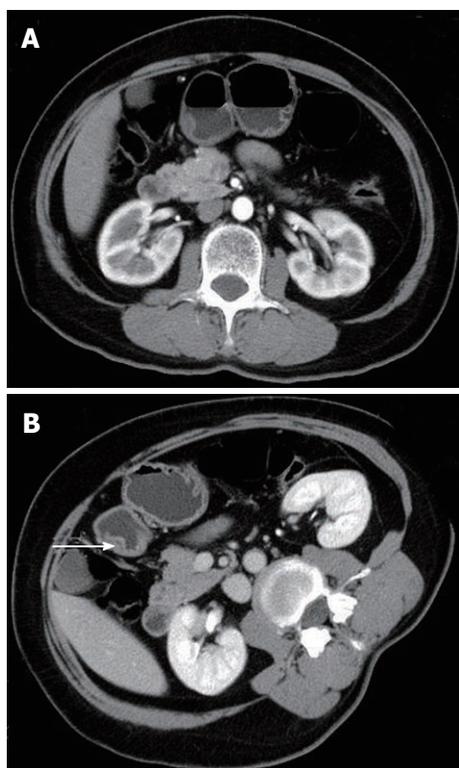


Figure 1 Heterotopic pancreas in the gastric antrum of a 59-year-old woman. A: Arterial phase image showing gastric antrum wall thickening. There is a circumscribed border of the lesion below the mucosal layer. The density at the center of the lesion is slightly lower than at the periphery. B: Each patient was asked to lie on their right side during venous phase scanning. The central area of the low-density region (white arrow) is obvious.

simpler to identify. However, two of the patients had non-specific symptoms, including carcinoid syndrome or cachexia associated with other systemic diseases. As these symptoms did not directly indicate a heterotopic pancreas, the lesions were more difficult to identify. They were discovered after physical examination and other tests. The 3-mo-old girl was jaundiced, and although CT scanning revealed the presence of a choledochal cyst, the heterotopic pancreatic tissue that indeed was later found to be

present in the mesentery was not detected (Table 1).

CT techniques

The CT values of the heterotopic pancreatic tissues were calculated, and these ranged from -47.8 to 43.3 Hu (Table 2). The density measured in four cases was indicative of soft tissue, and this density correlated with the fatty tissue in only one case. The lesions were frequently superficially lobulated, with circumscribed borders, and they protruded into the gastrointestinal cavity or peritoneal cavity. After carrying out contrast-enhanced CT, the CT value of each lesion was based on the arterial phase scanning data. The three soft tissue lesions were homogeneously enhanced to varying extents. The other two lesions were heterogeneously enhanced (Table 2).

The scanning data were analyzed in depth using the GE workstation. The lesions could be seen from any angle in addition to the traditional axial, coronal, and sagittal views. Post-processing software was used to reveal further detail. In two cases, there was a strip of low density, which could be seen in both the axial and sagittal images; this may have represented the duct of the heterotopic pancreas (Figures 1 and 2).

CTVE imaging of each entire lesion revealed structural features of the mass, such as a spherical shape and superficial lobulation, amongst others. This also aided in identifying the layer of the gastrointestinal wall in which the lesion was located, and it showed that the mucosa around the lesion was normal (Figure 3).

DISCUSSION

The incidence of heterotopic pancreas is low, with only 40% of patients experiencing symptoms and 60% of cases being found incidentally during surgery for other disorders^[6-9]. Although CT scanning is a highly sensitive technique, it can still be difficult to detect this abnormality. In this study, a heterotopic pancreas was detected in five of six patients by means of CT scanning. Moreover, contrast-enhanced CT scanning should always be per-

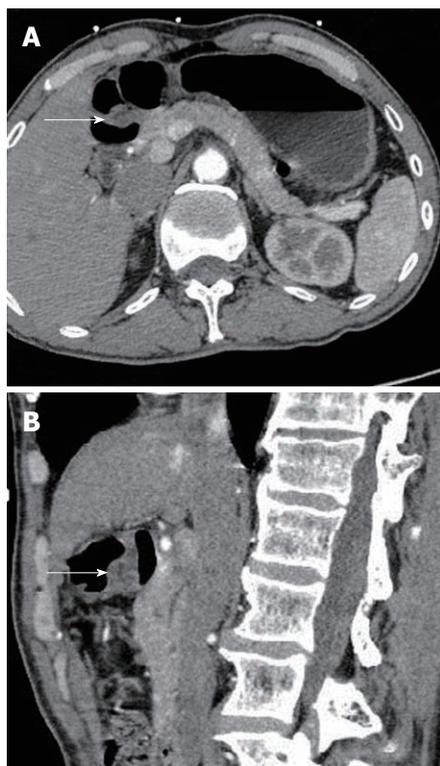


Figure 2 Heterotopic pancreas in the gastric antrum of a 68-year-old woman; the lesion is protruding into the stomach cavity. This heterotopic pancreas is of lower density than the pancreas, is heterogeneous, and slightly enhanced. In both the axial (A) and sagittal images (B), there is a low-density strip (white arrow) in the center of the lesion, which may represent the duct of the heterotopic pancreas. From the sagittal image (B), it can be seen that although the heterotopic pancreas tissue lies in the gastric antrum wall, the wall is still well defined. This suggests that the lesion does not violate the surrounding tissues and organs.

formed, when possible. Heterotopic pancreatic tissue was not observed in only one patient - the infant. For that patient, we postoperatively analyzed the CT images, but the lesion was still undetectable. As the intestines had expanded due to gas and liquid accumulation, as is commonly seen in infants, some of the intestines congregated at the mesenteric root.

In this study, 83.3% (five of six lesions) of the heterotopic pancreas lesions we observed occurred in the stomach, duodenum, and jejunum. In the stomach, 80%-90% of the lesions occurred in the antrum, within 5 to 6 cm of the pylorus^[1,10,11]. Two of the three lesions that occurred in the stomach were located in the antrum (Figure 1). Heterotopic pancreas rarely occurs outside the gastrointestinal wall in tissues such as the liver, lung, omentum, mesentery, umbilicus, mediastinum, and fallopian tube^[1,11-14].

Histologically, heterotopic pancreas is composed of ductal components, acinar cells, and islet cells. Because of differences in the proportions of the three components, however, the density of heterotopic pancreas tissue can vary^[15]. In our study, lesions in four cases were composed of solid soft tissue, which was the commonest type. With the borders being defined to a variable extent, the density was similar to that of the main pancreas in 75% (3/4)

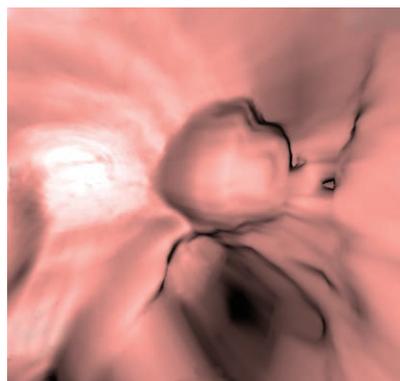


Figure 3 Heterotopic pancreas at the lesser curvature aspect of a 60-year-old man, the lesion is located below the mucosal layer. Computed tomography virtual endoscopy image showing that the lesion is round and superficially lobulated with a depression on one side.

of the cases of this type. Both the non-enhanced and contrast-enhanced CT images were analyzed, and the CT value of each lesion was measured (Table 2). These tissues were also homogeneously enhanced and were similar to the pancreas^[16]. Only one case had a heterogeneous enhancement density that was typical of fatty tissue, and this was initially misdiagnosed as a lipoma. Therefore, lesions of the gastrointestinal tract having a density equivalent to fat should be additionally screened for a potential diagnosis of heterotopic pancreas.

Besides the density types discussed above, there is also a less common mixed-density cyst/solid type that was not observed in our study. This has also been considered as a complication of heterotopic pancreas^[7,17]. Kim *et al.*^[14] reported that heterotopic pancreas with predominantly pancreatic acini shows a homogeneous enhancement pattern, whereas lesions with a mixed composition of acini and ducts show a heterogeneous enhancement. In our study, two cases showed a low-density strip of tissue with a well-defined border in the middle of the heterotopic pancreas lesion. In one case, the low-density strip was peripherally enhanced and so may have been the duct of the heterotopic pancreas^[13].

Further thin-slice image data were obtained using the multi-slice CT, showing more details of each lesion, such as the interior density, enhancement pattern, location, and the duct. The thin-slice images also yielded high-quality two-dimensional and three-dimensional representations. With selection of the optimal viewing angle for the two-dimensional image, the low-density strip was shown to a greater extent; the presence of this strip is thus one of the characteristics of a heterotopic pancreas. Using EUS, Ryu *et al.*^[18] also identified the anechoic duct structure in some cases of heterotopic pancreas. Other work has shown that EUS may be more useful than CT in visualizing the pancreatic ducts^[19]. In our study, however, the very small, narrow ducts were detectable only upon analysis of thin-slice two-dimensional contrast-enhanced images, and they were not detectable by EUS in the same patients. Therefore, we found that CT images were more sensitive

for detecting the heterotopic pancreas duct.

CTVE is a three-dimensional display technology used in the post-processing of CT scanning data to reconstruct three-dimensional cavity surface images of hollow organs, and it provides images that are similar in detail to those obtained by endoscopy (Figure 3). The mucosa around all lesions we observed was normal. The gastrointestinal mucosa seemed to be undamaged and enhanced^[14]. This was an important sign in the diagnosis of heterotopic pancreas.

The features of the heterotopic pancreas have been confirmed by endoscopy^[20]. The lesions we observed were superficially lobulated, but none of the cases in our study exhibited typical heterotopic pancreas features, such as central dimpling or umbilication^[21]. Notably, Ryu *et al.*^[18] also did not observe such umbilication. Hazzan *et al.*^[10] suggested that, although central umbilication of the lesion is one of the characteristic features of a heterotopic pancreas, it is difficult to diagnose because umbilication is often absent in tumors of less than 1.5 cm in diameter. This may explain why umbilication was not observed in our study, i.e., because the lesions were small.

In conclusion, CT scanning, contrast-enhanced CT scanning and CTVE provide useful information about heterotopic pancreas tissue and reveal some of its characteristic features. This combined-technique approach represents a novel way of recognizing and diagnosing the disease. Although these techniques have some limitations, they have been shown to be beneficial for preoperative diagnosis of heterotopic pancreas and therefore may influence the choice of surgical procedure. Resection of heterotopic pancreas tissue is advisable in order to avoid later complications and a second operation^[22,23,25].

COMMENTS

Background

Heterotopic pancreas has a low incidence, and most affected individuals are asymptomatic. Previous reports describe the identification of heterotopic pancreas through endoscopic ultrasonography, but few studies have focused on the potential benefits of computed tomography (CT) and contrast-enhanced images. With the improvement in multi-slice CT technology and the widely used post-processing software, this approach provides a new way to detect the features of heterotopic pancreas.

Research frontiers

In recent years, an increasing number of studies have focused on the application of contrast-enhanced CT scanning, post-processing software, and Computed tomography virtual endoscopy (CTVE) technology for the diagnosis of heterotopic pancreas.

Innovations and breakthroughs

CT scanning revealed the location and enhanced features of the lesion. CTVE showed the location, size, and shape of lesions as well as the organs with which they were associated; it also could visualize lumen stenosis and the surrounding mucosa. CTVE is also a reliable way to show the duct of heterotopic pancreas tissue. The advantages of this approach are that it is non-invasive and can reveal the extent of disease.

Applications

The findings of this study will be advantageous for the preoperative diagnosis of heterotopic pancreas. The approach also demonstrates a new use of CTVE.

Peer review

It is an interesting proposition, even though founded on retrospective analysis.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Li JY

A case of gas gangrene in an immunosuppressed Crohn's patient

Natalie Kiel, Vincent Ho, Andrew Pascoe

Natalie Kiel, Vincent Ho, Andrew Pascoe, Department of Gastroenterology, Princess Alexandra Hospital, Brisbane, 4102 Queensland, Australia

Author contributions: Kiel N, Ho V and Pascoe A wrote the case report.

Correspondence to: Dr. Natalie Kiel, MBBS, BSc, Department of Gastroenterology, Princess Alexandra Hospital, Brisbane, 4102 Queensland, Australia. natalie_kiel@health.qld.gov.au

Telephone: +61-73176-2111 Fax: +61-73176-7366
Received: February 19, 2011 Revised: March 26, 2011

Accepted: April 2, 2011

Published online: September 7, 2011

Abstract

Clostridium septicum (*C. septicum*) gas gangrene is well documented in the literature, typically in the setting of trauma or immunosuppression. In this paper, we report a unique case of spontaneous clostridial myonecrosis in a patient with Crohn's disease and sulfasalazine-induced neutropenia. The patient presented with left thigh pain, vomiting and diarrhea. Blood tests demonstrated a profound neutropenia, and magnetic resonance imaging of the thigh confirmed extensive myonecrosis. The patient underwent emergency hip disarticulation, followed by hemicolectomy. *C. septicum* was cultured from the blood. Following completion of antibiotic therapy, the patient developed myonecrosis of the right pectoral muscle necessitating further debridement, and remains on lifelong prophylactic antibiotic therapy.

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Key words: Crohn's disease; Inflammatory bowel disease; Sulfasalazine; Neutropenia; *Clostridium septicum*

Peer reviewer: Wolfgang R Stremmel, Professor, Department of Gastroenterology, University Hospital Heidelberg, Medizinische Universitätsklinik, Heidelberg 49120, Germany

Kiel N, Ho V, Pascoe A. A case of gas gangrene in an immunosuppressed Crohn's patient. *World J Gastroenterol* 2011; 17(33): 3856-3858 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i33/3856.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i33.3856>

INTRODUCTION

Clostridium septicum (*C. septicum*) gas gangrene is not uncommonly described in the literature, invariably in the immunosuppressed patient. Though historically described as a complication of war wounds, in the present time it is usually associated with postoperative infections^[1]. Crohn's disease is an inflammatory bowel disease with variable luminal and extra-luminal manifestations. Because of the autoimmune nature of the disease, treatment is immunomodulatory in nature and common side effects include marrow suppression. We describe the development of *C. septicum* gas gangrene in a patient with Crohn's disease, who was neutropenic as a result of treatment with sulfasalazine.

CASE REPORT

A 26-year-old man presented to the emergency department with the complaint of a few hours of feeling unwell with vomiting, diarrhea and pain in the left anterolateral thigh. His background history included longstanding diarrhea secondary to Crohn's colitis diagnosed by colonoscopy 2 mo previously. The colitis was predominantly cecal, and the patient had been commenced on sulfasalazine treatment. Sulfasalazine had however been stopped 2 wk prior to presentation due to lack of efficacy, and prednisone was substituted.

On initial examination the patient looked unwell and was tachycardic (150 bpm), though afebrile and normotensive. Respiratory and abdominal examinations were unremarkable, and the left anterolateral thigh was tender

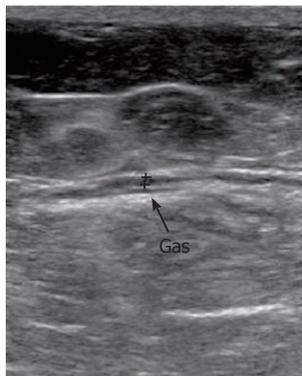


Figure 1 Ultrasound scan of left anterior thigh demonstrating gas. Princess Alexandra Radiology Department, 2007.

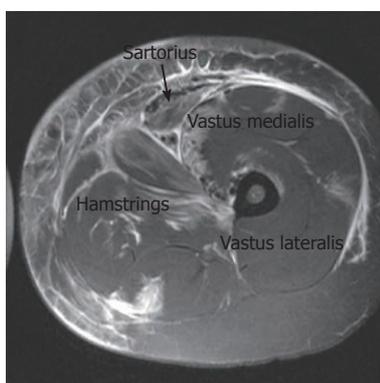


Figure 2 T2 fat suppressed magnetic resonance imaging of the left thigh, demonstrating changes of necrotizing myositis and gas formation. Princess Alexandra Hospital Radiology Department, 2007.

to palpation but otherwise normal in appearance. An initial ultrasound scan of the thigh also excluded any abnormality. Within 2 h the patient developed a fever to 38.5 °C and marked erythema of the left thigh with pustular formation. Initial laboratory investigations demonstrated a profound neutropenia (neutrophil count $0.2 \times 10^9/L$, white blood cell count $1.6 \times 10^9/L$) and myoglobinuria. An urgent repeat ultrasound scan of the anteromedial aspect of the left thigh demonstrated several small collections corresponding to pustule sites on the skin and visualized free gas near the obturator foramen (Figure 1). Magnetic resonance imaging confirmed extensive necrotic myositis and extensive gas formation in the left vastus medialis down to the extensor tendon and neurovascular bundle and in the rectus femoris (Figure 2). A presumptive diagnosis of infective myonecrosis was made. The patient underwent emergency left hip disarticulation and exploratory laparotomy. Two days later an abdominal computed tomography (CT) scan was performed for investigation of induration of the abdominal wall, and demonstrated cecal edema with free gas in the cecal wall. Right hemicolectomy was then performed for colitis and gangrene. *C. septicum* was cultured from blood samples taken at initial presentation. Antibiotic treatment of intravenous lincomycin, meropenem and penicillin was given.

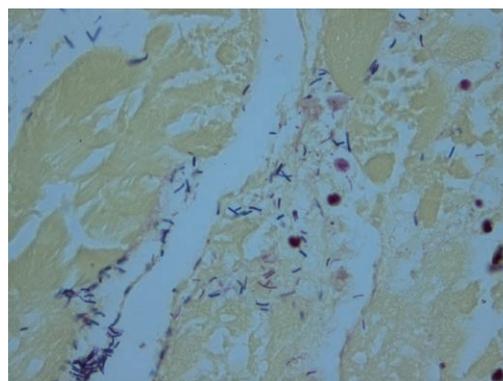


Figure 3 Necrotic chest wall muscle, with *Clostridium septicum* species. Pathology Queensland, Queensland Health-Princess Alexandra Hospital, 2007.

Routine hip stump washouts were performed and excluded progression of the necrotizing myositis. His recovery was complicated by a right pleural effusion requiring drainage. The patient was discharged from hospital on oral amoxicillin after a 3-wk stay. Two weeks after completion of the amoxicillin treatment the patient again presented with pain and erythema over the right pectoral muscle. A CT scan demonstrated an enlarged right pectoralis major muscle with inflammation of the overlying subcutaneous fat. The patient was taken to theatre where the necrotic pectoralis major muscle was debrided. *C. septicum* was cultured from the intraoperative specimen (Figure 3). Antibiotic treatment during the perioperative period was intravenous benzylpenicillin, and the patient was also treated with hyperbaric oxygen therapy postoperatively. He had an uneventful recovery, and now remains on lifelong oral ampicillin prophylaxis.

DISCUSSION

C. septicum is a large, spore-forming, gram-positive anaerobic bacillus^[2-4] found in the gastrointestinal tract of approximately 2% of the healthy population^[5]. *C. septicum* is typically found in the cecum and ileocecal area, where factors including poor vascular supply, local redox potential, pH and the osmotic and electrolyte environment provide an environment conducive to proliferation^[5-7]. The rapid proliferation and systemic toxicity that *C. septicum* causes is thought to be attributed to the production of four exotoxins^[4]. The alpha toxin causes intravascular hemolysis, necrosis of host tissue, and increases capillary permeability, thus producing tachycardia and hypotension^[2]. A hallmark of clostridial myonecrosis is a paucity of inflammatory cells in the affected tissue^[2,5,8]. The necrotic process spreads rapidly to adjacent healthy tissue, causing massive necrotizing gangrene within hours^[2].

C. septicum gas gangrene can be traumatic or non-traumatic. Traumatic gas gangrene historically complicated war wounds, and is now usually associated with postoperative infections^[1]. Non-traumatic clostridial infections nearly always occur in the immunosuppressed patient,

commonly in the setting of hematological or gastrointestinal cancer^[5,7]. Diagnosis in a patient with spontaneous myonecrosis is often difficult as systemic symptoms are vague, and early localized findings can be mistaken for cellulitis. However, in a novel presentation, the triad of pain, tachycardia out of proportion to fever and crepitus is highly suggestive of clostridial myonecrosis^[2,5].

We propose that in our patient, the ulcerated cecum allowed entry of *C. septicum* into the abdominal cavity, and this in concert with neutropenia secondary to sulfasalazine provided an environment predisposing to rapid proliferation of the bacilli.

Sulfasalazine is a medication commonly used in the treatment of inflammatory bowel disease, and a not uncommon complication is neutropenia. Mortality is high in *Clostridium* sepsis in the neutropenic milieu, and death usually occurs within 24 to 48 h^[4,8]. For this reason, early diagnosis followed by immediate, aggressive surgical debridement is critical to survival^[2,9]. It is therefore advisable that blood counts be monitored in patients who are commenced on this treatment, and that neutropenic patients receive emergency management should symptoms of sepsis arise.

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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.

Antonio Basoli, Professor, General Surgery "Paride Stefanini", Università di Roma-Sapienza, Viale del Policlinico 155, Roma 00161, Italy

Chris Briggs, MB ChB, MRCS(Ed), Department of Hepatobiliary and Pancreatic Surgery, K Floor, Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF, United Kingdom

Jeff Butterworth, MB, FRCP, Dr, Department of Gastroenterology, Shrewsbury and Telford Hospital NHS Trust, Mytton Oak Road, Shrewsbury, Shropshire SY3 8XQ, United Kingdom

Herwig R Cerwenka, Professor, Department of Surgery, Medical University of Graz, Auenbruggerplatz 29, A-8036 Graz, Austria

Patrick O Dwyer, MB, BCh, BAO, FRCS (1), MCh, FRCS (Glasg), University Department of Surgery, Western Infirmary, Glasgow G11 6NT, United Kingdom

Yasuhiro Fujino, MD, PhD, Director, Department of Surgery, Hyogo Cancer Center, 13-70 Kitaoji-cho, Akashi 673-8558, Japan

Fabio Grizzi, PhD, Laboratories of Quantitative Medicine, Istituto Clinico Humanitas IRCCS, Via Manzoni 56, 20089 Rozzano, Milan, Italy

Atsushi Irisawa, MD, PhD, Professor, Department of Gastroenterology, Fukushima Medical University Aizu Medical Center, 10-75, Aizuwakamatsu City, Fukushima 965-8555, Japan

Jae J Kim, MD, PhD, Associate Professor, Department of Medicine, Samsung Medical Center, Sungkyunkwan University

School of Medicine, 50, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

Weekitt Kittisupamongkol, MD, Hua Chiew Hospital, 665 Bumrungruang Road, Bangkok 10100, Thailand

I-Rue Lai, Assistant Professor, Department of Anatomy and Cell Biology, Medical College, National Taiwan University, 7, Chun-San S Rd, Taipei 106, Taiwan, China

Min-Hsiung Pan, PhD, Professor, Department of Seafood Science, National Kaohsiung Marine University, No.142, Haijhuang Rd, Nanzih District, Kaohsiung 81143, Taiwan, China

Nageshwar D Reddy, Professor, Asian Institute of Gastroenterology, 6-3-652, Somajiguda, Hyderabad 500 082, India

Robert V Rege, MD, Department of Surgery, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas, TX 75390-9031, United States

Nathan Subramaniam, PhD, Associate Professor, Membrane Transport Laboratory, Queensland Institute of Medical Research, 300 Herston Road, Herston, Brisbane, QLD 4006, Australia

Orhan Sezgin, Professor, Gastroenteroloji Bilim Dalı, Mersin Üniversitesi Tıp Fakültesi, Mersin 33190, Turkey

Scott Steele, MD, FACS, FASCRS, Chief, Colon and Rectal Surgery, Dept of Surgery, Madigan Army Medical Center, Fort Lewis, WA 98431, United States

Andi Utama, PhD, Molecular Epidemiology Division, Mochtar Riady Institute For Nanotechnology, Jl Boulevard Jend Sudirman 1688, Lippo Karawaci-Tangerang, Banten 15810, Indonesia

Robert Christiaan Verdonk, MD, PhD, Department of Gastroenterology and Hepatology, University Medical Centre Groningen, Hanzeplein 1, Groningen, 9700 RB, The Netherlands

Thomas Wex, PhD, Clinic of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University Magdeburg, Leipziger Str. 44, Magdeburg 39120, Germany

Events Calendar 2011

- January 14-15, 2011
 AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States
- January 20-22, 2011
 Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States
- January 27-28, 2011
 Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany
- January 28-29, 2011
 9. Gastro Forum München, Munich, Germany
- February 4-5, 2011
 13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany
- February 13-27, 2011
 Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia
- February 17-20, 2011
 APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver Bangkok, Thailand
- February 22, 2011-March 04, 2011
 Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada
- February 24-26, 2011
 Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland
- February 24-26, 2011
 2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil
- February 24-26, 2011
 International Colorectal Disease Symposium 2011, Hong Kong, China
- February 26-March 1, 2011
 Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada
- February 28-March 1, 2011
 Childhood & Adolescent Obesity:
- A whole-system strategic approach, Abu Dhabi, United Arab Emirates
- March 3-5, 2011
 42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States
- March 7-11, 2011
 Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States
- March 14-17, 2011
 British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom
- March 17-19, 2011
 41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany
- March 17-20, 2011
 Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States
- March 18, 2011
 UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States
- March 25-27, 2011
 MedicRes IC 2011 Good Medical Research, Istanbul, Turkey
- March 26-27, 2011
 26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States
- April 6-7, 2011
 IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States
- April 7-9, 2011
 International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy
- April 15-16, 2011
 Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany
- April 18-22, 2011
 Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States
- April 20-23, 2011
 9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea
- April 25-27, 2011
 The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia
- April 25-29, 2011
 Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States
- April 28-30, 2011
 4th Central European Congress of Surgery, Budapest, Hungary
- May 7-10, 2011
 Digestive Disease Week, Chicago, IL 60446, United States
- May 12-13, 2011
 2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom
- May 19-22, 2011
 1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain
- May 21-24, 2011
 22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venice, Italy
- May 25-28, 2011
 4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina
- June 11-12, 2011
 The International Digestive Disease Forum 2011, Hong Kong, China
- June 13-16, 2011
 Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy
- June 14-16, 2011
 International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia
- June 22-25, 2011
 ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain
- June 29-2, 2011
 XI Congreso Interamericano de Pediatría "Monterrey 2011", Monterrey, Mexico
- September 2-3, 2011
 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany
- September 10-11, 2011
 New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States
- September 10-14, 2011
 ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States
- September 30-October 1, 2011
 Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium
- October 19-29, 2011
 Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia
- October 22-26, 2011
 19th United European Gastroenterology Week, Stockholm, Sweden
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 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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ISSN and EISSN

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

Indexed and Abstracted in

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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 PMID: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 Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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 biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek 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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Write as mean \pm SD or mean \pm SE.

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World Journal of Gastroenterology®

Volume 17 Number 34
September 14, 2011



Published by Baishideng Publishing Group Co., Limited,
Room 1701, 17/F, Henan Building,
No. 90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
E-mail: baishideng@wjgnet.com
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ISSN 1007-9327



World Journal of Gastroenterology

www.wjgnet.com

Volume 17

Number 34

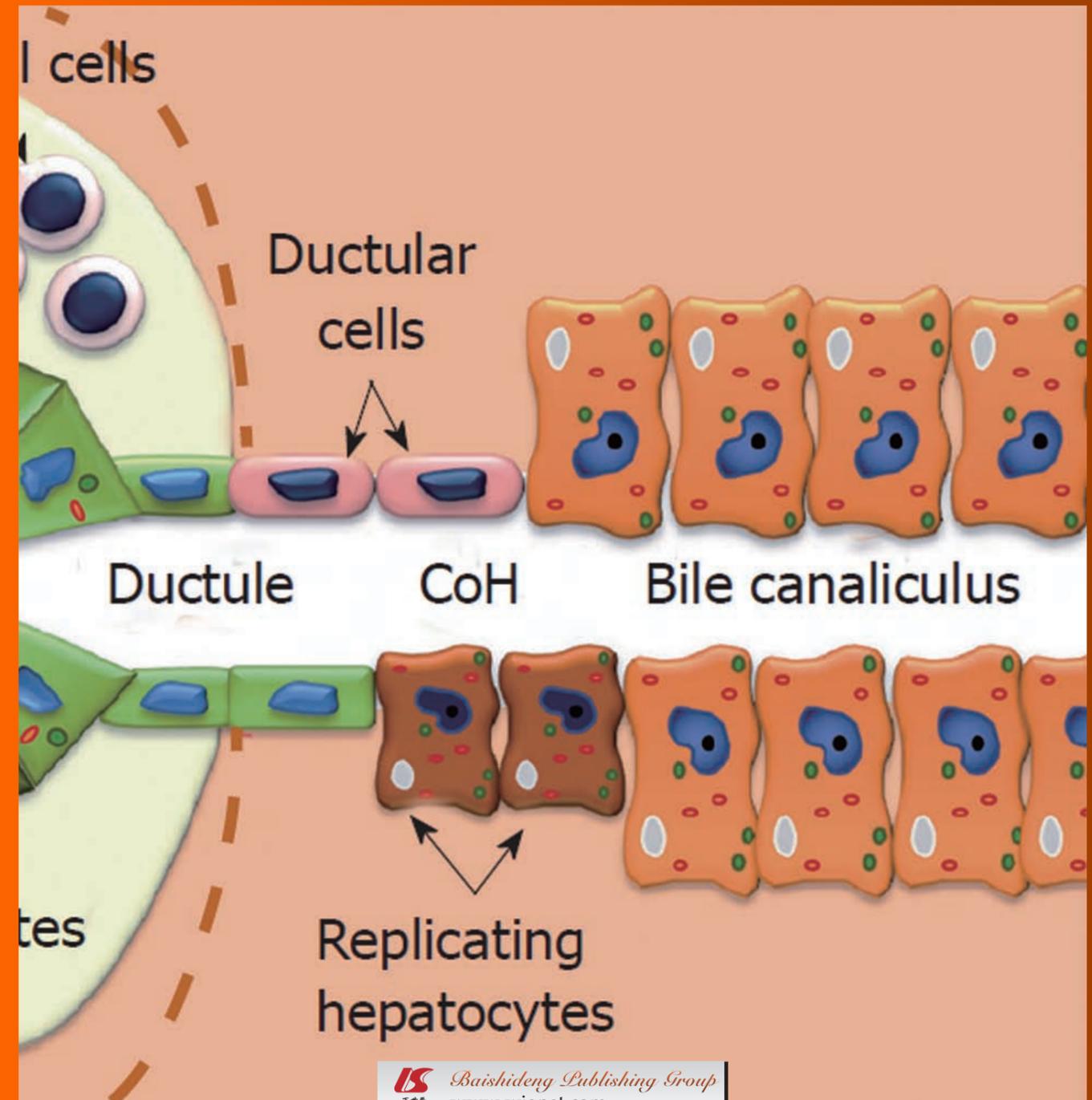
Sep 14

2011



World Journal of *Gastroenterology*

World J Gastroenterol 2011 September 14; 17(34): 3859-3956



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ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

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<http://www.wjgnet.com/1007-9327/full/v17/i34/3870.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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Telephone: +86-10-5908-0039
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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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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
September 14, 2011

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

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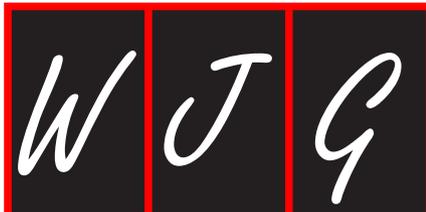
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Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
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ONLINE SUBMISSION
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Nutritional stimulation of the autonomic nervous system

Misha DP Luyer, Quirine Habes, Richard van Hak, Wim Buurman

Misha DP Luyer, Department of Surgery, Catharina Hospital Eindhoven, 5602 ZA Eindhoven, The Netherlands

Quirine Habes, Richard van Hak, Faculty of Medicine, Maastricht University, 6229 ER Maastricht, The Netherlands

Wim Buurman, Department of Surgery, Maastricht University, 6229 ER Maastricht, The Netherlands

Author contributions: Luyer MDP and Buurman W designed previous studies; Luyer MDP performed research; Habes Q, van Hak R and Luyer MDP wrote the paper; Buurman W read and corrected the manuscript.

Correspondence to: Misha DP Luyer, MD, PhD, Department of Surgery, Catharina Hospital Eindhoven, PO Box 1350, 5602 AZ Eindhoven, The Netherlands. misha.luyer@catharina-ziekenhuis.nl
Telephone: +31-6-40006809 Fax: +31-40-2443370

Received: December 31, 2010 Revised: June 21, 2011

Accepted: June 28, 2011

Published online: September 14, 2011

Abstract

Disturbance of the inflammatory response in the gut is important in several clinical diseases ranging from inflammatory bowel disease to postoperative ileus. Several feedback mechanisms exist that control the inflammatory cascade and avoid collateral damage. In the gastrointestinal tract, it is of particular importance to control the immune response to maintain the balance that allows dietary uptake and utilization of nutrients on one hand, while preventing invasion of bacteria and toxins on the other hand. The process of digestion and absorption of nutrients requires a relative hyporesponsiveness of the immune cells in the gut to luminal contents which is not yet fully understood. Recently, the autonomic nervous system has been identified as an important pathway to control local and systemic inflammation and gut barrier integrity. Activation of the pathway is possible *via* electrical or *via* pharmacological interventions, but is also achieved in a physiological manner by ingestion of dietary lipids. Administration of dietary lipids has been shown to be very effective in reducing the inflammatory cascade and maintaining intestinal barrier integrity in several experimental studies. This beneficial effect of nutrition on the in-

flammatory response and intestinal barrier integrity opens new therapeutic opportunities for treatment of certain gastrointestinal disorders. Furthermore, this neural feedback mechanism provides more insight in the relative hyporesponsiveness of the immune cells in the gut. Here, we will discuss the regulatory function of the autonomic nervous system on the inflammatory response and gut barrier function and the potential benefit in a clinical setting.

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Key words: Inflammation; Nutrition; Acetylcholine; Intestinal barrier; Innate immunity; Autonomic nervous system; Cholecystokinin

Peer reviewer: Anthony J Bauer, PhD, Associate Professor of Medicine, University of Pittsburgh, S-849 Scaife Hall, 3550 Terrace Street, 15261 Pittsburgh, PA, United States

Luyer MDP, Habes Q, van Hak R, Buurman W. Nutritional stimulation of the autonomic nervous system. *World J Gastroenterol* 2011; 17(34): 3859-3863 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i34/3859.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i34.3859>

INTRODUCTION

Disturbance of the inflammatory response in the gut is important in several clinical diseases ranging from inflammatory bowel disease (IBD) to postoperative ileus (POI)^[1,2]. Although a fierce response to pathogens, ischemia, trauma and other forms of injury is necessary, the inflammatory cascade needs to be tightly controlled to avoid local tissue damage or systemic effects such as shock, organ failure or even death^[3,4]. Especially in the gastrointestinal tract a delicate equilibrium is required; on one hand there needs to be a state of relative hyporesponsiveness to commensal bacteria, dietary antigens and biological toxins that are ingested along with nutrients, and on the other hand potential pathogens must be recognized and neutralized when necessary^[5].

An unrestrained inflammatory response is not desired since it may cause local damage to healthy tissue, cause acute and chronic inflammatory diseases and even evolve into critical systemic inflammatory syndromes such as sepsis.

Controlling the inflammatory response has been a therapeutic target for many gastrointestinal diseases for a long time. For IBD, control of the intestinal inflammatory response *via* an anti-inflammatory therapy using tumour necrosis factor- α (TNF- α) antibodies has been proven to be very successful in the clinical situation^[6,7]. For other syndromes such as POI, only experimental evidence is available that shows a positive effect of an anti-inflammatory therapy^[8].

In the last decade, a new anti-inflammatory pathway has been discovered involving the autonomic nervous system. Several experimental studies have shown that stimulation of the autonomic nervous system can dampen the inflammatory response and prevent loss of gut barrier integrity^[9-11]. In this way, activation of the autonomic nervous system can potentially ameliorate inflammation-based diseases and prevent complications. Both IBD and POI have been shown to be reduced effectively in experimental models using activation of the autonomic nervous system^[8,12]. A more physiological and less invasive way of activating this anti-inflammatory vagal pathway is by administration of nutrition^[13-15]. Nutrition and specifically dietary lipids activate the autonomic nervous system *via* afferent vagal nerve fibres and release of neuro-endocrine hormones^[16,17]. In this way nutrition may be used as therapy to prevent excessive inflammation and the accompanying local tissue damage. Although the beneficial effect of nutrition on inflammation has been recognized by many for a long time in several clinical settings, this vagal feedback mechanism gives more insight in the mode of action of nutrition and individual food components and helps to develop new nutritional therapies.

THE INFLAMMATORY RESPONSE

Release of proinflammatory cytokines by macrophages and neutrophils is essential in the initial and rapid innate immune response. Additionally, exposure to bacterial ligands elicit a complex orchestrated network of responses in which complement, chemokines, adhesion molecules, heat shock proteins and other late mediators play a role^[18]. One of the key mediators released early after exposure to bacterial structures is TNF- α ^[19]. This pleiotropic cytokine causes activation of macrophages and stimulates neutrophils. Inhibition of this cytokine has been shown to be therapeutic in IBD and rheumatoid arthritis, although it may also be detrimental in patients carrying certain pathogens while also in sepsis^[20]. Other rapidly released pro-inflammatory mediators include interleukin-6, interleukin-1 β and interleukin-8. The inflammatory response is often triggered by bacteria or bacterial products that have distinctive characteristics called pathogen-associated molecular patterns that

are recognized by immune cells *via* Toll-like receptors (TLRs)^[21]. There are several ligands for TLRs including endotoxin, the major constituent of Gram-negative bacteria (TLR4), bacterial DNA (TLR9) and peptidoglycans (TLR2). Activation of TLRs triggers several intracellular signalling pathways leading to translocation of the transcription factor nuclear factor- κ B (NF- κ B) to the nucleus and ultimately resulting in the release of inflammatory mediators^[22]. To prevent an exaggerated inflammatory response and to manage the collateral damage caused by release of proinflammatory mediators, several control systems are activated at all levels. This so-called “anti-inflammatory response”, consists of numerous anti-inflammatory mediators, including cytokines, neuromediators, hormones and stress molecules released to restore homeostasis. Interleukin-10 (IL-10) and transforming growth factor- β are important anti-inflammatory cytokines. For example, IL-10 has been shown to play a pivotal role in the intestinal recovery following surgery^[23]. Another important pathway in the anti-inflammatory response is triggered by the catabolism of heme by the enzyme heme-oxygenase 1. This results in induction of biliverdin which has been shown to protect against polymicrobial sepsis in cecal ligation and puncture^[24]. Furthermore, carbon monoxide is formed which has been shown to ameliorate development of postoperative ileus *via* reduction of the inflammatory response and induction of IL-10^[25].

To prevent an exaggerated inflammatory response several control systems are activated at all levels. This so-called “anti-inflammatory response”, consists of numerous anti-inflammatory mediators, including cytokines, neuromediators, hormones and stress molecules. IL-10 and transforming growth factor- β are the main identified anti-inflammatory cytokines.

The complex of these counter-regulatory mechanisms to severe infection of injury is also called the “compensatory anti-inflammatory response syndrome” (CARS). CARS was at first considered to be a global deactivation of the immune system following systemic inflammatory response syndrome. However, new insights suggest that it is rather a reprogramming of leucocytes leading to a compartmentalized control to prevent excessive inflammation upon infection and injury^[26].

INTESTINAL BARRIER INTEGRITY

The intestinal lumen is an important reservoir of bacteria that is strictly separated from the sterile environment of the host *via* a physical/anatomical and immunological barrier. Changes or defects in certain components of the intestinal barrier may lead to activation of the inflammatory system potentially leading to known gastrointestinal diseases such as inflammatory bowel disease^[27].

The physical/anatomical barrier of the gut is formed by a monolayer of epithelial cells, originating from multipotent stem cells present in the crypt. These cells differentiate into several subclasses, amongst which are absorptive enterocytes, that make up > 80% of all

small intestinal epithelial cells, Paneth cells and goblet cells^[28,29]. The intestinal cells are bound together with several protein complexes including occluding, claudin and zonula occludens proteins, also called tight-junctions^[30]. Breakdown of this barrier potentially leads to the translocation of luminal antigens, bacteria and their toxic products into the circulation^[31,32]. In the case of transmural damage to all intestinal layers (mucosa, submucosa, muscularis and serosa) luminal content may pass into the abdominal cavity leading to detrimental effects as sepsis.

The immunological barrier is formed by enterocytes that are considered to actively participate as innate immune sensors of microbial pathogens and commensal organisms. Host recognition of microbial components is achieved by pattern recognition receptors, like the cytoplasmic NOD-like receptors and membrane-bound TLRs. Crypt Paneth cells secrete defensins (e.g., antimicrobial peptides) into the villous crypt, thereby maintaining its sterility and regulating microbial homeostasis^[33]. It has also been shown that Paneth cells are equipped with the proper molecules to recognize and signal endotoxin, the major component of Gram-negative bacteria^[34]. Goblet cells secrete mucus (composed of glycoproteins and water) providing a filter overlying the intestinal epithelium and secrete trefoil peptides; small proteins needed for epithelial growth and repair. Gut-associated lymphoid tissue is present in the lamina propria and provides immune surveillance. Finally, sampling of luminal antigens occurs by M-cells and dendritic cells, which present antigens to T and B cells, thereby inducing the acquired immune system. This response includes secretion of large amounts of IgA by plasma cells. This secretory IgA covers the mucosal surface and has a major role in excluding antigen from passing the epithelium^[35].

Interestingly, the process of digestion and metabolism of nutrients requires a physiological breach of the intestinal barrier, without noticeable activation of the immune response. This relative hyporesponsiveness to luminal contents during the process of food uptake is not fully understood. The autonomic nervous system may be important in regulation of this process.

THE CENTRAL NERVOUS SYSTEM AND INNATE IMMUNITY

Excessive release of inflammatory mediators following activation of inflammatory cells by bacterial products is (amongst other pathways) controlled by the central nervous system. Supposedly, inflammatory mediators such as TNF- α and interleukins activate afferent vagal pathways leading to a variety of responses. The hypothalamic-pituitary-adrenal signalling pathway is activated causing an instantaneous release of serum corticosteroids that leads to inhibition of (excessive) inflammation. In addition to this afferent or sensory function during inflammation, the efferent vagal system is also involved in regulation of the inflammatory response^[36]. Previous studies have

shown that stimulation of efferent vagal fibres increases release of acetylcholine, the principal neurotransmitter of the vagus nerve. Acetylcholine subsequently binds to specific $\alpha 7$ nicotinic receptors ($\alpha 7$ nAChR) leading to activation of an intracellular pathway^[37]. It has been shown that the transcription factor STAT3 is phosphorylated by tyrosine kinase Jak2, leading to the anti-inflammatory effect *via* NF- κ B^[12]. Ultimately this results in a decreased release of both early inflammatory mediators such as TNF- α but also late mediators such as HMGB-1^[9,38,39]. Stimulation of this neural feedback loop is efficient in reducing the inflammatory response in several experimental models. Direct electrical stimulation or pharmacological stimulation of nicotinic receptors *via* agents such as CNI-1493 significantly reduces the systemic inflammatory response to endotoxic shock^[40]. Furthermore, activation of this neural anti-inflammatory pathway, the so-called cholinergic anti-inflammatory pathway reduces the inflammatory response and its sequelae during septic peritonitis and following hemorrhagic shock^[11]. Interestingly, recent evidence indicates that vagus nerve signalling has a dual effect in macrophages. Besides inhibiting cytokine secretion, the cholinergic nervous system also enhances endocytosis and phagocytosis of bacteria by macrophages^[41]. This effect on phagocytosis is caused by stimulation of the nAChR $\alpha 4/\beta 2$ rather than $\alpha 7$ nAChR. The spleen has also been implicated in the anti-inflammatory cholinergic pathway; however the underlying mode of action at this stage is elusive. The vagus nerve conveys signals from the brain to the immune cells residing in the spleen *via* the celiac-superior mesenteric plexus ganglia and the splenic nerve^[42]. The requirement of the nicotinic acetylcholine receptor $\alpha 7$ in this process is not yet clear.

NUTRITIONAL STIMULATION OF THE AUTONOMIC NERVOUS SYSTEM

Besides electrical or pharmacological stimulation, the autonomic nervous system can also be stimulated in a physiological way *via* nutrition, more specifically *via* dietary lipids and proteins/peptides. Ingestion of dietary lipids, proteins and peptides triggers release of cholecystokinin (CCK)^[43]. CCK binds to CCK-1 and CCK-2 receptors, leading to a rapid activation of the autonomic nervous system *via* the afferent vagal pathway^[44]. Subsequently the efferent vagal pathway is activated resulting in release of acetylcholine. Binding of acetylcholine to $\alpha 7$ nicotinic receptors on inflammatory cells then leads to a decreased release of inflammatory cytokines. Such a nutritional intervention with dietary fat has been proven to be very efficient in reducing the systemic inflammatory response, ameliorate tissue damage and preserve intestinal barrier function^[13-15]. Interestingly, the protective effects of dietary lipids were shown to be efficient when given before an inflammatory trigger. Even when the nutritional intervention is started after the inciting event it still is effective^[45]. This is especially of interest in

acute clinical settings such as trauma in which changes in intestinal barrier integrity are clinically important and may be related to associated complications^[46].

The fact that such a negative feedback mechanism exists seems logical and functional during feeding. During digestion and absorption of vital nutrients, a fierce immune response to temporally present bacterial toxins, antigens and destructive endogenous lysozymes accompanying nutrition needs to be avoided. Next to the dampening effect of lipid enriched nutrition on the inflammatory response, intestinal barrier integrity is also preserved in various experimental models. This beneficial effect on gut barrier integrity may be explained twofold. First of all, release of acetylcholine can prevent intestinal damage *via* a decreased release of inflammatory cytokines. Both TNF- α and IFN- γ have long since been known to modulate the epithelial barrier in the intestine^[47,48]. Interestingly, lipid rich nutrition preserves intestinal barrier function early on, suggesting that local inflammation may be of importance^[49]. Another route may be through enteric glia cells since ablation of these enteric glia cells has been shown to directly affect gut barrier function and may result in inflammation^[50]. The effects of lipid rich nutrition on these cells however needs to be further investigated.

NEW THERAPEUTIC STRATEGIES

Stimulation of the autonomic nervous system may be useful as a therapeutic target in acute inflammatory based conditions. There have been many experimental studies showing a beneficial effect of electrical or pharmacological stimulation of the autonomic nervous system. However, general activation of nicotinic receptors may also have a wide scope of unwanted effects on other cells or cell systems besides inflammatory cells, which have yet to be determined.

Enteral administration of dietary fat provides a physiologic way to stimulate the cholinergic anti-inflammatory pathway and administration of nutrition is considered to be safe. Early enteral nutrition is successfully implemented in fast-track programs such as the Enhanced Recovery After Surgery program in which both hospital stay and the complication rate following colorectal surgery are reduced^[51].

Surgical patients are an interesting intervention group since the timing of surgery (and therefore the trigger for the immune response) is predetermined. Dampening the postoperative local and inflammatory response is expected to have important effects on related complications such as POI, but potentially also on other parameters.

Future clinical trials with dietary lipids as a means to stimulate the anti-inflammatory cholinergic pathway will provide more clarity to these questions.

CONCLUSION

The autonomic nervous system is an endogenous path-

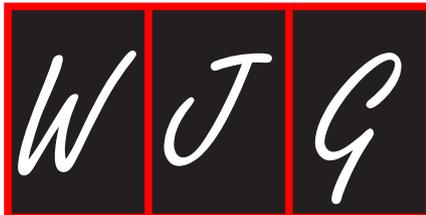
way that dampens the inflammatory response and regulates intestinal barrier function. Further delineation of this pathway may help to understand the relative hyporesponsiveness in the intestine to luminal contents. Furthermore, nutritional stimulation of this anti-inflammatory neurogenic feedback may be used as an important therapeutic target in several inflammatory based diseases.

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S- Editor Sun H L- Editor O'Neill M E- Editor Li JY



Update of cholangioscopy and biliary strictures

Marcus W Chin, Michael F Byrne

Marcus W Chin, Department of Gastroenterology and Hepatology, Vancouver General Hospital, Amy and Athelstan Saw Fellow, University of Western Australia, Perth 6009, Australia

Michael F Byrne, Department of Gastroenterology and Hepatology, Vancouver General Hospital, University of British Columbia, Vancouver V5Z1M9, Canada

Author contributions: Byrne MF and Chin MW contributed equally to this work; Chin MW and Byrne MF contributed research and wrote the paper.

Correspondence to: Michael F Byrne, Clinical Professor, Department of Gastroenterology and Hepatology, Vancouver General Hospital, University of British Columbia, 5135-2775 Laurel St Vancouver, British Columbia, Vancouver V5Z1M9, Canada. michael.byrne@vch.ca

Telephone: +1-604-8755640 Fax: +1-604-8755447

Received: August 16, 2010 Revised: January 15, 2011

Accepted: January 22, 2011

Published online: September 14, 2011

Abstract

Cholangioscopy remains another modality in the investigation of biliary strictures. At cholangioscopy, the "tumour vessel" sign is considered a specific sign for malignancy. Through its ability to not only visualise mucosa, but to take targeted biopsies, it has a greater accuracy, sensitivity and specificity for malignant strictures than endoscopic retrograde cholangiopancreatography guided cytopathological acquisition. Cholangioscopy however, is time consuming and costly, requires greater technical expertise, and should be reserved for the investigation of undifferentiated strictures after standard investigations have failed.

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Key words: Biliary strictures; Cholangioscopy; Endoscopic retrograde cholangiopancreatography; Endoscopy; Cholangiocarcinoma

Peer reviewer: Yoshi Ueno, MD, Division of Gastroenterology, Tohoku University Hospital, Sendai 980-8574, Japan

Chin MW, Byrne MF. Update of cholangioscopy and biliary strictures. *World J Gastroenterol* 2011; 17(34): 3864-3869 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i34/3864.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i34.3864>

INTRODUCTION

Multiple modalities are now available for the investigation of obstructive jaundice: endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance cholangiopancreatography, endoscopic ultrasound (EUS), computed tomography (CT) and transabdominal ultrasound (TUS). TUS is accurate in identifying biliary obstruction, but is less accurate in identifying the aetiology^[1-3]. Whilst there have been refinements in techniques in the other aforementioned imaging modalities, making a conclusive diagnosis in the setting of a biliary stricture or intraductal tumour may be difficult^[4,5]. Biliary strictures may be caused by benign and malignant tumours, as well as inflammatory processes. Management strategies with optimal outcomes are clearly dependent on knowing the correct diagnosis. Whilst features suggestive of malignancy are assessed for on cholangiography and EUS, these criteria are known to be non specific^[6] and histopathology remains the gold standard for diagnosis.

Conventional tissue sampling during ERCP is routinely performed in the investigation of biliary strictures; however it remains sub-optimal in making a diagnosis. Brush cytology remains the mainstay of obtaining a cytological sample from a biliary stricture at ERCP. It is simple to perform and reported to have few complications^[7]. Brush cytology performed at the time of ERCP has a sensitivity of 30% to 57%^[8-10] and is thought to be limited by the poor cellular yield.

Cholangioscopy involves direct visualisation of the biliary tree *via* a fibre optic or video scope. It may be performed percutaneously or "peroral", where a scope is passed down the therapeutic channel of a duodenoscope, and has become an additional modality in the investiga-

tion and management of biliary disease. Initially, the percutaneous approach was favoured because these cholangioscopes were larger than the operating channel of a conventional therapeutic duodenoscope. Subsequently, cumbersome mother daughter systems were developed to visualise the biliary tree *via* the ampulla. Peroral cholangioscopy (POCS) was first described in the in 1976 by Kawai *et al*^[11]. Initially the domain of specific tertiary hospitals, the clinical use of cholangioscopy is expanding. Our focus is to review the utility of cholangioscopy in biliary strictures: in particular details of cholangioscopes available, the evidence available in relation to the diagnostic realms of cholangioscopy, cholangioscopy assisted biopsy and the utility of chromocholangioscopy (CC) and POCS with narrow band imaging (NBI).

INSTRUMENTS

Peroral cholangioscopes

Peroral cholangioscopes are passed down the 4.2 mm working channel of a therapeutic duodenoscope to visualise the biliary tree. There are currently devices available from Olympus, Pentax, and Boston Scientific.

Olympus and Pentax provide reusable fibre optic and video cholangioscopes. There are four different sized diameter per-oral cholangioscopes available - 2.6, 2.8, 3.1 and 3.4 mm. The 2.6 and 2.8 mm diameter cholangioscopes have a 0.75 mm working channel that allows the passage of a 0.025 inch guide wire. The 2.8, 3.1 and 3.4 mm diameter cholangioscopes with a 1.2 mm working channel permit use of 1.9-3 French electrohydraulic lithotripsy fibers, 0.035 inch guide wire and biopsy forceps. These cholangioscopes come in 187, 190 and 200 cm lengths and have bi-directional (up-down) movement.

These scopes consist of a dial for two way tip deflection, air/water buttons and suction channels. The core of the cholangioscope is predominantly fibre optic cables through which the image is transmitted from the tip of the endoscope to the eyepiece, with a light guide for illumination, angulation wires for tip deflection, an air/water nozzle and a working channel.

There are newer 3.4 and 5.3 mm cholangioscopes available using charge couple device (CCD) technology and a NBI system incorporated into a video cholangioscope (Olympus Inc., CHF-B260 and CHF-BP260). They are currently still undergoing FDA approval and have not yet been used in North America^[12,13].

The Spyglass Direct Visualization System (Microvasive Endoscopy, Boston Scientific Co., Natick, Mass, United States) is composed of a reusable optical probe that traverses a disposable access and delivery catheter (Figure 1). The 3.4 mm diameter disposable catheter consists of the optical probe port, an irrigation port and 1.2 mm accessory channel. The 0.77 mm, 6000 pixel, reusable optic probe, is a collection of light fibers and optical fiber bundles. This system offers 4-way deflected steering, and a specific miniature biopsy forceps (Spybite[®]) which is commercially

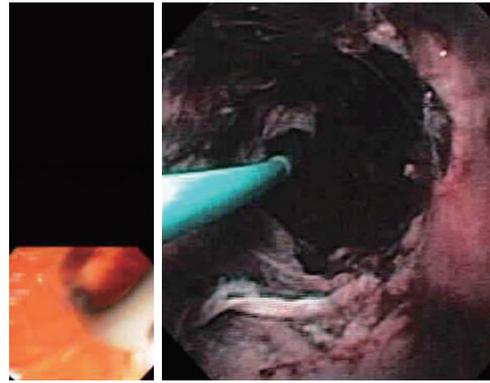


Figure 1 Video cholangioscopy of benign common hepatic stricture secondary to primary sclerosing cholangitis.

available with this system.

Percutaneous/intraoperative cholangioscopes

Percutaneous/intraoperative cholangioscopes used are usually shorter (35, 38, 45 and 70 cm) working length, wider diameter (4.8, 4.9 and 6.0 mm) and have a larger accessory channel (2.0, 2.2 and 2.6 mm). This wider channel facilitates instrumentation with more therapeutic devices - intraductal lithotripsy fibers, baskets, and forceps.

Paediatric endoscopes

Paediatric endoscopes have also been used to directly visualise the biliary tree intubated under fluoroscopic and endoscopic guidance, and the use of a biliary placed guidewire or balloon catheter^[14,15]. These techniques overcome the disadvantages of using the “mother daughter” system which is relatively expensive in capital expenditure, time consuming, cumbersome, usually requires two endoscopists, is fragile and has a smaller working channel. This method has been reported with a variable success (45.5%-100%) of biliary intubation, but is currently not routinely performed^[14,15].

A double balloon enteroscope overtube (TS 13140, total length 1450 mm, Fujinon Corp., Omiya, Japan) has been used in combination with an Olympus paediatric gastroscope (GIF-N230 or N260; Olympus Optical Co, Ltd, Tokyo, Japan) to perform cholangioscopy. The gastroscope was used to intubate the duodenum, the overtube advanced over the gastroscope and post-insufflation used as an anchor whilst the gastroscope intubated the biliary tree under endoscopic and fluoroscopic guidance. In this single case series of 12 patients, the device had a success of 83.3%^[16]. Lithotripsy and biopsies were able to be performed through the working channel of the paediatric gastroscope.

Wilson Cook Inc. (Winston Salem, NC, United States) has released a cholangioscopy access balloon. These are 315 cm long 4 Fr catheters with either a 15 or 20 mm balloon that is inserted into the biliary tree through the duodenoscope. The balloon is then insufflated, securing its position, and the duodenoscope is exchanged for a paediatric

atric gastroscope with tension on the catheter to prevent looping. After intubation of the biliary tree, the balloon may be deflated if biopsies are to be taken or therapeutic intervention needs to be performed. At this current time, there are no clinical trials evaluating the use of this device and it awaits FDA approval for clinical use.

Peroral vs percutaneous cholangioscopy

Percutaneous cholangioscopy (PTC) was often the favoured method of cholangioscopy. Wider, shorter and more maneuverable scopes enabled biopsies for diagnosis and therapeutic devices to be advanced through the working channel in a less cumbersome manner. The procedure itself requires only one endoscopist. The drawbacks of PTC are that it is performed through an operatively placed T-tube tract or a percutaneous transhepatic biliary drain (PTBD). To use the T-tube, 3 to 5 wk must be waited for the maturation of the tract, with inherent time delays. To perform PTCs *via* the PTBD, 2 to 4 wk must be allowed for a tract to mature after an internal/external 7 to 8 French pigtail catheter is initially deployed, with sequential dilatation commencing after several days to eventually deploy a 16 French diameter catheter across the tract. The final step before introduction of the cholangioscope is a pre-procedure dilation. A percutaneous plastic peel-away sheath (Wilson Cook Inc, Winston Salem, NC, United States) or metal sheath (Olympus Co., Tokyo, Japan) may be used for direct access if these time delays are not feasible. Drawbacks of this approach are the necessity for long hospitalization, cumbersome management of a drainage catheter and possible complications of bleeding/hemobilia, seeding metastasis along the sinus tract of percutaneous transhepatic drainage and risk of intraperitoneal metastasis^[17].

POCS is usually performed with two experienced endoscopists and has a long procedure time. Alternatively a specially designed external cholangioscope fixation device or nurse/assistant may be used. ERCP is usually performed to delineate the biliary tree and pathology, and the cholangioscope is placed down the working channel of the duodenoscope and may be inserted freehand through the previously sphincteromised ampulla or it may be inserted over a long 450 cm guidewire that has been backloaded into the channel of the daughter scope. The cholangioscope is fragile and judicious use of the elevator is advised, with the latter method trying to overcome the need for vigorous use of the elevator. Maneuverability is technically more challenging due to the length of the POCS, and two way tip deflection and limitations also arise from traversal of the duodenoscope. Passage of the biopsy forceps can also be difficult due to the length and angles of the cholangioscope required to arrive at the target lesion.

Cholangioscopic findings in biliary strictures

Numerous studies have evaluated the use of cholangioscopy in undifferentiated strictures^[18-22]. In a case series of 111 patients with biliary tumours/strictures, they aimed

to correlate cholangioscopic findings with histopathology from cholangioscopic guided biopsies or surgically resected specimens^[23]. They classified the cholangioscopic findings of cholangiocarcinoma into three categories: nodular, papillary and infiltrative types^[23]. Nodular type cholangiocarcinoma is described to have nodular mucosa causing luminal narrowing, with the mucosa being irregular with intense neovascularization. This type of cholangiocarcinoma is short in length. Papillary type cholangiocarcinoma is described to have papillary mucosal projections, superficially spreading and neovascularization is usually not a feature. These papillary projections usually caused obstruction and were intermingled with pus and sludge. The authors describe a mucin secreting cholangiocarcinoma, that on cholangioscopy appear similar to papillary type cholangiocarcinoma with papillary or villous mucosal projections. The mucin secretion is thought to cause marked proximal ductal dilatation. Infiltrative type cholangiocarcinoma was not usually associated with a mass lesion. This malignancy has a smooth tapered narrowing, with the mucosa being white with subtle elevations on the margin of tumour vessels. Neovascularization was not described to be as predominant as with the nodular type cholangiocarcinoma. There have been no studies to prospectively evaluate if the cholangiographic type of cholangiocarcinoma prognosticates the clinical outcome.

Benign strictures were described in this case series to have a smooth surface mucosa and tapered luminal narrowing with no definite neovascularization^[23]. Biliary papillomatosis consists of papillary projections into the lumen with intervening normal areas of mucosa, and is thought to predispose to papillary adenocarcinoma. The authors conclude that it is difficult to cholangiographically differentiate papillary adenoma from papillary adenocarcinoma; however they suggest that the degree of luminal obstruction may correlate to the risk of overt malignancy.

ASSESSMENT OF NEOVASCULARIZATION OR THE "TUMOUR VESSEL" SIGN

The "tumour vessel" is an abnormally proliferating and tortuous vascular structure on the mucosa adjacent to the stricture. Synonymous terms include "capillary sign", neovascularization and vascular dilatation. A prospective study of patients with biliary strictures without a luminal mass in or around the stricture diagnosed at ERCP or percutaneous transhepatic cholangiography, evaluated the sensitivity and specificity of the "tumour vessel" sign for malignancy as seen at PTC^[24]. In total, 63 patients were enrolled in the study. Malignancy was confirmed when malignant cells were seen in PTC directed biopsies and in the surgically resected specimen. A stricture was deemed benign if the resected specimen had no evidence of malignant cells or, after the 1 year follow up, there was no clinical or radiological evidence of disease progression.

They found the sensitivity of this sign for malignancy to be 61% (25/41) and specificity was 100%. PTC directed biopsies had a sensitivity of 80% (33/41) and specificity of 100%. Concordance between two endoscopists for the “tumour vessel” sign was 100%. Six of eight strictures with a positive “tumour vessel” sign had false negative biopsies. The majority of these malignant strictures were infiltrative type cholangiocarcinomas, which is thought to spread beneath the epithelium, and the abundant fibrosis may lead to higher rates of falsely negative biopsies. For this particular reason, the authors suggested cholangioscopy be considered in patients with non diagnostic cytological specimens gathered at ERC in particular to assess for this specific sign.

THE CLINICAL UTILITY OF CHOLANGIOSCOPY

In a case series of 97 patients with biliary filling defects and strictures, Fukuda *et al*^[20] evaluated the additional benefit of POC to ERCP and biopsies or brushings. Patients were excluded if there was an ampullary or pancreatic (based on CT or TUS) mass or in subjects with negative cytology who were not clinically followed up for a minimum of 12 mo. Criteria used to assess strictures for malignancy included: (1) the presence of tumour vessels; (2) easy oozing; and (3) irregular surface.

Patients were deemed to have malignancy if they had positive surgical specimens, evidence of malignant cytology by other means, or clinical progression at follow up. Of 76 strictures, 38 were malignant and 28 benign. ERCP and tissue sampling correctly identified 22 of the 38 malignant strictures giving sensitivity, specificity, accuracy, and a positive and negative predictive value of 57.9%, 100%, 78.1%, 100% and 68.6%, respectively. In comparison, the addition of POCS to ERCP and tissue sampling correctly identified all 38 malignant strictures and 33 of the 38 benign strictures. This improved the sensitivity to 100%; however the specificity was 87.2%. The five false positive strictures identified on POCS had abnormal tortuous dilated vessels present, which was previously thought to be specific for malignancy. The authors conclude that POCS without POCS directed biopsy is a useful adjunct to ERCP in the management of biliary strictures^[20].

Shah *et al*^[19] assessed the clinical utility of POCS and cholangioscopic assisted/directed biopsies in the investigation of indeterminate biliary strictures. Sixty-two patients were referred with a combination of non diagnostic cytology taken at ERCP and/or non specific imaging by CT, magnetic resonance imaging or positron emission tomography scanning. Eighteen patients had a final diagnosis of malignancy and 16 were diagnosed with cholangioscopy. One of the malignancies missed was proximal to a benign appearing biliary stricture secondary to primary sclerosing cholangitis and diagnosed intraoperatively in the caudate lobe. The other malignancy was a hilar cholangiocarcinoma diagnosed in the explanted liver, 7 mo after cholangioscopy with negative cholangioscopic

assisted and directed biopsies. Based on cholangioscopy, two patients were incorrectly diagnosed with malignancy. Both had negative cholangioscopic assisted and directed biopsies and normal intraoperative biopsies. In the 16 patients correctly diagnosed with malignancy at cholangioscopy, biopsies were positive in 10 patients (63%). Overall the sensitivity for detecting malignancy by cholangioscopy with and without biopsy based on this cohort was 89%, specificity 96%, positive predictive value 89% and negative predictive value 96%. These studies suggest that cholangioscopy without biopsy has a relatively high sensitivity and specificity for malignant strictures as compared with ERCP and cytopathological acquisition. Whilst there are no head to head studies comparing ERCP and cholangioscopy, it is not unreasonable to consider cholangioscopy in the setting of a non diagnostic ERCP.

IMAGE ENHANCED VIDEO CHOLANGIOPANCREATOSCOPY

New techniques currently being evaluated in the investigation of biliary strictures include CC and NBI.

Chromocholangioscopy

Chromoendoscopy is commonly used in assessment, particularly of the borders of gastrointestinal malignancies. Methylene blue chromocholangioscopy has been evaluated in two studies in patients with biliary strictures^[25,26]. Hoffman *et al*^[26] performed per oral chromoendoscopy by injecting 15 mL of 0.1% methylene blue down the working channel of the cholangioscope. Excess dye was then removed with suction after 2 min. In 55 patients who underwent chromoendoscopic cholangioscopy for biliary strictures or filling defects, dye spray added on average 18 min to the procedure (range 10–45 min). Chromocholangioscopic images were correlated with cholangioscopic directed biopsies. In this pilot study, different staining patterns of methylene blue were seen in normal, inflammatory (diffuse uptake) and dysplastic mucosa (irregular uptake). In particular, the authors suggested the uptake of stain in inflammatory tissue helped to distinguish fibrotic from inflammatory strictures, especially in patients with primary sclerosing cholangitis. The authors report that most lesions were identifiable with white light cholangioscopy^[26]. Whilst this study demonstrated clear difference in patterns, this has not been repeated, nor is chromocholangioscopy practice widespread.

Narrow band imaging

The NBI system developed by Olympus medical system is based on modifying/narrowing the bandwidth of spectral transmittance resulting in optical colour separation. The filter is placed in the optical illumination system and removes all light wavelengths except for two narrow wavelengths. The central wavelengths of each band are 415 nm and 540 nm. This is available on two video cholangioscopes (CHF-B260 and CHF-BP260). The shorter band is thought to give information about

the capillary and pit patterns of the superficial mucosa, whilst the 540 nm wavelength gives more information about thicker capillaries in slightly deeper tissues. To date, published literature in relation to narrow band imaging cholangioscopy is limited to case reports and small case series^[12,27-30]. The largest case series is of 21 lesions in 12 patients who underwent POCS with narrow band imaging^[12]. Their aim was to comparatively assess the clinical feasibility of using POCS with NBI, and the ability of POCS with NBI to identify biliary lesions in comparison to conventional white light POCS. In particular they assessed the ability to determine distal and if possible, proximal margins, and surface vasculature. In this small case series, only two POCS with conventional white light cholangioscopies were rated as “excellent” in comparison with 12 lesions with NBI. NBI identified four strictures not seen with standard POCS. The authors suggest observation of surface structure and mucosal vessels was as good as or better than, conventional observation and that video cholangioscopy with NBI may be helpful in differentiating benign from malignant strictures and bile duct tumours showing superficial spread^[12]. A limitation of NBI cholangioscopy is that bile and blood both appear as dark red fluid, possibly limiting views. These preliminary findings need to be further evaluated with randomized controlled studies.

CONCLUSION

Cholangioscopy remains another modality in the investigation of biliary strictures. Through its ability to visualise mucosa and take targeted biopsies, it has a greater accuracy, sensitivity and specificity for malignant strictures than ERCP guided cytopathological acquisition^[19,20]. Cholangioscopy however, is time consuming and costly, requires greater technical expertise, and should be reserved for the investigation of undifferentiated strictures after standard investigations have failed. There is still a paucity of literature with regards to image enhanced cholangioscopy and its role, which remain promising, in imaging technologies that require further investigation.

ACKNOWLEDGMENTS

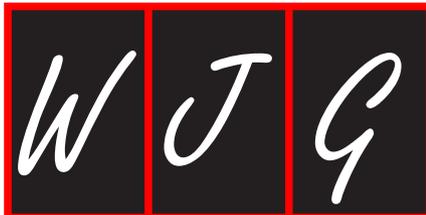
The authors thank Professor Todd Baron, Mayo Clinic for Figure 1.

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S- Editor Tian L L- Editor Rutherford A E- Editor Zheng XM



Therapeutic application of stem cells in gastroenterology: An up-date

Patrizia Burra, Debora Bizzaro, Rachele Ciccocioppo, Fabio Marra, Anna Chiara Piscaglia, Laura Porretti, Antonio Gasbarrini, Francesco Paolo Russo

Patrizia Burra, Debora Bizzaro, Francesco Paolo Russo, Department of Surgical and Gastroenterological Sciences, Gastroenterology Section, University of Padua, 35128 Padova, Italy
Rachele Ciccocioppo, First Department of Internal Medicine, IRCCS Policlinico San Matteo Foundation, University of Pavia, 27100 Pavia, Italy

Fabio Marra, Department of Internal Medicine, University of Florence, 50134 Florence, Italy

Anna Chiara Piscaglia, Antonio Gasbarrini, Department of Internal Medicine, Gemelli Hospital "Gastrointestinal and Liver Stem Cell Research Group" (GILSteR), Catholic University of Rome, 00168 Roma, Italy

Laura Porretti, Center of Transfusion Medicine, Cell Therapy and Cryobiology, IRCCS Fondazione Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, 20122 Milano, Italy

Author contributions: Burra P, Bizzaro D, Ciccocioppo R, Marra F, Piscaglia AC, Porretti L, Gasbarrini A and Russo FP wrote and approved the paper.

Correspondence to: Patrizia Burra, MD, PhD, Department of Surgical and Gastroenterological Sciences, Gastroenterology Section, University of Padua, Via Giustiniani 2, 35128 Padova, Italy. burra@unipd.it

Telephone: +39-049-8212892 Fax: +39-049-8760820

Received: December 29, 2010 Revised: March 7, 2011

Accepted: March 14, 2011

Published online: September 14, 2011

Abstract

Adult stem cells represent the self-renewing progenitors of numerous body tissues, and they are currently classified according to their origin and differentiation ability. In recent years, the research on stem cells has expanded enormously and holds therapeutic promises for many patients suffering from currently disabling diseases. This paper focuses on the possible use of stem cells in the two main clinical settings in gastroenterology, i.e., hepatic and intestinal diseases, which have a strong impact on public health worldwide. Despite encouraging results obtained in both regenerative medicine and immune-mediated conditions, further

studies are needed to fully understand the biology of stem cells and carefully assess their putative oncogenic properties. Moreover, the research on stem cells arouses fervent ethical, social and political debate. The Italian Society of Gastroenterology sponsored a workshop on stem cells held in Verona during the XVI Congress of the Federation of Italian Societies of Digestive Diseases (March 6-9, 2010). Here, we report on the issues discussed, including liver and intestinal diseases that may benefit from stem cell therapy, the biology of hepatic and intestinal tissue repair, and stem cell usage in clinical trials.

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Key words: Stem cells; Cell therapy; Regenerative medicine; Liver disease; Inflammatory bowel disease

Peer reviewer: Toshihiro Mitaka, Professor, Pathophysiology, Department of Cancer Research Institute, South-1, West-17, Chuo-ku, Sapporo 060-85567, Japan

Burra P, Bizzaro D, Ciccocioppo R, Marra F, Piscaglia AC, Porretti L, Gasbarrini A, Russo FP. Therapeutic application of stem cells in gastroenterology: An up-date. *World J Gastroenterol* 2011; 17(34): 3870-3880 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i34/3870.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i34.3870>

INTRODUCTION

In recent years stem cells (SCs) have become increasingly important in all fields of modern medicine. Different types of SCs are eligible for cell therapy, such as mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs) and adult liver stem/progenitor cells (LPCs). Although the clinical usefulness of the related lines of research remains to be ascertained in human trials, there is fascinating potential for growth in the use of SCs as a

therapeutic option. It is now well known that adult SCs are the self-renewing progenitors of numerous body tissues, classified according to their origin and ability to differentiate, and functionally responsible for the development and regeneration of tissues and organs, including the gastrointestinal tract and liver^[1]. Embryonic stem cells would probably be the best cells to use in clinical research, but their use raises many ethical controversies, therefore the researchers' attention has shifted instead to adult stem cells as a potential therapeutic tool.

SCs have recently gained great importance in gastroenterology and hepatology regarding both the pathogenesis and the treatment of liver diseases and inflammatory or immune-mediated bowel diseases, two clinical fields with a great impact for people worldwide.

But the other side of the presence of SCs in the gastrointestinal tract and liver needs to be carefully considered since the self-renewing properties characteristic of SCs may sometimes give them a key role in the processes of carcinogenesis^[2].

Discussion on the therapeutic applications of SCs in gastroenterology (and other fields) was first prompted by a previously-published paper^[3]. The Italian Society of Gastroenterology (SIGE) consequently sponsored a workshop on SCs in Verona during the Congress of the Federation of Italian Societies of Digestive Diseases (FISMAD) (March 6-9, 2010). The present paper reports on the issues analyzed, ranging from which liver and intestinal diseases may benefit from stem cell therapy to the biology of liver and intestinal tissue repair, and stem cell usage in clinical trials.

AUTOLOGOUS STEM CELLS IN THE TISSUES REPAIR PROCESS

Regeneration

The liver and gastrointestinal epitheliums are known to have great regenerative potential in response to injuries and normal cell turnover^[4,5].

The intestine consists of rapidly-proliferating tissue, in which cell turnover occurs every 2-7 d under normal circumstances, and even more rapidly following tissue damage^[3]. This rapid regenerative potential is possible thanks to a consistent proliferation of progenitor cells that occurs in crypts. Indeed, unlike most other mammalian tissues, the stem cells of the intestine are strictly compartmentalized in crypts. Two hypotheses exist regarding the exact identity and localization of SCs. The principal hypothesis, developed in the late 1950s and experimentally supported by Potten in the 1977^[6], is the so-called “+4 position model”. According to this theory, SCs are found directly above the Paneth cells, in position 4 starting from the bottom of the crypt. From there, the SCs move up and differentiate into Goblet cells, enterocytes and enteroendocrine cells, or they move down to become Paneth cells^[7]. The second, and more recent, hypothesis is the “SCs zone model”, which is based on the identification of the crypt base columnar (CBC) cells

hidden between the Paneth cells^[8]. In 2007, Berker and colleagues demonstrated that CBC cells express a peculiar marker, the *Lgr5*, a Wnt target gene that encodes an orphan G protein-coupled receptor characterized by a large leucine-rich extracellular domain and seven transmembrane domains^[9]. Using lineage tracing experiments, they demonstrated that the *Lgr5*⁺ cells are multipotent, giving rise to all the different intestinal epithelial cells types, and are very long-lived. Similar observations were made also in the colon, suggesting that the CBC *Lgr5*⁺ cells are authentic intestinal SCs^[10]. Consequently, *Lgr5* can be identified as a definitive marker of crypt SCs^[11,12].

Other than the resident intestinal SCs, recent studies have suggested that mesenchymal cells derived from bone marrow (BM) have a crucial role in intestinal repair and fibrosis^[13-15]. Studies conducted on both experimental animals and humans given sex-mismatched BM-derived SCs (BM-SCs) transplants showed that a population of myofibroblasts (MFs) derived from the male donor populated the mucosa in the female host intestine, and the sub-epithelial compartment in particular^[16]. Given the importance of these MFs in orchestrating epithelial cell turnover and function, it may be that they have a positive therapeutic effect on gastrointestinal functions. Further studies demonstrated that donor-derived cells were able to repopulate the host's intestinal epithelium with cells expressing all four lineage markers (goblet cells, Paneth cells, enteroendocrine and enterocytes^[4]). The donor BM-SCs were also able to differentiate into all the cell types needed for neo-angiogenesis (i.e. pericytes, endothelial cells and vascular smooth muscle cells), thus contributing to tissue repair. However, the mechanisms by which SCs take effect in gut injury are still under debate. Some researchers believe that BM-SCs differentiate into intestinal SCs or progenitor epithelial cells, while others have demonstrated that a fusion with resident cells takes place. Protection against injury can be achieved even in the absence of SC differentiation, giving the impression that SCs aid native tissues via cell-cell interaction or by releasing protective substances as they transit through injured tissues^[4].

In the liver, regeneration mainly involves mature hepatocytes, highly differentiated cells with a long lifespan that can re-enter the cell cycle and restore the liver mass in response to parenchymal loss^[5]. If the hepatocytes' replication is impaired, or experimentally inhibited, then regeneration can be accomplished by the activation, expansion, and differentiation of LPCs putatively located within the canals of Hering (CoH). LPCs are responsible for the so-called “ductular reaction” in humans, which corresponds to the oval cell (OC) response seen in specific rodent models of liver injury^[17]. Numerous studies have shown that both OCs and LPCs are highly clonogenic and bipotent cells (capable of differentiating into hepatocytes and cholangiocytes), and that they co-express biliary and hepatocytic markers, as well as HSC-associated antigens, such as CD34 and c-kit^[18]. LPCs are

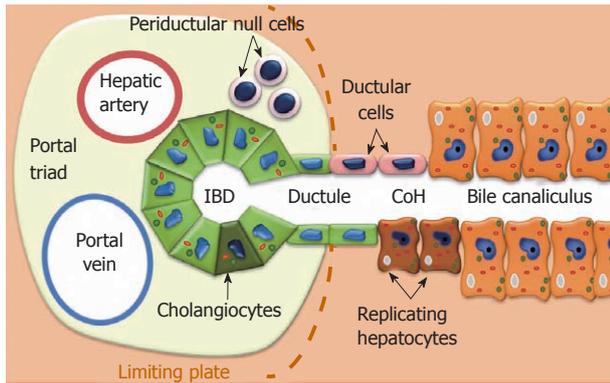


Figure 1 Hepatic cell populations with stemness potential and their location within the liver. IBD: Inflammatory bowel diseases; CoH: Canal of Hering.

a heterogeneous population with a variable stemness potential and notable phenotypic discrepancies, depending on the experimental conditions^[19]. Using a functional assay, Kuwahara *et al.*^[20] found 4 possible hepatic cell populations with a stemness potential: (1) replicating hepatocytes at the parenchymal/stromal interface; (2) ductular cells of the CoH; (3) cholangiocytes of the intralobular bile ducts; and (4) periductular null cells (devoid of hepatocytic and biliary markers). The asymmetrically-dividing cells that populate these sites may represent some form of lineage hierarchy within the LPC population: periductular null cells might give rise to the cytokeratin-positive cells of the CoH, which in turn could generate the intraductal cells and periductular hepatocyte-like cells^[21] (Figure 1).

In some cases the LPC are supported in the regeneration process by stem cells mobilized from BM. Various studies have in fact reported that stem cells from BM can contribute to liver regeneration through their release into the circulation, migration to the liver and differentiation into hepatocytes. However, the extents to which this occurs and the mechanisms involved remain highly controversial issues^[1,22].

Estimates of liver repopulation by HSCs vary widely, ranging from 0.01% to 40%^[23-34]. Petersen *et al.*^[24] originally reported that BM-SCs transplanted into sublethally irradiated rats repopulate the BM and then migrate to the liver and “transdifferentiate” into hepatocytes by entering the liver OC progenitor pathway. BM-SCs are believed to have the ability to expand clonally and to have a bipotential capacity that enables them to differentiate into both hepatocytes and bile duct epithelial cells^[35,36]. This mechanism was generally accepted until studies by Wang *et al.*^[37] using lacZ marking showed that BM cells did not enter the OC pool in wild-type mice treated with DDC, nor did they contribute to liver repopulation by OCs in mouse recipients. Dabeva *et al.*^[38] also showed that BM cells transplanted into rats contributed less than 1% to the OCs expanded after inducing damage with 3 different methods: (1) 2-acetylaminofluorene/partial hepatectomy (PH); (2) retrorsine/PH; or (3) D-galactosamine-induced liver injury. The debate is still open.

Fibrosis

Fibrosis is the progressive accumulation in the tissues of fibrillary extracellular matrix (ECM), but - for its pathophysiological role to be fully understood - it should be seen as a process of chronic wound healing taking place in a tissue continually subjected to injury.

Intestinal fibrosis is a potentially severe complication of inflammatory bowel diseases (IBD), such as Crohn’s disease, and its pathophysiology is still unclear. The presumably key events in the development of IBD-associated fibrosis are the exposure of MFs to inflammatory mediators, and the subsequent production of ECM and tissue remodeling^[29]. Intestinal fibrosis is characterized by myofibroblast accumulation secondary to their local proliferation, migration and recruitment from the BM. Different studies have shown that BM-SCs frequently engraft in the intestine and differentiate into MFs in the lamina propria. *In situ* hybridization was used to detect Y-chromosomes in these cells and their myofibroblastic phenotype was confirmed by their immunostaining positivity for alpha-smooth muscle actin (α -SMA) and negativity for desmin, the mouse macrophage marker F4/80, and the hematopoietic precursor marker CD34. These results were confirmed in mice as early as 1 wk after BM transplantation, and were also seen 2 and 6 wk after cell transplantation, indicating that transplanted BM cells are capable of withstanding a sustained turnover of the MF cells in the lamina propria^[39].

Intestinal MFs can also derive from alternative sources however, such as circulating fibrocytes and the process of “epithelial-mesenchymal transition (EMT)”. Fibrocytes are BM-derived circulating mesenchymal progenitors that co-express hematopoietic and mesenchymal cell markers, and produce ECM components^[30]. In inflammatory processes, fibrocytes are released from the BM and migrate to the sites affected where they differentiate into epithelial, endothelial, neuronal and mesenchymal cells^[30]. In several systems, significant numbers of fibroblasts may be generated by the transformation of non-mesenchymal into mesenchymal cells in a process termed EMT^[40], during which epithelial cells lose their expression of E-cadherin and other components of epithelial cell junctions and acquire a mesenchymal cell phenotype^[41]. This process has a role in the genesis of the fibroblasts that contribute to fibrosis in adult tissues.

In the liver, fibrosis is a multicellular, integrated process requiring a close cross-talk between hepatocytes, cholangiocytes, and non-parenchymal cells (including infiltrating inflammatory cells, Kupffer cells, hepatic stellate cells and sinusoidal endothelial cells)^[42]. Nearly all forms of chronic liver disease can cause fibrosis, though its rate of progression and likelihood of leading to cirrhosis differs in the various etiologies.

All forms of fibrogenesis develop in the context of tissue damage, where hepatocytes and non-parenchymal cells produce signals that target hepatic stellate cells and other fibrogenic MFs, leading to the accumulation of ECM. The generation of reactive oxygen species and

non-oxidant products of oxidative stress exacerbates the hepatocellular damage, promoting inflammation and Kupffer cell activation. Oxidative stress also directly provides pro-fibrogenic stimuli to hepatic MFs^[43].

Hepatic stellate cell activation is considered the major source of MFs in liver damage, but other ECM-producing cells contribute to liver fibrosis, including fibroblasts and portal tract MFs, smooth muscle cells localized in the vessel walls, and MFs located around the centrilobular vein^[42]. Recent studies have demonstrated, moreover, that epithelial cells (both hepatocytes and bile duct epithelial cells) have the ability to acquire myofibroblastic features in the process of EMT, as in the intestine^[44], although the extent to which this process contributes to the development of fibrosis remains controversial.

The role of BM-SCs in the pathogenesis of liver fibrosis has recently been the object of considerable interest. It is usually impossible to track the lineage of cells in humans, although this was done in a study by Forbes *et al.*^[45] in a series of male patients with sex-mismatched liver transplants who subsequently developed graft fibrosis, and in one female patient who developed cirrhosis after receiving a BM transplant from a male. The authors used Y chromosome tracking to identify the origin of the cells participating in liver fibrosis. Substantial numbers of scar-associated MFs in fibrotic areas were found to derive from BM. Using a mouse model of liver fibrosis in which sex-mismatched BM transplants were performed, the same group found clear evidence of a BM contribution to the MFs in fibrotic scars^[46], and provided evidence that the BM contributes to both the macrophage and stellate cells populations in the injured liver^[47]. By subfractionating the BM-SCs compartment, it was demonstrated that, although HSCs contribute to the inflammatory cell infiltrate, the BM-derived MF-like cells originate from MSCs. Intriguingly BM-SCs are widely distributed in the scar tissue in advanced fibrosis. This suggests that, whatever the origin and topography of the injury in chronic disease, BM-derived MFs gradually begin to replace local MF recruitment. Evidence of a functional role of BM-derived MFs was provided by transplanting BM from mice bearing a reporter transgene for collagen, before inducing fibrosis, showing that the recruited MFs transcribe this gene. Also, when wild-type mice were transplanted with BM from a transgenic mouse that develops a characteristic liver scarring pattern (because it expresses a form of collagen I not susceptible to degradation by matrix metalloproteinases), CCl₄ administration induced liver scarring, with characteristics similar to those seen in the BM donor mouse. These data indicate that the transfer of genetically modified BM alters the phenotype of the liver fibrosis, making it reflect the genotype of the BM donor rather than that of the recipient mouse. The same study also provided conclusive evidence that recruited cells contribute directly to fibrosis through the expression, synthesis, and secretion of collagen^[47].

Despite the available evidence for the contribution of BM-SCs, the matter is still widely disputed. Indeed,

a recent paper revealed an unexpectedly limited role of BM-derived cells in collagen production in two mechanistically distinct models of liver fibrosis^[48]. Although some of the BM-derived cells exhibited a mesenchymal morphology resembling that of MFs, the number of BM-derived α -SMA-positive cells was much smaller than previously reported. More importantly, specific and quantitative analyses of collagen type I alpha 2 promoter activation, using a combination of enhanced green fluorescent protein and luciferase reporter genes, clearly showed that BM-derived cells produce little, if any, type I collagen during hepatic fibrogenesis.

STEM CELLS AS A THERAPEUTIC TOOL FOR LIVER AND INTESTINAL DISEASES

Stem cell therapy in intestinal diseases

It is well recognized that inflammatory and immune-mediated bowel diseases, such as Crohn's disease and celiac disease, are due to a dysregulation of the immune response in genetically susceptible individuals. Both these conditions have a strong impact on public health due to their increasing prevalence and incidence in Western populations.

Medical therapy for these disorders has improved dramatically in the last decade with the introduction of targeted biological therapies, the optimization of older therapies and a better understanding of the mucosal immune system and genetics involved in the pathogenesis of IBD. Nevertheless, a considerable number of patients remain refractory to therapy, or become unresponsive to, or intolerant to therapy^[49]. Unconventional strategies have consequently been investigated, identifying the use of SCs as an effective alternative approach to IBD.

The possibility that SCs might represent an effective treatment in IBD and celiac disease initially emerged from several case reports of remission being induced in patients undergoing hematopoietic stem cell transplantation (HSCT) for concomitant hematological malignancies. Theoretically, allogeneic HSCT could be beneficial by replacing the genetic predisposition to IBD, and autologous HSCT might also offer the advantage of a more intense immunosuppression than would otherwise be given, thereby clearing the body of committed lymphocyte clones. On this basis, two phase I clinical trials were carried out on a total of 16 patients who underwent autologous HSCT for refractory Crohn's disease^[50,51]. The results were promising, with remission observed in 14/16 patients, but the adverse effects were far from negligible. As for celiac disease, autologous HSCT was used in a phase I clinical trial to treat 7 patients suffering from refractory celiac disease type II, prompting a significant reduction in the number of aberrant T cells and an improvement in both clinical and serological parameters in 6/7 cases after a mean follow-up of 15.5 mo^[52]. In contrast, when 15 patients with enteropathy-associated T cell lymphoma as a complication of celiac disease were treated with autologous HSCT in three phase I trials, 10

patients died of progressive disease, indicating that the HSCT afforded no benefit in prognostic terms^[53-55].

MSCs can be isolated from various tissues, such as BM, adipose or muscle tissue, fetal tissues and perivascular tissue, and they can support hematopoiesis and differentiation towards adipogenic, osteogenic^[56], and myogenic lineages^[57]. These cells are identified by their expression of a particular panel of surface molecules, e.g., CD105, CD73, CD90, and the absence of CD14, CD34, CD45, and HLA-DR. They elicit no proliferative response from alloreactive lymphocytes because of the negligible levels of extracellular MHC class I and II determinants (though they are present intracellularly). MSCs are also endowed with important immunomodulatory functions in all the cells involved in both the innate and adaptive immune responses^[58]. *In vitro* studies have demonstrated that MSCs can inhibit or suppress several T cell functions, such as proliferation after mitogen and antigen stimulation and inducing cell cycle arrest, probably *via* the release of chemokines, nitric oxide and the enzyme indoleamine 2, 3-dioxygenase. MSCs also do not preferentially target any T cell subset and their inhibition can also extend to B cells, NK cells, and dendritic cells. More precisely, dendritic cells cultured in the presence of MSCs have an impaired T cell stimulatory activity in a mixed lymphocyte reaction, with a shift from predominantly pro-inflammatory Th1 to anti-inflammatory Th2 cells, thus skewing the immune response to T-cell tolerance. Combined, these results support a role for MSCs in preventing rejection after organ transplantation and in the treatment of immune-mediated diseases.

A parallel series of *in vivo* studies conducted on animal models of gastrointestinal injury (gastric and colonic ulcers, and IBD), such as dextran sulfate sodium- and trinitrobenzene sulfoxide-induced colitis, showed beneficial effects of MSCs after both systemic infusion and topical injection^[59-61]. These studies showed that MSCs preferentially homed onto areas of mucosal injury, promoted tissue repair and neo-angiogenesis, reduced inflammation, and restored the immune cell balance.

To date, over 60 MSC clinical trials have been registered and/or are underway according to the website (www.clinicaltrials.gov) in the fields of immune-mediated and cardiovascular diseases, orthopedics, and organ transplantation. As for gastrointestinal diseases, MSCs were used in a single systemic infusion in an open-label phase 2 trial testing Prochymal (*ex vivo* cultured human MSCs, Osiris Therapeutics) for the treatment of refractory Crohn's disease. All 10 patients treated had a statistically significant, mean 105-point reduction in their CDAI score by day 28, and there appeared to be a positive correlation between the dose of cells infused and the clinical response, with patients on high doses achieving a better response^[62]. The most impressive results, however, emerged from a multicenter, phase II trial in which patients with severe, acute GvHD refractory to conventional therapies were treated with two MSC infu-

sions, resulting in an overall survival rate of 53% two years later^[63].

Given the immunomodulatory function of MSCs and their ability to home onto sites of tissue injury, the Ciccocioppo group in Pavia demonstrated that BM-derived MSCs from Crohn's disease patients can be isolated and expanded, and that this cell population exhibits the same biological characteristics as those from healthy controls. MSCs from Crohn's disease patients may therefore be considered for use in cell therapy in an autologous setting.

In general, the convenient isolation procedure, the lack of significant immunogenicity (which allow for allogeneic transplantation without using immunosuppressive drugs), the absence of ethical controversies, the potential to differentiate into tissue-specific cell types with a trophic activity, and their immunosuppressive and immunomodulatory effects make MSCs particularly interesting with a view to their potential uses in regenerative therapy in many gastrointestinal diseases^[64].

Stem cell therapy in liver diseases

Liver disorders affect hundreds of millions of patients worldwide. Liver failure can be defined as the inability of the liver to perform its normal, physiological synthetic and metabolic functions due to severe hepatic injury. Classically, liver failure is divided into acute (ALF) and chronic forms (CLF). ALF is characterized by the sudden onset of hyperbilirubinemia, hepatic encephalopathy and coagulopathy with no underlying liver disease; this remains a dramatic, unpredictable condition, with high morbidity and mortality rates^[65]. CLF usually occurs in the context of hepatic cirrhosis, which can be the result of many possible causes, including excessive alcohol consumption, chronic hepatitis B or C, autoimmunity, or metabolic disorders. The natural course of chronic liver disease is often complicated by acute episodes of potentially reversible decompensation, triggered by a precipitating event, such as infection or upper gastrointestinal bleeding; this situation is frequently called acute-on-chronic liver failure (AoCLF)^[66].

With the help of intensive care and artificial liver support, a substantial proportion of ALF patients may recover spontaneously, but orthotopic liver transplantation (OLT) is the only curative option for end-stage liver diseases and remains the final resort of proven benefit for ALF too, raising the one-year survival rate of ALF patients from 50% to 75%^[67]. Organ shortage remains a major limitation however, and alternative solutions are being examined. Hepatocyte transplantation, or the use of these cells in bioartificial livers^[68,69], have attracted attention in the past three decades, but their exploitation in clinical practice still poses considerable problems, including the difficulty of obtaining hepatocytes and particularly of maintaining their viability and differentiated function when cultured *in vitro*. These issues have been investigated with the aim of improving results^[70,71], but the use of SCs and/or growth factors seems to be

a more appealing and applicable solution for promoting liver repair^[3,72,73].

The possible therapeutic value of BM-SCs was first investigated by intraportally transplanting autologous CD133⁺ cells in patients with liver cancer undergoing portal embolization prior to extensive liver resection (LR)^[74]. A significant improvement in Child-Pugh score and albumin levels was reported in 9 cirrhotic patients given a portal vein infusion of unsorted autologous BM-SCs^[75]. Improved liver function after LR was also recently documented in patients with cirrhosis and hepatocellular carcinoma (HCC) after they underwent autologous BM-SCs transplantation prior to surgery^[76].

Other clinical approaches rely on the administration of G-CSF in combination with leukocyte apheresis and reinfusion of mobilized HSC. The feasibility, safety and BM-SCs mobilization patterns following G-CSF treatment has been assessed in patients with cirrhosis^[77]. Yannaki *et al*^[78] reported on the successful use of boost infusions of mobilized CD34⁺ cells after a standard G-CSF regimen in 2 patients. A significant biochemical and histopathological improvement was achieved by infusing G-CSF-mobilized CD34⁺ HSCs in a patient with drug-induced ALF^[79]. In a phase I clinical trial on 5 patients with AoCLF, administering G-CSF and then reinfusing the CD34⁺ cells improved liver function in more than 50% of cases during a 60-d follow-up^[80]. The same patients were then monitored for up to 18 mo, during which time the procedure was judged to be safe and the beneficial effects lasted around 12 mo^[81]. In 9 patients with alcohol-related cirrhosis, the reinfusion of CD34⁺ HSCs (collected after G-CSF mobilization and expanded *in vitro*) was well tolerated and beneficial to liver function^[82]. In another trial, 40 patients with HBV-related cirrhosis were randomized to receive G-CSF alone or in combination with the reinfusion of peripheral blood monocytes in the hepatic artery. During a 6-mo follow-up, a significant biochemical and clinical improvement was seen in both groups^[83]. Finally, G-CSF has been used alone to treat end-stage liver diseases in a few small clinical trials: overall, the procedure proved safe and was well tolerated, though these studies were too small to draw any conclusions regarding patient survival or treatment efficacy^[77,84-86]. It is worth noting, however, that G-CSF administration was associated with the induction of endogenous liver SCs proliferation within 7 d in the largest randomized trial published to date, conducted by Sphar *et al*^[86].

Most of the above-mentioned clinical trials have their limitations, having been conducted on small groups of patients, with no controls, and using outcome parameters that are easily biased, as mentioned elsewhere^[87,88]. It seems likely that the main role for SC-based therapies in hepatology will be as a bridge to transplantation, or as a way to maintain patients who are not eligible for OLT, using repeated infusions of SCs and/or growth factors to stabilize their liver function. Some conceptual and technical issues nonetheless continue to restrict the diffusion of such treatments in clinical practice^[87,89] and, until these open questions have been properly answered,

SC-based therapies for liver diseases should be limited to well-designed and adequately powered clinical trials.

THE DARK SIDE OF STEM CELLS: THE CANCER STEM CELLS THEORY

The cellular origin of most solid tumors is largely unknown, but different tumor subtypes seem to reflect different origins at the time of cancer initiation. Cells within the tumor population also often exhibit different phenotypic and functional characteristics, which lend heterogeneity to the tumor mass^[90].

At least two models have been proposed to account for the heterogeneity and inherent differences in tumor-generating capacity of SCs, including the cancer stem cells (CSCs) and the clonal evolution models^[91,92]. In the CSCs model, based on a cell hierarchy within the tumor, only a minority of tumor cells can generate a tumor, based on their self-renewal properties and enormous proliferative potential. The clonal evolution model, on the other hand, postulates that genetic and epigenetic changes can occur stochastically over time in individual cancer cells, giving them a selective advantage in proliferating and forming a tumor mass. These two models are not mutually exclusive in cancers following a stem cell model, because CSCs would be expected to evolve via the clonal evolution of transformed SCs^[2]. The hypothesis that stem cells could be targets of malignant transformation has led to an awareness of the similarities between CSCs and normal SCs, including their surface marker phenotype and molecular machinery relating to self-renewal and differentiation. In the last ten years, evidence has been accumulating to indicate that CSCs are not only involved in the perpetuation of hematopoietic tumors, but also play a part in various solid cancers, including those of the breast, brain, prostate, colon, and liver.

Colorectal cancer is the second leading cause of cancer-related death in the western world. A number of studies have produced evidence of the existence of colon CSCs and demonstrated that the tumorigenic cell population of colorectal cancer can be isolated from its expression of specific cell surface biomarkers. The existence of colon CSCs was first reported by the Dick and De Maria research groups^[93,94], each of which identified a small population of cancer cells capable of initiating tumor growth in immunodeficient mice and staining positive for the marker CD133. This marker (also known as prominin-1) was initially identified as a marker of *Drosophila* neuroblasts^[95]. Although it is expressed on different types of stem cell and may play a part in cell polarity, it has no known key role in stem cell function. CD133 is reportedly a potential marker of CSCs in various tumors^[96], but its role as a marker of colorectal cancer was recently questioned by Shmelkov *et al*^[97], who showed that both CD133-positive and CD133-negative metastatic colon cancer cells can initiate a tumor. For the time being it is not clear whether all reported markers really do mark CSCs or merely lead to their enrichment,

and whether these markers are directly linked to stem cell functions. So the search for the perfect CSC marker, which could be useful as a therapeutic target, goes on.

The cellular origin of HCC has long been debated, but whether it originates from mature hepatocytes or stem/progenitor cells, or both, remain to be seen. It has been suggested that intrahepatic stem cells can give rise to human HCC and cholangiocarcinoma (CC)^[98], since oval cell activation has been demonstrated in rodent models of HCC and CC^[99,100]. A role for intrahepatic stem cells in carcinogenesis is also supported by a histological subtype of liver malignancy that displays features of both HCC and CC (HC-CC), combined with the presence of numerous liver progenitor cells^[101,102].

Putative CSCs have recently been isolated from both cancer cell lines and primary HCC tissue using different cell surface markers specific for normal stem cells (Table 1). CD133 seems to play a crucial part in HCC too. Cells positive for CD133 have been reported to exhibit a greater tumorigenicity than the corresponding CD133-negative cells in HCC cell lines^[103,104]. Another frequently-used CSCs marker for isolating liver CSCs is EpCAM^[105,106]. As mentioned earlier, the classification of HCC patients based on EpCAM expression has a prognostic significance. In addition, the prospectively isolated EpCAM cells exhibited an adult SC-like gene expression profile. Given that the heterogeneity of human liver cancer may be related to its CSC origin, hepatic CSCs cannot be identified by the expression of a single marker. A clear liver CSCs profile has yet to be established, and Colombo *et al.*^[107] recently implemented a long-term culture system for isolating and characterizing human liver CSCs. Different HCC cell lines and clones from single HCC specimens were obtained, probably generated from different clonogenic cells, some of which had SC-like features. These HCC cell lines revealed a different morphology, antigen profile, proliferative potential, and *in vitro* antitumor drug resistance. The progenies of clone-initiating cells tend to reproduce the original population over time, suggesting the existence of hierarchically different cells and thus supporting the CSCs model. On the other hand, other HCC cell lines, which showed less aggressive features *in vivo* and were unable to generate different clones *in vitro*, seem to support the clonal evolution model. Taken together, these preliminary data appear to indicate that liver cancer heterogeneity can be explained by a model incorporating both the cancer stem cell hypothesis and clonal evolution mechanisms.

CONCLUSION

Stem cells may have the potential for replacing cells lost as a result of many devastating diseases, such as acute and chronic liver diseases and inflammatory or immune-mediated bowel diseases. There is little doubt that this potential benefit underpins the huge interest in stem cell research. Nonetheless, despite encouraging results obtained in regenerative medicine and the promise of

Table 1 Immunophenotypes of putative hepatocellular carcinoma stem cells reported in literature

Phenotype	Ref.
EpCAM ⁺ cells	[106,108]
CD133 ⁺ cells; CD133 ⁺ /44 ⁺ cells	[103,109]
CD133 ⁺ /ALDH ⁺ cells	[110]
AFP ⁺ /CD56 ⁺ /c-Kit ⁺ cells	[111]
CD90 ⁺ /CD44 ⁺ cells	[112]
Side population	[113]

future therapies, more studies are needed to thoroughly understand the biology of stem cells, overcome barriers related to immune response, and assess their oncogenic properties. Moreover, some conceptual and technical issues still restrict the diffusion of such treatments in clinical practice. There is still no consensus on the best methods to use for stem cell purification, the ideal route of delivery, amount of cells to infuse, and timing of infusions. Moreover, the risk of malignant transformation and/or pro-fibrogenic effects of SC-based therapies cannot be ruled out, and this imposes the need for a careful evaluation and longer follow-up periods to ascertain the safety and efficacy of SCs in therapeutic applications. Finally, while stem cell experimentation is appealing to researchers and medical specialists, it is also arousing fervent ethical, social and political debate.

Adopting scientific methods based on randomized and controlled trials should produce the necessary results on the real therapeutic role of stem cells. Indeed, the latest findings and ongoing technical improvements entitle researchers to have high hopes for the near future. The scientific researchers and physicians of today continue to hope that the mythological idea of regeneration in the story of Prometheus can be made real by developing therapies to restore lost, damaged or aging cells and tissues in the human body^[3]. Much has been done, but there is still a lot to do. The future is open and promising.

ACKNOWLEDGMENTS

The authors are grateful to all the members of the “Stem Cells in Gastroenterology” Study Section of the SIGE who contributed to a unique and stimulating debate. We also gratefully acknowledge the support of the SIGE and its highly professional staff. “Stem Cells in Gastroenterology” Study Section of the SIGE: Pietro Andreone (Bologna), Diletta Arcidiacono (Padua), Michele Barone (Bari), Vincenzo Boccaccio (Pavia), Massimiliano Cadamuro (Padua), Andrea Cappon (Padua), Tatiana Chioato (Padua), Carolina Ciacci (Naples), Federico Colombo (Milano), Gino Roberto Corazza (Pavia), Alfredo Di Leo (Bari), Luca Fabris (Padua), Isabel Freitas (Pavia), Silvia Gaia (Turin), Stefania Lorenzini (Bologna), Maurizio Parola (Turin), Daniele Prati (Lecce), Maria Luisa Russo (Pavia), Cristina Ubezio (Pavia), Lorenzo Valfrè di Bonzo (Turin), Giovanni Zanellati (Pavia).

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S- Editor Sun H L- Editor Rutherford A E- Editor Li JY



Current trends in management of hepatitis B virus reactivation in the biologic therapy era

Claudio M Mastroianni, Miriam Lichtner, Rita Citton, Cosmo Del Borgo, Angela Rago, Helene Martini, Giuseppe Cimino, Vincenzo Vullo

Claudio M Mastroianni, Miriam Lichtner, Rita Citton, Cosmo Del Borgo, Helene Martini, Infectious Diseases Unit, Fondazione Eleonora Lorillard Spencer Cenci, Sapienza University, SM Goretti Hospital, 04100 Latina, Italy
Angela Rago, Giuseppe Cimino, Hematology Unit, Sapienza University, SM Goretti Hospital, 04100 Latina, Italy
Vincenzo Vullo, Department of Public Health and Infectious Diseases, Sapienza University, Policlinico Umberto I, 00161 Rome, Italy

Author contributions: Mastroianni CM, Lichtner M, Citton R, Del Borgo C, Rago A and Martini H contributed to manuscript conception, preparation and writing; Cimino G and Vullo V reviewed the manuscript.

Correspondence to: Claudio M Mastroianni, MD, PhD, Infectious Diseases Unit, Fondazione Eleonora Lorillard Spencer Cenci, Sapienza University, SM Goretti Hospital, Via Canova 2, 04100 Latina, Italy. claudio.mastroianni@uniroma1.it

Telephone: +39-773-6553741 Fax: +39-773-6553735

Received: August 30, 2010 Revised: January 12, 2011

Accepted: January 19, 2011

Published online: September 14, 2011

the use of prophylactic antiviral therapy. In this article, we discuss current trends in the management of HBV reactivation in immunosuppressed patients receiving biologic therapy, such as rituximab, alemtuzumab and TNF- α antagonists.

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Key words: Hepatitis B virus; Virus reactivation; Rituximab; Tumor necrosis factor- α antagonists; Biologic agents; Antiviral drugs

Peer reviewer: Rakesh Aggarwal, Additional Professor, Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India

Mastroianni CM, Lichtner M, Citton R, Del Borgo C, Rago A, Martini H, Cimino G, Vullo V. Current trends in management of hepatitis B virus reactivation in the biologic therapy era. *World J Gastroenterol* 2011; 17(34): 3881-3887 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i34/3881.htm>
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Abstract

Hepatitis B virus (HBV) reactivation represents an emerging cause of liver disease in patients undergoing treatment with biologic agents. In particular, the risk of HBV reactivation is heightened by the use monoclonal antibodies, such as rituximab (anti-CD20) and alemtuzumab (anti-CD52) that cause profound and long-lasting immunosuppression. Emerging data indicate that HBV reactivation could also develop following the use of other biologic agents, such as tumor necrosis factor (TNF)- α inhibitors. When HBV reactivation is diagnosed, it is mandatory to suspend biologic treatment and start antiviral agents immediately. However, preemptive antiviral therapy prior to monoclonal antibody administration is crucial in preventing HBV reactivation and its clinical consequences. Several lines of evidence have shown that risk of HBV reactivation is greatly reduced by the identification of high-risk patients and

INTRODUCTION

Hepatitis B virus (HBV) infection remains a major public health issue, and represents an important cause of liver-related morbidity and mortality. It is estimated that one third of the world's population has been infected with HBV and 350 million people worldwide are affected by chronic HBV infection. HBV reactivation is a well-known phenomenon in chronic HBV carriers and it represents a life-threatening complication following cytotoxic or other immunosuppressive anticancer therapy^[1-3]. The liver damage due to HBV reactivation is characterized by two pathogenetic phases. Initially, during the stage of intense immunosuppressive therapy, there is markedly enhanced viral replication, as reflected by increases in serum levels

of HBV DNA, which results in widespread infection of hepatocytes. Following completion of immunosuppressive therapy and subsequent restoration of immune function, there is a rapid cytotoxic-T-cell-mediated destruction of HBV-infected hepatocytes, which is clinically characterized by development of hepatitis, hepatic failure, and even death.

The risk of clinical manifestations is mainly seen in overt carriers of HBV, but hepatitis might be observed also in subjects with occult HBV infection, who are positive for markers of previous exposure to the virus. The most commonly reported types of chemotherapy related to HBV reactivation are those used for the treatment of hematological malignancy, especially malignant lymphoma^[4,5]. Recently, the risk of HBV reactivation has been heightened by the use of biologic therapy, such as rituximab (anti-CD20) and alemtuzumab (anti-CD52) that cause profound and long-lasting immunosuppression^[6]. Emerging data indicate that HBV reactivation could also develop following the use of other monoclonal therapies, such as tumor necrosis factor (TNF)- α inhibitors^[7,8].

In the present article, we discuss the current trends for the management of HBV reactivation in immunosuppressed patients receiving biologic therapy, such as rituximab, alemtuzumab and TNF- α blockers.

HBV REACTIVATION: DEFINITION AND CLINICAL IMPLICATIONS

HBV reactivation is usually defined as an increase in HBV viral replication in patients with chronic or past HBV infection. The chance of HBV reactivation is closely linked to the serological profile of the infected patient^[9]. The risk is highest in patients who are positive for HBV surface antigen (HbsAg) (the so-called overt carriers of HBV)^[10]. Nevertheless, HBV reactivation can occur also in HBsAg-negative patients who have only markers of previous exposure to HBV [hepatitis B core antibody (HBcAb)-positive with or without hepatitis B surface antibody (HBsAb)] (the so-called occult carriers of HBV)^[11,12]. In such patients, a low level of HBV replication persists in the liver and in peripheral blood mononuclear cells for several years. The timing of HBV reactivation in patients undergoing monoclonal antibody therapy is not uniform. In overt carriers with high HBV viral load, hepatitis may occur during immunosuppressive treatment. However, in most cases, reactivation occurs following the cessation of biological treatment^[10]. At present, the most reliable and easy test used in clinical practice to diagnose HBV reactivation is the demonstration of an increase in serum HBV DNA levels. Serological tests, such as serum IgM HBcAb, are not sufficiently specific to discriminate between acute HBV infection and reactivation in patients with chronic HBV infection.

HBV reactivation may be symptomatic or asymptomatic. Hepatitis should be ascribed to HBV reactivation if it is preceded or accompanied by enhanced HBV viral replication (demonstrable increase in HBV DNA by at

least 10-fold, or an absolute increase to $> 10^8$ IU/mL). The time interval between the peak of HBV DNA viral load and hepatitis onset is variable. Classical features of hepatitis, including fatigue, jaundice, ascites, hepatic encephalopathy and coagulopathy, may be present. Patients with pre-existing cirrhosis are more likely to develop liver failure. Although some subjects can recover spontaneously, the mortality of HBV reactivation is reported as 5%-40%^[3].

IMMUNOSUPPRESSION AND RISK OF HBV REACTIVATION FOLLOWING MONOCLONAL ANTIBODY THERAPY

In recent years, the effective management of various hematological malignancies and immune-mediated inflammatory diseases has included the use of monoclonal antibody therapy. Rituximab (anti-CD20), alemtuzumab (anti-CD52) and TNF- α inhibitors represents a modern therapeutic approach that has greatly improved the prognosis and outcome of several malignant and non-malignant conditions. However, these biologic agents induce a profound immunosuppression and their use has been reported to be associated with development of severe infections, including HBV reactivation^[13,14].

Rituximab (anti-CD20 monoclonal antibody)

Rituximab therapy represents one of the most important advances in the treatment of lymphoproliferative disorders in the past 30 years. Rituximab is the archetype of this new class of compounds that is being currently used for the treatment of CD20-positive B-cell lymphoma, chronic lymphocytic leukemia (CLL) and autoimmune conditions, such as immune thrombocytopenia and collagenopathy. It is a chimeric murine-derived monoclonal antibody that is engineered by grafting the variable regions that target the CD20 antigen from a murine anti-CD20 antibody into human constant regions^[15]. Binding of rituximab to B cells activates complement and promotes C3b deposition in close proximity to cell-bound rituximab. In addition to antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity, rituximab also induces apoptosis through activation of caspase-3 and sensitizes cells to pro-apoptotic stimuli, which appears to be crucial in rituximab-induced cell killing. Treatment with rituximab is not commonly associated with severe opportunistic infections, while the risk of HBV reactivation is significantly higher if compared to conventional chemotherapy. There are increasing reports of HBV reactivation after rituximab therapy, either when used alone or in combination with chemotherapy^[3,16,17]. HBV infection has been reported to be the most common viral infection in patients affected by lymphoma and treated with rituximab. Aksoy *et al.*^[18] have shown that rituximab-related HBV infections are associated with a mortality rate of 50% compared to 33% observed in patients with other infections. In a recent retrospective

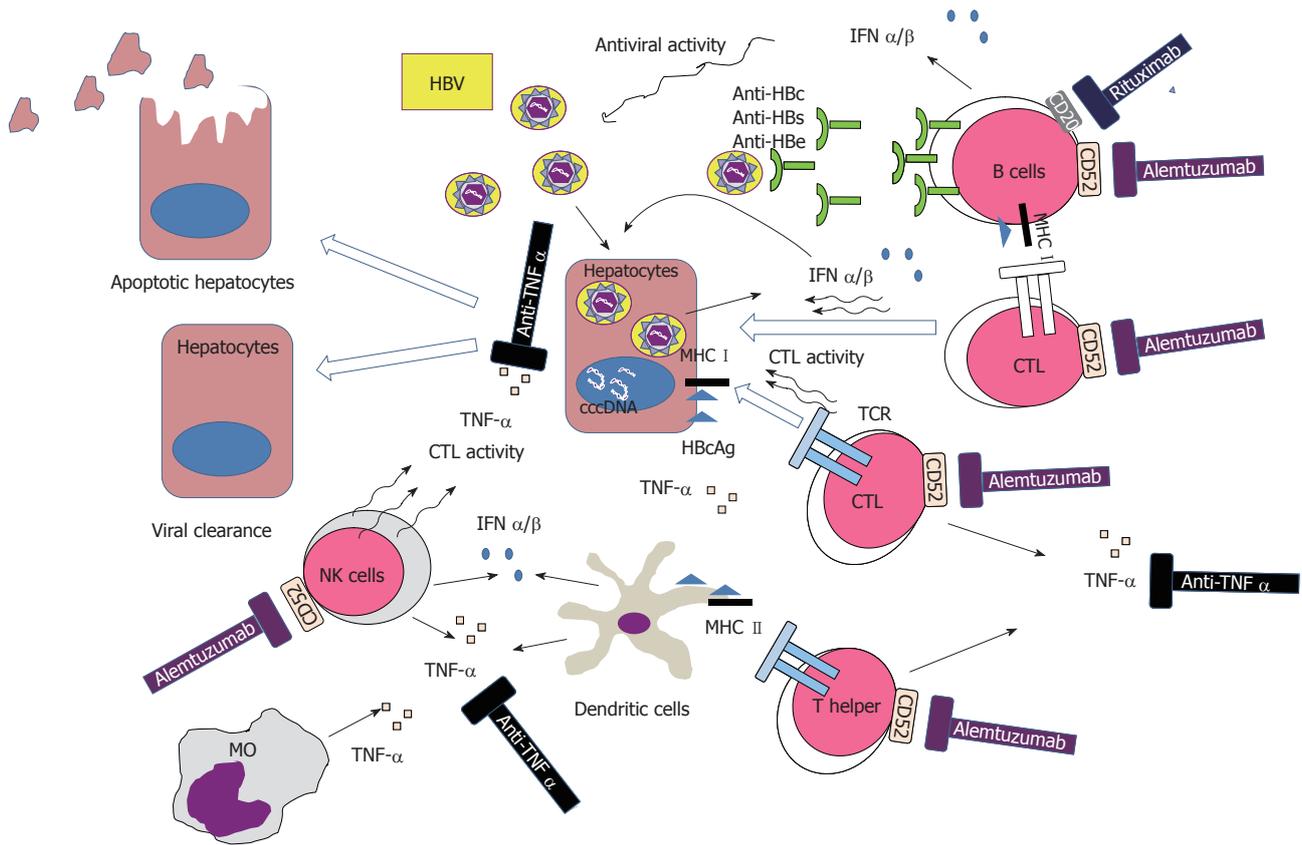


Figure 1 Pathogenetic hypothesis of hepatitis B virus reactivation following monoclonal antibody treatment. IFN: Interferon; TNF: Tumor necrosis factor; MHC: Major histocompatibility complex; NK: Natural killer; MO: Monocytes; TCR: T-cell receptor; CTL: Cytotoxic T lymphocyte; HBV: Hepatitis B virus; HBcAg: HBV core antigen.

study, 80% of HBV carriers without lamivudine prophylaxis experienced HBV-related hepatitis, including one fatal hepatic failure. Moreover, about 4% of HBsAg-negative patients developed *de novo* HBV hepatitis and two died of fulminant hepatitis^[10]. The profound and durable depletion of circulating population of B cells induced by the drug lead to dysregulation in host immunity to HBV and represents the main pathogenetic factor involved in viral replication and reactivation^[19]. The control of HBV infection is mediated mainly by HBV-specific cytotoxic T lymphocytes; nevertheless, B lymphocytes are still essential for antigen presentation. The failure in antigen presentation related to the prolonged depletion of B cells by rituximab may allow the HBV to escape the cytotoxic T lymphocyte control, hence leading to development of viral hepatitis reactivation (Figure 1).

Alemtuzumab (anti-CD52 monoclonal antibody)

Alemtuzumab is a humanized chimeric lymphocytotoxic monoclonal antibody that recognizes the antigen CD52, a 21- to 28-kDa heavily glycosylated membrane-anchored glycoprotein that is abundantly expressed on B and T cells, as well as on natural killer cells and macrophages). Typically, granulocytes, platelets, erythrocytes, and hematopoietic stem cells (HSCs) lack CD52 expression. CD52 is expressed on all CLL cells and indolent lymphomas. CD52 is not shed, internalized, or modulated and is

therefore an ideal antigen for targeted immunotherapy. However, the ubiquitous expression of CD52 on lymphocytes and monocytes is predictive of the increased neutropenia, lymphopenia and infectious complications observed with alemtuzumab therapy. Alemtuzumab was first used in the allogeneic HSC transplantation as *ex vivo* treatment of donor HSC or *in vivo* as part of combination chemotherapy or for the conditioning regimen of patients undergoing HSC transplantation^[20], with the aim to prevent graft-*vs*-host disease. More recently, alemtuzumab was approved for the treatment of high-risk patients with CLL. Most treated patients manifest profound peripheral-blood lymphopenia by 2-4 wk, which may persist for > 1 year. The effector mechanisms of alemtuzumab are not fully understood but may include ADCC, complement-mediated cell lysis, and induction of apoptosis (Figure 1). Alemtuzumab therapy is known to be associated with a high risk of serious bacterial, viral and fungal infections and should be used with great caution^[21]. The risk of severe infection, e.g., *Pneumocystis jirovecii* pneumonia, adenovirus infection and parvovirus B19 infection have also been reported. Although the risk of HBV reactivation after anti-CD52 monoclonal-antibody-mediated immunosuppression is not well defined, there have been multiple reports on the development of HBV reactivation after alemtuzumab therapy. In particular, alemtuzumab-containing chemotherapy regimens are as-

sociated with a high risk (29%) of reactivation of occult HBV infection and severe HBV-related hepatitis^[14,21,22]. Considering the degree and duration of immunosuppression induced by alemtuzumab, chronic HBV-infected patients should be treated with pre-emptive anti-HBV therapy before commencement of an alemtuzumab-containing chemotherapy regimen.

TNF- α antagonists

Emerging data suggest a potential risk of HBV reactivation in patients treated with anti-TNF- α agents^[8]. Currently, five TNF- α antagonists are approved in the United States for treatment of various immune-mediated inflammatory diseases, such as rheumatoid arthritis, psoriasis, psoriatic arthritis, ankylosing spondylitis, and Crohn's disease. Four are antibodies directed against TNF- α : infliximab, a chimeric mouse/human monoclonal antibody; adalimumab and golimumab, humanized monoclonal antibodies; and certolizumab, a pegylated Fab fragment of humanized monoclonal antibody. The fifth drug, etanercept is a protein that blocks the TNF- α receptor on human cells. The critical role of TNF- α in mediating inflammation, particularly granulomatous inflammation, has led to an increased susceptibility to infections by intracellular pathogens, such as *Mycobacterium tuberculosis*, *Mycobacterium avium* complex, *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Coccidioides* species, and possibly *Pneumocystis*, *Listeria*, and *Aspergillus*^[23,24]. To date, no consensus exists about the safety of anti-TNF- α in patients with chronic HBV infection. The 2008 American College of Rheumatology recommendations contraindicate the use of anti-TNF- α in patients with hepatitis^[25]. A recent consensus statement on biologic agents for treatment of rheumatic diseases recommends that patients should be screened for HBV before anti-TNF- α initiation^[26]. Specific warnings about HBV reactivation have been added to the American label by the FDA, recommending that antiviral therapy should be used in patients in whom HBV infection is diagnosed during anti-TNF- α therapy. Various case reports have suggested that TNF- α inhibition facilitates HBV reactivation and replication, with fulminant hepatic failure or fatal outcomes^[8]. HBV reactivation resulting in acute or subfulminant hepatitis is more likely in active HBV carriers. The risk of viral reactivation in occult HBV carriers seems to be significantly lower, and it is seen especially in patients with intense immunosuppression. In a large study of 88 patients with potential occult HBV infections exposed to TNF- α blockers, a small number of cases of HBV reactivation was observed^[27]. It is currently unknown if the risk of HBV reactivation is homogeneous within the family of TNF- α inhibitors. Among different anti-TNF- α agents, infliximab seems to be more frequently associated with viral reactivation, although the reasons for this are still unclear^[28]. In chronic HBV infection, TNF- α has a dual role: it protects the hepatocytes by decreasing transcriptional activity of the HBV core promoter gene; and the cytokine can augment hepatocyte apoptosis and eventual liver fibrosis through different mechanisms. Thus, paradoxically,

TNF- α blockade may be potentially beneficial, because long-term inhibition of this cytokine could have a protective effect on the hepatocytes; sparing them from injury and the liver from progressive fibrosis. However, failure to secrete appropriate amounts of TNF- α and impairment in the circulating CD8⁺ T-cell responses is associated with decreased clearance of HBV (Figure 1). This phenomenon raises concern regarding the safety of TNF- α inhibitors in patients with underlying liver disease^[7].

ANTIVIRAL STRATEGIES FOR MANAGEMENT OF HBV REACTIVATION

When HBV reactivation is diagnosed, it is mandatory to suspend all chemotherapy and start treatment with antiviral agents immediately. However, pre-emptive antiviral therapy prior to monoclonal antibody administration is crucial in preventing HBV reactivation and its clinical consequences. Several lines of evidence have shown that risk of HBV reactivation is greatly reduced by the identification of high-risk patients and the use of prophylactic antiviral therapy^[9,29]. The early identification of patients at risk of HBV reactivation before they receive monoclonal antibody therapy is crucial for avoiding the serious morbidity associated with disease reactivation. Virological screening is recommended in all patients at risk of HBV reactivation, who are planned to receive monoclonal antibody therapy (Figure 2). Testing for HbsAg, HBcAb and HBsAb could allow identification of patients with chronic, occult or resolved infection^[30]. HBsAg-positive patients, including both the active carriers (detectable HBV DNA with the presence of liver damage) and the inactive carriers (undetectable HBV DNA with no liver damage), should be treated immediately, in order to block viral replication and disease progression before monoclonal antibody therapy is given.

Recommendations for standard management are less clear in patients with occult HBV infection (HBsAg-negative/HBcAb-positive with or without HBsAb and undetectable HBV DNA). Indeed, in these patients, the risk of disease reactivation is significantly lower if compared with HBsAg-positive individuals. Therefore, it has been proposed that HBsAg-negative patients with positive HBcAb and undetectable HBV DNA should be strictly monitored by measurement of alanine aminotransferase (ALT) and serum HBV DNA; in case of HBV reactivation, as assessed by significant increase in HBV-DNA levels, they should be promptly treated with antiviral drugs, possibly before ALT elevation. Nevertheless, this approach is not universally accepted because a delay in the administration of antiviral treatment might expose the patient to the risk of severe hepatic failure during occult HBV infection^[29]. In our opinion, considering the relative safety of newer oral antiviral agents, prophylactic treatment represents a reasonable strategy; especially in high-risk patients receiving a rituximab-based regimen (Figure 2).

The optimal timing and duration of antiviral prophylaxis should be individualized, taking into consideration

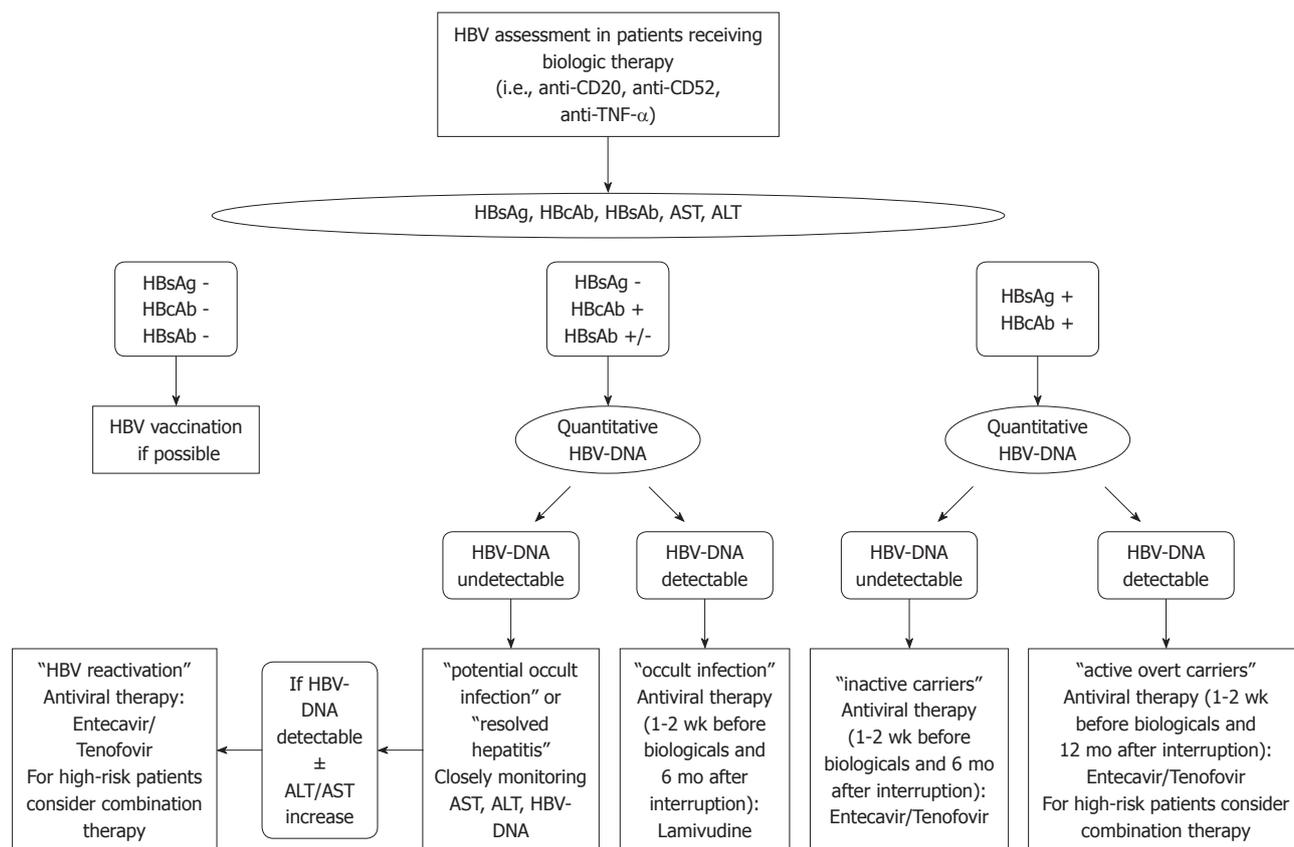


Figure 2 Proposed algorithm for the management of hepatitis B virus-infected patients requiring biologic therapy. HBV: Hepatitis B virus; TNF- α : Tumor necrosis factor α ; HbsAg: HBV surface antigen; HBcAb: Hepatitis B core antibody; HBsAb: Hepatitis B surface antibody; ALT: Alanine transaminase; AST: Aspartate transaminase.

viral and host factors. Starting antiviral treatment before immunosuppressive regimen and proceeding until the immune system has fully restored might be the most reasonable approach. In general, antiviral drugs should be initiated 1-2 wk before monoclonal antibody therapy and should be provided for at least 6 mo after the biologic treatment is stopped (Figure 2). Nevertheless, close monitoring of HBV DNA and ALT is strongly recommended after stopping prophylaxis to prevent severe HBV flare.

SELECTION OF ANTIVIRAL REGIMEN

Drugs used for treatment of HBV infection include interferon therapy and nucleos(t)ide analogs. Standard interferon (IFN)- α or pegIFN- α is contraindicated for treatment of HBV reactivation following biologic therapy.

Currently, five oral nucleos(t)ide antiviral drugs are approved for the treatment of chronic HBV infection: lamivudine, adefovir, entecavir, tenofovir, and telbivudine. The most commonly used antiviral drug in HBV reactivation is lamivudine^[31,32]. However, prognosis of patients undergoing a chemotherapeutic regimen including rituximab remains severe despite lamivudine therapy. Furthermore, lamivudine efficiency is hampered by the high rate of drug resistance mutations within HBV polymerase, which are associated with treatment failure^[33]. Lamivudine monotherapy can also select HBV strains associated with

resistance to entecavir^[34] and adefovir^[35]. To date, current treatment guidelines no longer recommend lamivudine monotherapy as primary treatment for chronic hepatitis B^[30]. This drug may have still a role in the prevention of HBV reactivation in patients with occult HBV infection (Figure 2). Nevertheless, considering the high rate of mortality associated with HBV reactivation during monoclonal antibody therapy, the use of more potent and effective antiviral drugs needs to be evaluated. Adefovir has been used in patients with established HBV reactivation and individuals treated with lamivudine prophylaxis who have developed drug resistance. However, primary treatment failure occurs in $\geq 10\%$ of patients treated with adefovir, and viral resistance occurs in nearly 30% of patients with hepatitis B e antigen-negative disease after 5 years of therapy^[36]. Entecavir and tenofovir are more potent and effective antiviral drugs that now represent the current preferred options for the management of treatment-naïve patients with chronic HBV infection^[2]. These new-generation HBV polymerase inhibitors exhibit very low rates of resistance in nucleoside-naïve patients when given as monotherapy. To date, there is no or very limited experience on the use of these drugs in the management of HBV reactivation in immunosuppressed patients undergoing monoclonal antibody therapy^[37]. There are a few case reports on telbivudine safety in HBV reactivation but there are no reports on tenofovir efficacy in

HBV reactivation.

The best evidence for antiviral prophylaxis of HBV reactivation is still for lamivudine (Figure 2), but there are few data on the use of entecavir. In two recent case reports, entecavir was successfully used as a first-line treatment for HBV reactivation following chemotherapy containing rituximab^[38,39]. However, in another study, it has been shown that entecavir increases mortality of patients with HBV reactivation, by increasing lactic acidosis and encephalopathy^[40]. Recently, we described a case of HBV reactivation in a patient with non-Hodgkin's lymphoma following a rituximab-based regimen: he was successfully treated with a combination antiviral treatment including entecavir and tenofovir^[41]. Antiviral treatment with entecavir was immediately initiated when the patient had HBV reactivation, and a rapid reduction of viral replication with normalization of transaminases was obtained. However, after a few months, the patient again presented with virological and biochemical breakthrough; probably related to the persistent and severe immune impairment of T- and B-cell function. We obtained full viral suppression and stable normalization of liver enzymes only after starting a combination strategy that included entecavir and tenofovir. Anti-HBV combination therapy has been considered in HBV-infected patients with decompensated cirrhosis and following liver transplantation^[57]. In our opinion, the combined use of antiviral drugs should be further evaluated for the management of HBV reactivation; especially in patients with severe impairment of the immune system (Figure 2), although safety data in different groups of patients are needed.

CONCLUSION

HBV reactivation represents an important medical issue in HBV-infected patients receiving biological treatment with monoclonal antibodies, such as rituximab, alemtuzumab or TNF- α antagonists. The identification of high-risk patients with active, inactive and occult HBV infection and the use of prophylactic antiviral treatment is crucial in avoiding the serious morbidity associated with HBV disease reactivation. Finally, studies examining the safety and efficacy of the latest generation anti-HBV drugs in sequential monotherapy, or in combination, for the management of HBV reactivation should be strongly encouraged.

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



Distribution, function and physiological role of melatonin in the lower gut

Chun-Qiu Chen, Jakub Fichna, Mohammad Bashashati, Yong-Yu Li, Martin Storr

Chun-Qiu Chen, Department of Gastrointestinal Surgery, Shanghai Tenth People's Hospital, Tongji University School of Medicine, 200072 Shanghai, China

Chun-Qiu Chen, Jakub Fichna, Mohammad Bashashati, Martin Storr, Division of Gastroenterology, Department of Medicine, University of Calgary, 3330 Hospital Dr NW, T2N 0N1 Calgary, Alberta, Canada

Yong-Yu Li, Department of Pathophysiology, Tongji University School of Medicine, 200092 Shanghai, China

Martin Storr, Division of Gastroenterology, Department of Medicine, University of Munich, Marchioninstr 15, 81377 Munich, Germany

Author contributions: Storr M designed the project; Chen CQ, Fichna J, Bashashati M and Li YY performed literature research; Chen CQ, Fichna J, Bashashati M and Storr M wrote the paper.

Correspondence to: Martin Storr, MD, Division of Gastroenterology, Department of Medicine, University of Munich, Marchioninstr 15, 81377 Munich, Germany. gidoc@gmx.com

Telephone: +49-89-70950 Fax: +49-3212-1027208

Received: January 11, 2011 Revised: March 18, 2011

Accepted: March 25, 2011

Published online: September 14, 2011

Abstract

Melatonin is a hormone with endocrine, paracrine and autocrine actions. It is involved in the regulation of multiple functions, including the control of the gastrointestinal (GI) system under physiological and pathophysiological conditions. Since the gut contains at least 400 times more melatonin than the pineal gland, a review of the functional importance of melatonin in the gut seems useful, especially in the context of recent clinical trials. Melatonin exerts its physiological effects through specific membrane receptors, named melatonin-1 receptor (MT1), MT2 and MT3. These receptors can be found in the gut and their involvement in the regulation of GI motility, inflammation and pain has been reported in numerous basic and clinical studies. Stable levels of melatonin in the lower gut that are unchanged following a pinealectomy suggest local synthesis and, fur-

thermore, implicate physiological importance of endogenous melatonin in the GI tract. Presently, only a small number of human studies report possible beneficial and also possible harmful effects of melatonin in case reports and clinical trials. These human studies include patients with lower GI diseases, especially patients with irritable bowel syndrome, inflammatory bowel disease and colorectal cancer. In this review, we summarize the presently available information on melatonin effects in the lower gut and discuss available *in vitro* and *in vivo* data. We furthermore aim to evaluate whether melatonin may be useful in future treatment of symptoms or diseases involving the lower gut.

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Key words: Melatonin; Ileum; Colon; Receptor; Motility; Inflammatory bowel disease; Clinical trial

Peer reviewers: John R Grider, PhD, Professor, Department of Physiology & Biophysics, Virginia Commonwealth University, PO Box 980551, Richmond, VA 23298, United States; Angelo A Izzo, Professor, Department of Experimental Pharmacology, University of Naples Federico II, Via D Montesano 49, 80131 Naples, Italy

Chen CQ, Fichna J, Bashashati M, Li YY, Storr M. Distribution, function and physiological role of melatonin in the lower gut. *World J Gastroenterol* 2011; 17(34): 3888-3898 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i34/3888.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i34.3888>

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine), discovered in 1917, is found in humans, animals, plants and microbes. It is a lipophilic compound diffusing rapidly through biological membranes and is involved in many regulatory processes, such as biological rhythms, intestinal reflexes, protection against inflammation, metabolism and repro-

duction. Additionally, melatonin may act as a mediator of inter-organ communication, e.g., between gut and liver^[1].

In animals, melatonin was first reported in the bovine pineal gland in the year 1958, and in the gut it was first identified in the human appendix in 1974^[2]. Melatonin is synthesized in the pineal gland and secreted in a circadian pattern, with highest amounts released during nighttime^[3]. The light/dark information regulating the secretion of melatonin by the pineal gland is received in the supra-chiasmatic nuclei *via* retinal photosensitive ganglionic cells. Melatonin released into the bloodstream acts as an endocrine hormone and controls biological functions with circadian rhythms, e.g., the sleep-wake cycle. Melatonin is also involved in the regulation of food intake and digestion^[4].

Following the detection of the melatonin-synthesizing enzymes N-acetyltransferase and hydroxyindole-O-methyltransferase in the gut^[5], the possibility of additional extra-pineal melatonin synthesis was considered^[6]. Melatonin produced in the gut is believed to act as a paracrine hormone which can be secreted in both a continuous or a cyclic fashion. Melatonin is also synthesized by a variety of other extra-pineal cells, such as bone marrow cells, lymphocytes, mast cells and epithelial cells, and it is unclear to what extent melatonin from these sources contributes to gut melatonin levels. Release of melatonin from all these extra-pineal sources seems to be independent of the photoperiod^[7-9].

Melatonin has been studied in different areas of medicine in numerous clinical trials. In the lower gut, the roles of melatonin are complex and largely uncharacterized. This review will focus on the gastrointestinal (GI) localization of melatonin, the role of melatonin in the lower gut and the mechanisms involved. Animal and benchside research, as well as translational human research, will be discussed with a special emphasis on summarizing all available human data related to the lower gut.

MELATONIN PRESENCE IN THE LOWER GUT

Bubenik *et al.*^[10] were the first to report the presence of melatonin in the mucosa of the gut. This observation was later confirmed by studies using immunohistochemistry and radioimmunoassay techniques^[11]. In the mammalian gut, melatonin exhibits striking differences in regional distribution, with the highest levels in the rectum and the colon and the lowest levels in the jejunum and the ileum. These regional differences in tissue distribution were confirmed in other species including rabbit, mouse and human^[12]. Moreover, specific antibodies against melatonin in rat identified melatonin-like immunoreactivity in all parts of the gut, and after administering exogenous melatonin the most pronounced accumulation of melatonin was seen in the colon and the rectum^[13]. Furthermore, melatonin was detected in luminal fluids of the gut. This melatonin may originate from food, mucosal sources, or organisms populating the gut. Finally, luminal melatonin may also be of biliary origin, but at the present time its

sources have not been elucidated.

Melatonin is synthesized in the enterochromaffin (EC) cells throughout the gut^[14,15], and the EC cells have been reported to be the major source of L-tryptophan-induced increase of circulating melatonin. Interestingly, the distribution of melatonin is comparable to the density of EC cells in the gut. Oral administration of L-tryptophan caused a rapid and dose-dependent elevation of circulating melatonin in rats or chickens^[16]. L-tryptophan-induced melatonin synthesis was greater following oral than following intraperitoneal administration. This indicates that L-tryptophan is a crucial precursor in gut melatonin synthesis.

Some melatonin detected in the gut is of pineal origin through accumulation from circulating sources, and the digestive tract, especially in the lower gut, might act as a store for pineal-derived melatonin particularly at nighttime^[17]. Melatonin levels in the gut are independent of pineal production, since in rats pinealectomy had no influence on gut melatonin concentrations^[18]. Interestingly, at any time of the day or night, the gut contains at least 400 times more melatonin than the pineal gland, once again emphasizing the functional importance of melatonin in the gut. No photoperiodic cyclical secretion of melatonin was observed in the gut, which is in contrast to the typical secretion pattern for melatonin from the pineal gland. In diabetic rats, lower melatonin levels were observed in the pancreas, the kidneys and the duodenum, but no change of melatonin level was detected in the colon, when compared to non-diabetic control rats^[19]. The relevance of these observations has yet to be determined.

Melatonin concentrations in the gut vary depending on age. In the postnatal rat, GI melatonin levels peaked at birth and then declined to stable levels at the age of 21 d^[13]. This decline in melatonin concentrations was more pronounced in the jejunum, ileum and colon compared to the stomach^[20]. However, later in life the levels of melatonin increase; it has been shown that melatonin concentration in the mucosa of the ileum and distal colon is 126% higher in older mice (22-24 mo) compared to younger mice (2-5 mo)^[21]. Remarkably, the same study provided evidence that most of the daytime levels of melatonin in the blood are of GI origin. It was also shown in pigs that the serum melatonin levels correlate well with the levels of melatonin in the lower gut^[22]. Food restriction increases melatonin concentrations in the gut and in the brain in mice^[23]. These distinct changes in melatonin levels suggest that there may be a physiological role for melatonin in the regulation of digestion and in the control of food intake. On the contrary, the melatonin levels in the lower gut may be influenced by luminal contents and may thus depend on the movement of digesta, but this notion remains speculative at the present stage.

MELATONIN RECEPTORS ARE LOCALIZED IN THE ILEUM AND THE COLON

Melatonin exerts some of its physiological effects through

activation of specific membrane receptors. According to their pharmacological properties, these receptors have been classified as Mel1A, Mel1B and Mel1C^[24,25]. Two of these, Mel1A and Mel1B, were recently renamed melatonin-1 receptor (MT1) and MT2 receptors. Both MT1 and MT2 receptors are members of the G-protein coupled receptor family and share a common seven transmembrane structure. MT1 and MT2 show high homology at the amino acid level, with a 55% overall homology and a 70% homology within the transmembrane domains. They also share some specific short amino acid sequences, suggesting that they represent a specific subfamily^[26,27]. However, MT1 and MT2 receptors activate very distinct intracellular signaling pathways^[28,29]. It has been shown that the MT1 melatonin receptor is coupled to G proteins that mediate adenylate cyclase inhibition and phospholipase C beta activation. The MT2 receptor couples to a number of signal transduction pathways, including phosphoinositol production, inhibition of adenylate cyclase and the inhibition of soluble guanylate cyclase pathway^[30-32]. Luzindole acts as an antagonist at both receptors and is used in numerous studies^[33-35]. 4-P-PDOT is a selective antagonist at MT2^[36]. For the MT1 receptor no highly selective antagonist has been reported yet.

The third melatonin binding site MT3 (formerly Mel1C) is an enzyme named quinone reductase 2 (QR2). MT3 can be blocked by prazosin. Activation of MT3/QR2 by melatonin may explain the protective effect of melatonin against oxidative stress in different animal models, since MT3/QR2 has potent antioxidant properties.

The melatonin receptors display regional tissue and cell specific variations, reflecting the overall complexity of melatonin signaling^[37]. Functional assays, as well as receptor binding studies, have demonstrated the presence of high affinity binding sites for melatonin on cell membranes, for example in the hypothalamus, medulla oblongata, hippocampus, cerebellum, parietal cortex and striatum of rats. Interestingly, the density of these binding sites varies depending on the time of the day and physiological conditions, including age^[38,39].

Furthermore, there exists a group of nuclear melatonin receptors. These nuclear receptors for melatonin belong to the retinoid Z receptor (RZR) or retinoid orphan receptor (ROR) subfamilies, which include three subtypes (α , β , γ), encoded by three different genes^[40,41]. An interaction between membrane and nuclear melatonin receptors was suggested by the observation that the expression of ROR/RZR mRNA is decreased in blood mononuclear cells with reduced MT1 receptor expression. Finally, melatonin can directly interact with intracellular proteins such as calmodulin, calreticulin or tubulin, extending the list of potential sites of binding and action for melatonin^[42-44].

All three MT receptors can be found in the gut and the data on localization are summarized in Table 1. The subcellular distribution of melatonin binding is highest in the nuclear fraction, followed by the microsomal and the mitochondrial fractions, and is lowest in the cytosolic fraction.

Melatonin MT1 receptor mRNA has been detected

Table 1 Localization of melatonin membrane receptors in the ileum and the colon of rodents

	MT1	MT2	MT3
Ileum	+	+	+
Colon	+	+	+
Mucosa	+	No data	No data
Muscularis mucosae		+ (i); + (c)	
Submucosa		+ (i); + (c)	
Muscularis propria		+ (c)	

MT: Melatonin receptor; i: Ileum; c: Colon.

in rat small and large intestine. The highest MT1 mRNA expression was found in the rat duodenum, with lower expression in the jejunum and ileum. No circadian changes were found in MT1 mRNA expression in gut tissues. In the duck gut, there was found to be a significant variation in the densities of 2-iodo (¹²⁵I)-melatonin binding sites in different regions of the gut, with the following descending order of density: ileum, jejunum > duodenum, colon > cecum > esophagus^[45]. Short-term fasting increased the expression of MT1 in the sub-epithelial layer of the rat small and large intestine, but no changes in MT1 expression were detected in other gut layers. During long-term fasting this increase in MT1 expression persisted only in distal colon, while in the remainder of the colon and in the small intestine MT1 expression returned to normal levels^[46-48].

A study using tissues from rat pancreas, stomach, duodenum and colon found the highest levels of MT2 in the colon by using western blot analysis^[49]. In the same study, the most intense MT2 immunoreactivity was observed in the muscularis mucosae and in the circular and longitudinal muscle layers of rat gut. Detection of MT2 receptors in the gut muscle layers suggests an involvement of MT2 in the regulation of intestinal motility. Comparable to MT1, the expression of MT2 receptors does not vary with food intake.

Pharmacological studies suggested the presence of the melatonin MT3 receptor in guinea pig colon^[50] and later MT3 was found in monkey (*Macaca fascicularis*) gut^[51]. However, presently it is unclear in which gut layer MT3 is expressed.

In addition, in blood vessels of both rodent and human colon, a high density of melatonin-binding sites was reported. *In vitro* preparations of arterial smooth muscle of the porcine colon relax in response to melatonin and melatonin receptor agonists, although these effects were seen at rather high concentrations of melatonin^[52]. Based on *in vitro* experiments on rat arteries, it has been suggested that a vasoconstrictive effect of melatonin is mediated *via* MT1 and a vasodilatory effect is mediated *via* MT2 receptors^[53]. The effects of melatonin in the gut may be dose-dependent and reflect actual MT1/MT2 ratio in muscle layers of gut segments.

Whereas data are available for the localization and expression of MT receptors in the gut, the presence of nuclear melatonin binding sites remains unresolved. One

study has suggested that melatonin nuclear receptors are present in murine colon cancer cells, but the relevance of this observation and localization remains unclear^[54].

ACTIONS OF MELATONIN IN THE ILEUM AND THE COLON

In contrast to the central nervous system, the function of melatonin in the gut is less clear. In the gut it seems that melatonin plays significant roles in regulating intestinal motility, the immune system, GI secretion, and the release of peptides involved in energy balance such as peptide YY^[55]. Melatonin was also shown to protect the colon in different pathophysiological conditions; frequently these protective effects involve activation of antioxidative mechanisms or the regulation of blood vessel tone and thus modification of perfusion^[56,57]. Another effect of melatonin is the alteration of gut flora and potential anti-microbiotic actions; melatonin was shown to influence *E. coli* O157:H7 growth *in vitro* and *in vivo* in infected wethers^[58].

Motility

Melatonin is known to be involved in the regulation of GI motility. Melatonin is produced in EC cells of the GI tract and has high lipophilicity, and therefore may diffuse into deeper layers through mucosa and submucosa, to finally act in the muscularis mucosae or the myenteric plexus. In these actions, muscular and neuronal sites are involved. Contractile and relaxant effects of melatonin in the GI tract have been reported in numerous species^[59-61]. The involved sites of action and the mechanism of melatonin action in the GI tract are not poorly characterized. Involvement of melatonin receptors and/or ion channels located on GI smooth muscle cells and/or neurons have been suggested and details are discussed below.

Melatonin alters GI motility by activating melatonin receptors. The most likely sites of melatonin action in the GI smooth muscle cells are the membrane-bound melatonin receptors and there is strong evidence that MT₂ receptors are involved. *In vivo* animal studies showed that melatonin exerted both excitatory and inhibitory effects on the gut depending on the dose of melatonin. Small doses of melatonin accelerated the intestinal transit in rats, while high doses reversed this effect. These effects were blocked by luzindole, suggesting the involvement of intestinal melatonin receptors^[62].

Early *in vitro* research showed that melatonin reduces the force of spontaneous contractions of ileum and colon segments of rat intestine, while the frequency of intestinal contractions remained unchanged^[61]. In the GI tract, the cyclic generation of electrical currents is one fundamental mechanism of coordinated smooth muscle contraction. Slow waves and spiking activity are organized in myoelectric migrating complexes (MMC). Depending on the report, endogenous and exogenous melatonin inhibits pre- and postprandial irregular spiking activity of intestinal motility. Furthermore, pinealectomy suppressed the regular phase of MMCs, and adminis-

tration of exogenous melatonin could restore a regular phase MMC activity in rat ileum^[63]. These changes may depend on the action of melatonin on the GI neurons. In one study focusing on gastric emptying, melatonin partly inhibited gastric motility by activating sympathetic neurons. In the stomach, melatonin also reduces nitrergic myenteric innervation^[64]. In electrophysiological experiments it was shown that the nitrergic component of the smooth muscle inhibitory junction potential was reduced by melatonin and this may be a consequence of direct inhibition of nitric oxide synthase (NOS) activity by melatonin at enteric synapses. Other studies suggest that the effect of melatonin may be related to the blockade of nicotinic channels by melatonin, or due to an interaction between melatonin and Ca²⁺-activated K⁺ channels^[65]. Furthermore, it was demonstrated that the inhibitory effect of melatonin is apamin-sensitive and thus involves Ca²⁺-activated K⁺ channels^[66]. One study also showed that the melatonergic attenuation of acetylcholine-induced contractions of intestinal strips from goldfish is dependent on extracellular calcium^[67].

Moreover, a beneficial effect of melatonin in reversing lipopolysaccharide-induced motility disturbances, which involves a reduction in lipid peroxidation and an increase of mitogen-activated protein kinase activation, nuclear factor kappaB (NF-κB) activation, inducible NOS (iNOS; NOS-2) expression and finally nitrite production^[68], was reported. Additionally, melatonin was shown to modulate the cholecystokinin action on ileal motility and to reduce the duration of cholecystokinin stimulatory effects on GI smooth muscle in rats^[69].

Other possible sites of melatonin action are 5-HT receptors. One study suggested that high doses of melatonin in the GI tract interact with cholecystokinin-2 and 5-HT₃ receptors on the vagal afferent fibers, and thus induce vago-vagal inhibitory reflexes^[70]. In some reports, the relaxant effect of melatonin through 5-HT receptor antagonism was proven^[71], but other pathways may also be involved. Recently, it was demonstrated that melatonin can inhibit the activity of the serotonin transporter, which controls the reuptake of 5-HT in intestinal epithelial cells and inhibits NK₂ receptor-triggered 5-HT release from guinea pig colonic mucosa by acting at a MT₃ melatonin receptor located directly on the mucosal layer^[72]. These actions may at the same time affect gut secretion.

Secretion

Melatonin is involved in the regulation of intestinal ion transport. Exogenous melatonin reduced diarrhea in rats with colitis, but the involved mechanisms have not been fully elucidated^[73]. In the colon, melatonin is thought to play a role in regulating Cl⁻ secretion^[74]. Melatonin can affect the expression of COX-2 and iNOS and melatonin modulates secretion elicited by prostaglandin E₂ and sodium nitroprusside in rat distal colon. Some of these secretory effects seem to be localized in the colonic epithelium and involve cAMP pathways, while others involve the enteric neuronal system^[75]. According to these studies,

melatonin is a physiological modulator of ion transportation in the lower gut and many mechanisms are involved.

Immune system

Melatonin has numerous effects on the immune system. It increases natural killer cell activity and Th2 cell-mediated immune responses^[76,77]. Melatonin was reported to regulate gene expression of several cytokines including IL-2, IL-2R and IFN- γ released by human CD4 T cells^[78,79]. The effects on other functions of the immune system, such as lymphoproliferation and cytokine production by human lymphocytes, have also been studied. Melatonin protects human and murine CD4⁺ T cells from apoptosis by inhibiting CD95 ligand mRNA and protein up-regulation in response to TCR/CD3 stimulation^[80]. Additionally, the melatonin/IL-2 relationship may be particularly relevant for immune tolerance. Melatonin can affect T-cell tolerance *via* IL-2^[81]. At the same time, melatonin acts as an immunomodulator and these effects are mediated by melatonin receptors located on immunocompetent cells^[82]. Melatonin synthesized in human lymphocytes is involved in the physiological regulation of IL-2/IL-2R expression through mechanisms comprising both membrane and nuclear melatonin receptors^[83].

The majority of melatonin effects described for lymphocytes seem to be mediated through MT1 receptors^[84]. However, some evidence shows that melatonin-induced enhancement of immune function is also mediated *via* MT2 receptors^[85]. Antagonists at the MT2 receptor or the nuclear RZR/ROR were found to reduce human lymphocyte IL-2 production, proving the involvement of these binding sites in IL-2 production^[86].

Experimental inflammation

By preserving the mucosal cell integrity and inhibiting the accumulation of neutrophils, melatonin exerts protective effects against inflammation in the gut^[87]. Melatonin was shown to reduce the severity of intestinal inflammatory pathologies such as colitis in animal models^[88]. Pentney *et al.*^[89] reported that daily melatonin administration reduced the severity of dextran sodium sulphate (DSS)-induced colitis in mice. In these experiments, serum melatonin levels were more than 10 times higher in mice that received DSS, as compared to controls. It is presently unclear what causes the significant improvement of inflammation in melatonin-treated mice, as no receptor antagonists were employed in this study and no downstream mechanisms were investigated. Melatonin has been reported to reduce the severity of experimental colitis in mice and rats and though *in vitro* and *in vivo* studies suggest numerous pathways involved, the exact mechanism of action remains unclear^[90]. In experimental colitis in rats, melatonin reduced colon injury by influencing numerous events including the enzyme activities of matrix metalloproteinase-9 (MMP-9), MMP-2 and caspase-3, by suppressing the activities of cyclooxygenase-2 (COX-2) and iNOS, inhibiting the expression of NF- κ B and acting as a radical scavenger^[91-95].

Moreover, the regulation of macrophage activity^[96] and the reduction of bacterial translocation in trinitrobenzene sulfonic acid (TNBS)-induced colitis have been reported^[97]. Melatonin treatment also causes a substantial reduction of *FasL* gene activation, which is known to induce a pro-inflammatory response characterized by a release of IL-1b, macrophage inflammatory protein-1a (MIP-1a), MIP-1b and MIP-2. Blocking the action of these cytokines has been shown to delay the onset of experimental colitis, to suppress inflammation and to ameliorate colonic damage^[98]. But melatonin does not exert unanimously protective effects. Marquez *et al.*^[99] reported that acutely administered melatonin is protective against TNBS-induced colitis in rats, whereas chronic melatonin treatment exaggerates colitis. Future studies are needed to clarify the full extent of melatonin protection against colitis and to characterize the involved mechanisms.

ROLE OF MELATONIN IN DISEASES INVOLVING THE ILEUM AND THE COLON

Irritable bowel syndrome

Irritable bowel syndrome (IBS) is a functional GI disorder characterized by abdominal pain and is diagnosed following the Rome III criteria. Multiple factors are involved in the pathophysiology of IBS; amongst others IBS has been associated with abnormal GI motor functions, visceral hypersensitivity, as well as psychosocial factors^[100,101].

Some studies suggest a possible role of melatonin in the pathophysiology of IBS. For example, disturbances in melatonin metabolism and secretion may be involved in different GI diseases including IBS^[102]. In a clinical trial involving patients with IBS, the beneficial effects of melatonin were obvious in the relief of symptoms such as abdominal pain, abdominal distension and abnormal sensation of defecation^[103]. Melatonin may exert its beneficial effects in IBS through effects on the central nervous system, *via* an enhancement of the cellular and humoral immune systems, or by antagonizing corticoid- and serotonin-mediated effects^[104,105]. However, melatonin does not influence sleep pattern or psychological well-being in patients with IBS. Recently, it has been shown that the antinociceptive effects of melatonin are not mediated through melatonin receptors, but through a supra-spinal process linked to the central opioidergic system, as pre-treatment with naltrexone or luzindole blocked the antinociceptive effect of melatonin in TNBS-treated rats^[106].

According to recent clinical trials, melatonin may be a future therapeutic option for IBS management (Table 2). In one placebo-controlled, randomized clinical trial in 40 patients with IBS, daily administration of melatonin 3 mg orally at bedtime for two weeks significantly alleviated abdominal pain^[107]. Patients treated with melatonin for two weeks significantly increased rectal thresholds towards balloon pressure and volume, ameliorating rectal sensitivity to pain and urgency. In another clinical trial, 17 female

Table 2 Clinical trials using melatonin in patients with irritable bowel syndrome

Authors	n	Study design	Dose	Conclusion
Lu <i>et al.</i> ^[110]	17	Randomized, crossover placebo-controlled (8 wk) ¹	3 mg/od	CTT did not change significantly in IBS patients with melatonin treatment
Saha <i>et al.</i> ^[109]	18	Randomized, placebo-controlled (8 wk) ¹	3 mg/od	Significant symptomatic benefit on bowel symptoms, extra-colonic symptoms, and quality of life
Lu <i>et al.</i> ^[108]	17	Randomized, crossover placebo-controlled (8 wk) ¹	3 mg/od	Significant symptomatic benefit on IBS scores, anxiety, well-being, and depression scores
Song <i>et al.</i> ^[107]	40	Randomized, placebo-controlled (2 wk) ¹	3 mg/od	Significantly attenuated abdominal pain and reduced rectal pain

¹Treatment duration; od: Once daily; CTT: Colonic transit time; IBS: Irritable bowel syndrome.

IBS patients were randomized to receive either melatonin 3 mg or placebo at bedtime for 8 wk, followed by a 4-wk washout period^[108]. Improvements in mean IBS scores were significantly greater during treatment with melatonin compared to placebo. Additionally, sleep, anxiety and depression scores improved. Saha *et al.*^[109] randomly assigned 18 IBS patients to receive either melatonin 3 mg or placebo at bedtime for 8 wk and they found that melatonin significantly improved overall IBS scores and quality of life scores. All these trials suggest that melatonin has beneficial effects in patients with IBS, but larger clinical trials in patients with IBS are needed. Another clinical trial was interested in colonic transit time (CTT) in IBS patients. These patients were randomized and received either melatonin 3 mg or placebo daily for 8 wk^[110]. Neither in healthy controls, nor in IBS patients, were stool texture or CTT changed, but the tests used may not be sensitive enough to detect motility or secretory changes and thus these data need to be interpreted cautiously.

In some of these clinical trials it has been shown that the beneficial effects of melatonin in IBS may be related to its action on gut sensory pathways. In this context it would be interesting to know whether melatonin alters visceral hypersensitivity or whether it acts as a general analgesic and would reduce rectal sensations in healthy volunteers as well. It is presently not clear whether this melatonin effect on rectal sensation is short-lasting or holds over longer periods of time. Although only a few clinical studies have shown its efficacy, melatonin appears to have a significant role in reduction of abdominal distension and rectal pain in treatment of IBS.

Inflammatory bowel disease

Despite the numerous animal studies showing protective effects of melatonin in colitis models, there are only limited clinical data available on the therapeutic role of melatonin in inflammatory bowel disease (IBD). To our knowledge, there are three published case reports of the self-administration of melatonin in IBD (Table 3). However, no clinical trials have been performed in IBD patients.

In one case report, after the self-administered use of melatonin as a self directed treatment for jet lag on international flights, the patient observed that his ulcerative colitis (UC) symptoms were virtually absent^[111,112]. Once his flare-ups were more troublesome requiring continu-

ous topical mesalamine therapy, he self-administered melatonin 3 mg/d, and according to the report he was symptom-free for a period of 3 mo. His symptoms recurred within 1 wk of running out of melatonin tablets. In contrast, other cases showed melatonin exacerbated symptoms associated with UC or Crohn's disease^[113,114]. One patient decided to take melatonin capsules (3 mg) at bedtime. Two months later, the patient started to experience the symptoms of active UC, including bloody mucous diarrhea. He continued taking melatonin and received corticosteroids orally and rectally. Since the symptoms did not calm down, the patient was hospitalized and stopped consuming melatonin; 48 h later there was a complete remission of the UC symptoms. Another patient decided to take melatonin capsules (3 mg) at bedtime. Four days later, the patient started to experience the symptoms of active Crohn's disease, such as diarrhea and abdominal cramps. She then stopped taking melatonin, and 24 h later there was a complete remission of symptoms. Clinical trials should be performed to evaluate a possible beneficial or detrimental effect of melatonin in IBD; presently available literature is inconclusive, though basic studies strongly suggest beneficial effects.

Colon cancer

Following the identification of melatonin binding sites in human colon tissue from patients with carcinoma of the rectum and the colon, a possible role of melatonin in colorectal cancer was addressed in several studies. ¹²⁵I-melatonin binding sites were identified in the mucosa and the submucosa of the human colon and radioimmunoassays revealed melatonin concentrations of 467 ± 99 pg/g tissue in non-cancer control patients, while daytime melatonin concentrations in the colon of patients with colorectal carcinoma were 3147 ± 87.8 pg/g tissue^[115]. The relevance of the diurnal variation of melatonin levels to colon cancer has yet to be determined. Colorectal carcinoma patients showed significant decrements in the peak amplitude of melatonin secretion, as well as a reduction in overall melatonin output^[116]. Some studies suggest that melatonin may be involved in cancer risk or protection from cancer development^[117]. For example, following pinealectomy, increased colonic crypt cell proliferation was reported in rats, suggesting melatonin pathways being involved in carcinogenesis in the co-

Table 3 Case reports of melatonin self administration in patients with ulcerative colitis and Crohn's disease

Authors	Age, gender, disease	Treatment	Dose	Result
Maldonado <i>et al</i> ^[113]	56, male, UC	Added melatonin to the otherwise unchanged drug treatment (salazosulfapyridine, corticosteroids)	3 mg/od	Two months later, the patient started to experience the symptoms of active UC, including bloody diarrhea
Jan <i>et al</i> ^[111] Mann ^[112]	47, male, UC	Added melatonin to an existing medication of mesalamine due to ongoing bloody diarrhea	3 mg/od	Symptoms resolved fast (2-3 d) and the beneficial effect was long lasting
Calvo <i>et al</i> ^[114]	35, female, CD	After becoming pregnant, the patient interrupted the treatment with melatonin, corticoids and salazosulfapyridine and symptoms of CD emerged again	3 mg/od	Recurrence of diarrhea and abdominal cramps within 4 d

od: Once daily; UC: Ulcerative colitis; CD: Crohn's disease.

lon^[118]. Another study in rats showed that small bowel crypt cell hyperplasia occurred several weeks after pinealectomy, but again the exact mechanisms were not identified. Recently, melatonin showed a great potential to control the preneoplastic patterns induced by constant light in the colon^[119].

The suggested colon cancer controlling mechanism of melatonin involves inhibition of tumour angiogenesis, modulation of the mitotic and apoptotic indices, and maintenance of the intracellular level of glutathione^[120,121]. Although no effects of melatonin on *in vitro* cell growth were found, a statistically significant and progressive suppression of *de novo* DNA synthesis was found following melatonin application^[122]. Other melatonin effects related to the control of tumour growth are the modulation of estrogen receptors, direct effects on the cell cycle, influence on several growth factors, increasing of gap junctions and enhancing the level of antioxidants^[123,124]. The anti-oxidative and anti-inflammatory actions of melatonin, changing the oxidative status and reducing the production of nitric oxide by cultured colon cancer cells, may also be directly involved in the onco-static properties of melatonin^[125]. Some studies suggest that for colon adenocarcinoma, membrane-bound and nuclear melatonin receptors are involved in these onco-static actions^[126,127]. Melatonin binds to receptors on T helper cells and monocytes, stimulating the production of IFN γ and interleukins 1, 2, 6 and 12, which in turn up-regulates immune responses resulting in a restoration of immunodeficiency states^[128]. Melatonin in this context also modulates the expression of NF- κ B, TNF- α , IL-1 β and STAT3^[129]. The activation of lymphocytes and monocytes/macrophages by melatonin is one of the mechanisms by which melatonin as an immunosurveillant prevents tumor development^[130,131]. For example, patients with advanced GI carcinoma treated with a combination of IL-2 and melatonin exhibited a significantly higher number of lymphocytes, T lymphocytes, NK cells and CD4⁺ cells than those receiving IL-2 alone^[132].

In clinical trials, melatonin was shown to have cytoprotective effects that may be involved in increasing the efficacy of cancer chemotherapy and improving survival. Melatonin co-treatment was also shown to reduce the adverse toxicities of chemotherapy and radiotherapy in

several studies, including in patients with colorectal carcinoma^[133,134]. For example, the efficacy of weekly low-dose CPT-11 in pretreated metastatic colorectal cancer patients may be enhanced by a concomitant daily administration of melatonin (20 mg/d, orally)^[135]. Other clinical studies showed that melatonin co-treatment with IL-2, *Aloe vera* or fish oil partly enhanced the effect of chemotherapy and reduced the toxicity in colorectal carcinoma^[136-139]. However, melatonin did not have any protective effect on irradiation-induced lymphocytopenia in patients with colorectal carcinoma (Table 4)^[140].

Clinical trials using melatonin in the context of colorectal cancer are small and unfortunately not of high quality. Presently, these studies have to be carefully interpreted and the studies seem, if anything, to be hypothesis-generating. Controlled clinical trials are needed to establish the potential role of melatonin in cancer treatment.

CONCLUSION

Melatonin found in the lower gut comes largely from intestinal sources, such as the EC cells and, to only a minor extent, from extra-intestinal sources such as the pineal gland. Melatonin levels in the ileum and the colon are dependent on food intake and digestion, but in contrast to systemic melatonin levels, the GI melatonin level is independent of light or the circadian rhythm.

Melatonin regulates the motility of the lower gut by acting on membrane melatonin receptors and all known MT1-3 were found to be localized in the GI tract, though their exact involvement in the regulation is not fully characterized. Additionally, actions of melatonin on 5-HT receptors have been reported, adding to the complexity of melatonin involvement in the regulation of GI function.

Melatonin was recently suggested to be a promising future drug for IBS treatment. Presently available basic and clinical data indicate that it is particularly effective in alleviating hypersensitivity and pain in patients with IBS, but larger clinical trials, ideally double-blinded and placebo-controlled, are needed.

Melatonin is furthermore involved in immunomodulatory functions throughout the GI tract. The protective actions of melatonin in mouse models of intestinal inflammation or in models of GI cancer are promising

Table 4 Clinical trials using melatonin in patients with colorectal cancer

Authors	n	Disease	Study design	Dose	Results and conclusion
Lissoni <i>et al</i> ^[140]	18	Rectal cancer	Randomized to melatonin, melatonin + 5-methoxytryptamine or melatonin + IL-2, 5 wk	20 mg/od	Melatonin had no effect on radiation-induced lymphocytopenia
Lissoni ^[133]	152	CRC	Randomized to oxaliplatin/5-Fu or CPT-11/FS/5-Fu with or without melatonin	20 mg/od	Melatonin significantly reduced the occurrence of cachexia, thrombocytopenia, neurotoxicity and asthenia
Persson <i>et al</i> ^[139]	8	CRC	Randomized to fish oil or melatonin (4 wk) followed by 4 wk fish oil with melatonin	18 mg/od	Melatonin had no effect on serological inflammation markers
Cerea <i>et al</i> ^[135]	30	CRC	Randomized to CPT-11 or CPT-11 plus melatonin 9 wk	20 mg/od	Disease-control higher in CPT-11 + melatonin group
Lissoni <i>et al</i> ^[120]	7	CRC	Daily melatonin for at least 2 mo	20 mg/od	Melatonin may control tumor growth by reducing VEGF secretion
Lissoni <i>et al</i> ^[134]	25	CRC	Randomized to 5-Fu/FS or 5-Fu/FS + melatonin. 5 cycles of 28 d	20 mg/od	Melatonin reduces toxicity and increases efficacy of 5-Fu/FS chemotherapy
Lissoni <i>et al</i> ^[138]	8	CRC	Randomized to melatonin or melatonin + <i>Aloe vera</i> tincture until progression	20 mg/od	Melatonin + <i>Aloe vera</i> stabilized disease and increased survival in end-stage patients
Barni <i>et al</i> ^[137]	50	CRC	Randomized to BSC or BSC combined with low-dose IL-2 + melatonin 4 wk	40 mg/od	Low-dose IL-2 + melatonin induced tumor regression and prolonged survival in second-line treatment
Lissoni <i>et al</i> ^[136]	19	CRC	Randomized to IL-2 or IL-2 + melatonin 4 wk	40 mg/od	Melatonin enhanced the activity of IL-2, induced tumour regression, prolonged progression-free survival and overall survival

CRC: Colorectal cancer; od: Once daily; BSC: Best supportive care; VEGF: Vascular endothelial growth factor.

and warrant further research. The translation of these observations to humans is less well characterized.

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Preclinical evaluation of azathioprine plus buthionine sulfoximine in the treatment of human hepatocarcinoma and colon carcinoma

Borja Hernández-Breijo, Jorge Monserrat, Sara Ramírez-Rubio, Eva P Cuevas, Diana Vara, Inés Díaz-Laviada, M Dolores Fernández-Moreno, Irene D Román, Javier P Gisbert, Luis G Guijarro

Borja Hernández-Breijo, Sara Ramírez-Rubio, Eva P Cuevas, M Dolores Fernández-Moreno, Irene D Román, Luis G Guijarro, Molecular Hepatic Toxicology Unit, Biochemistry and Molecular Biology Department, University of Alcalá, Networked Biomedical Research Center of Hepatic and Digestive Diseases (CIBEREHD), Alcalá de Henares 28871, Spain

Jorge Monserrat, Medicine Department, University of Alcalá, Alcalá de Henares 28871, Spain

Diana Vara, Inés Díaz-Laviada, Neuroendocrinology of Cannabinoid/Vanilloid System Unit, Biochemistry and Molecular Biology Department, University of Alcalá, Alcalá de Henares 28871, Spain

Javier P Gisbert, Gastroenterology Unit, Hospital de la Princesa, Institute of Health Research Princesa, Networked Biomedical Research Center of Hepatic and Digestive Diseases (CIBEREHD), Madrid 28006, Spain

Author contributions: Hernández-Breijo B performed the majority of the *in vitro* and *in vivo* experiments; Monserrat J performed cytometry; Ramírez-Rubio S and Cuevas EP performed *in vitro* experiments; Vara D performed the *in vivo* experiments; Fernández-Moreno MD, Román ID performed analysis of glutathione levels and reviewed the manuscript; Díaz-Laviada I and Gisbert JP reviewed the manuscript; Guijarro LG designed the study and wrote the manuscript.

Supported by Grants from SAF2008-05355 and CCG07-UAH/BIO-2085

Correspondence to: Dr. Luis González Guijarro, Professor, Biochemistry and Molecular Biology Department, University of Alcalá, Alcalá de Henares 28871,

Spain. luis_guijarro@telefonica.net

Telephone: +34-91-885-4865 Fax: +34-91-885-4585

Received: January 10, 2011 Revised: March 26, 2011

Accepted: April 2, 2011

Published online: September 14, 2011

localized application into HepG2 tumor *in vivo*.

METHODS: Different hepatoma and colon carcinoma cell lines (HepG2, HuH7, Chang liver, LoVo, RKO, SW-48, SW-480) were grown in minimal essential medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution and maintained in a humidified 37 °C incubator with 5% CO₂. These cells were pretreated with BSO for 24 h and then with AZA for different times. We examined the effects of this combination on some proteins and on cellular death. We also studied the efficacy and the safety of AZA (6 mg/kg per day) and BSO (90 mg/kg per day) in HepG2 tumor growth *in vivo* using athymic mice. We measured safety by serological markers such as amino-transferases and creatine kinase.

RESULTS: The *in vitro* studies revealed a new mechanism of action for the AZA plus BSO combination in the cancer cells compared with other thiopurines (6-mercaptopurine, 6-methylmercaptopurine, 6-thioguanine and 6-methylthioguanine) in combination with BSO. The cytotoxic effect of AZA plus BSO in HepG2 cells resulted from necroptosis induction in a mitochondrial-dependent manner. From kinetic studies we suggest that glutathione (GSH) depletion stimulates c-Jun amino-terminal kinase and Bax translocation in HepG2 cells with subsequent deregulation of mitochondria (cytochrome c release, loss of membrane potential), and proteolysis activation leading to loss of membrane integrity, release of lactate dehydrogenase and DNA degradation. Some of this biochemical and cellular changes could be reversed by N-acetylcysteine (a GSH replenisher). *In vivo* studies showed that HepG2 tumor growth was inhibited when AZA was combined with BSO.

CONCLUSION: Our studies suggest that a combination of AZA plus BSO could be useful for localized

Abstract

AIM: To evaluate the efficacy and the safety of azathioprine (AZA) and buthionine sulfoximine (BSO) by

treatment of hepatocellular carcinoma as in the currently used transarterial chemoembolization method.

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Key words: Azathioprine; Buthionine sulfoximine; Transarterial chemoembolization; Glutathione; Apoptosis; Necrosis

Peer reviewer: Dario Conte, Professor, GI Unit-IRCCS Osp. Maggiore, Chair of Gastroenterology, Via F Sforza, 35, Milano 20122, Italy

Hernández-Breijo B, Monserrat J, Ramírez-Rubio S, Cuevas EP, Vara D, Díaz-Laviada I, Fernández-Moreno MD, Román ID, Gisbert JP, Guijarro LG. Preclinical evaluation of azathioprine plus buthionine sulfoximine in the treatment of human hepatocarcinoma and colon carcinoma. *World J Gastroenterol* 2011; 17(34): 3899-3911 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i34/3899.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i34.3899>

INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) is increasing, and currently there are approximately 1 million new cases annually worldwide^[1]. Classical curative options available are surgical resection or liver transplantation^[2]. For unresectable HCC, an effective treatment is transarterial chemoembolization (TACE)^[3]. In TACE, the branch of the hepatic artery supplying the tumor is occluded at the time of arteriography by injection of lipidol and/or gelatin-sponge particles, which results in necrosis of the tumor. To increase that effect, chemotherapeutic agents (e.g., doxorubicin, epirubicin, mitomycin c, cisplatin or 5-fluorouracil) are often mixed with lipidol^[3]. A significant number of patients benefit from treatment with TACE^[3]. For further progress in this encouraging treatment, other antitumor drugs are needed. One of the problems in treating HCC is chemoresistance, therefore the majority of systemic therapies have been disappointing. For years researchers have observed that an elevation in intracellular glutathione (GSH) has been associated with resistance to irradiation and to chemotherapy, and correspondingly, the diminution of GSH levels is associated with sensitization to both types of therapy^[4]. Azathioprine (AZA) and buthionine sulfoximine (BSO) are medicines which decrease GSH levels by different mechanisms. Azathioprine belongs to the family of thiopurines with cytostatic activity, which is used in the treatment of autoimmune diseases, organ transplantation and in acute lymphoblastic leukemia^[5]. The first step in AZA activation consumes GSH, leading to release of 6-mercaptopurine from the imidazole moiety in an enzymatic reaction catalyzed by several glutathione-S-transferase (GST) isoforms^[5]. BSO is a competitive inhibitor of γ -glutamylcysteine synthetase (GCS), the rate-limiting step in the biosynthetic pathway

of GSH production^[6]. With the aim of treating tumors that overexpress glutathione (e.g., hepatocarcinoma), several groups undertook phase I clinical studies with BSO to evaluate whether modulation of GSH could be clinically useful^[7]. In these preliminary studies, the drug proved to be well tolerated and safe, which prompted us to study the effect of BSO combined with AZA: (1) on cell death of HCC cell lines (Hep G2, Huh7 and Chang cells) in culture; and (2) on the volume of the tumor obtained by injection of HepG2 into athymic mice *in vivo*. These results were compared with colon cancer cell lines (Lovo, SW-480, RKO and SW-48).

MATERIALS AND METHODS

Materials

AZA was a gift from UCB Pharma SA (Madrid, Spain). Antibodies against phospho-p54/46 JNK (c-jun N-terminal kinase), p-p38 MAPK (mitogen-activated protein kinase), JNK, p38 MAPK and α -tubulin were from Cell Signaling Technology Inc. (Beverly, Massachusetts) and antibodies against Bax, Bid, Bad, Bcl-2, cytochrome c, procaspase-3, GCS, nucleoporin and poly (ADP-ribose) polymerase (PARP) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). p-38 MAPK inhibitor [SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole], JNK inhibitor (SP600125, 1,9-pyrazoloanthrone) were from Calbiochem (Barcelona, Spain). 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), DL-buthionine-[S,R]-sulfoximine (BSO), 6-mercaptopurine and 6-methylmercaptopurine were from Sigma Chemical Co. (Madrid, Spain). 2-amino-6-mercaptopurine and 2-amino-6-methylmercaptopurine were from ICN Biomedicals Inc. (Ohio, United States). Z-VAD-mfk caspase-3 inhibitor was from Bachem (Heidelberg, Germany). Ac-DEVD-AMC was from Alexis Biochemicals (San Diego, California). OxyBlot™ Protein Oxidation Kit was from Millipore (Billerica, Massachusetts). All other reagents were of the highest grade of purity available.

Cell culture

HepG2, HuH7 and Chang cells, LoVo, SW-480, RKO and SW-48 cells were from ATCC and were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution and maintained in a humidified 37 °C incubator with 5% CO₂.

Metabolic activity and cell viability

The MTT reduction assay was performed as described previously^[8]. Cell viability was determined by the trypan blue exclusion assay as previously described^[9].

Analysis of GSH

Intracellular GSH levels were determined fluorometrically as described previously^[10].

Lactate dehydrogenase release assay

Cellular supernatants were mixed with lactate dehydro-

genase (LDH) substrate (0.2 mol/L Tris-HCl buffer, pH 8.2, containing 2.5 mg/mL L-lactate, 2.5 mg/mL NAD⁺, 0.1% (v/v) Triton X-100, 1-methoxyphenazine methosulfate and MTT). The formazan formed was measured at a wavelength of 570 nm and reference of 655 nm^[8].

DNA fragmentation gel assay

The study of DNA fragmentation was performed as previously described^[11].

Western blotting

Total cell protein was extracted as described previously^[11]. The proteins were determined by the Bradford method and were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membranes overnight at 25 V and 4 °C. The immunoblots were developed as previously described^[11].

Cytometry

Mitochondrial impairment was estimated using a JC-1 probe (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide/chloride) as previously described^[12]. Cell cycle analysis using propidium iodide staining was performed as described previously^[12].

Caspase-3 activity analysis

Cells were lysed in 10 mmol/L Tris-HCl buffer (pH 7.5) containing NaH₂PO₄/NaHPO₄ (10 mmol/L), Triton-X-100 (1%), NaCl (130 mmol/L) and sodium pyrophosphate (10 mmol/L). The cell lysates were incubated for 60 min at 37 °C in a 20 mmol/L HEPES buffer (pH 7.5) containing, glycerol (10%), DTT (2 mmol/L) and the specific fluorogenic substrate Ac-DEVD-AMC. Cleavage of the fluorescent caspase-3 substrate was monitored using a fluorescence plate reader at excitation/emission wavelengths of 355/460 nm.

Analysis of in vivo tumor growth inhibition

Nude mice were subcutaneously inoculated with 10⁷ HepG2 cells. After solid tumor formation (i.e., tumor volume 100 mm³), the tumor-bearing nude mice were randomized into four experimental groups, each one with eight mice. These groups were treated by peritumoral injection with 0.1 mL dimethyl sulfoxide (DMSO) (5%), BSO (90 mg/kg per day), AZA (6 mg/kg per day) or BSO (90 mg/kg per day) plus AZA (6 mg/kg per day), respectively. The BSO treatment was daily, while the AZA treatment was given on alternate days for a total of 12 d. Tumor size was measured every day. At the end of the experiment, all the animals were sacrificed. Plasma levels of creatine kinase, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also determined, following the procedures stated by the provider (Spinreact).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism package (GraphPad Software Inc., San Diego,

CA). Values are reported as mean ± SE and evaluated by the analysis of variance. The statistical significance was set at $P < 0.05$.

RESULTS

Effect of AZA treatment on viability and metabolic activity of HepG2 cells

We treated HepG2 cells for 24 h, 48 h or 72 h with DMSO (0.2%) or AZA (75, 150, 300, 600 or 1000 μmol/L) dissolved in 0.2% DMSO. Figure 1A shows that AZA decreased cell viability (measured by Trypan blue exclusion assay) at 600 μmol/L and 1000 μmol/L without significant changes in cell viability at lower drug concentrations. Similarly, azathioprine significantly inhibited metabolic activity (measured by the MTT assay) in a dose- and time-dependent manner (Figure 1B). The metabolic activity was more sensitive to the effect of AZA than the cell viability. In fact with AZA at 300 μmol/L we observed a loss in metabolic activity without changes in cell viability compared with control cells (DMSO). The comparison of MTT and Trypan blue exclusion assays, suggest that AZA produced inhibition of cell proliferation and/or loss of mitochondrial activity up to 300 μmol/L and induced toxicity at higher concentrations (600 μmol/L and 1000 μmol/L). In any case, our results show that HepG2 cells are very resistant to AZA treatment unlike human hepatocytes in culture (data not shown).

Sensitization of HepG2 cells to azathioprine by BSO pretreatment

When HepG2 cells were pretreated for 24 h with BSO (500 μmol/L) and then co-treated with AZA (0, 75 μmol/L, 150 μmol/L, 250 μmol/L, 300 μmol/L, 600 μmol/L or 1000 μmol/L) for 24 h (Figure 1C), metabolic activity noticeably decreased (IC₅₀ = 80 μmol/L) with respect to those cells treated with AZA alone (IC₅₀ = 800 μmol/L). The pretreatment with BSO potentiated the efficiency of azathioprine 10-fold in decreasing the metabolic activity of HepG2 cells. In the same way, HepG2 pretreated cells for 24 h with BSO (500 μmol/L) and then co-treated with AZA (100 μmol/L) for 3 h, 6 h, 12 h or 24 h showed a time-dependent inhibition of metabolic activity, in contrast to those cells that did not receive BSO (Figure 1D). Pretreatment with BSO significantly decreased basal levels of GSH (Figure 1E). Kinetic studies underlined that AZA treatment decreased GSH content in a time-dependent manner both in BSO-pretreated cells and in control cells (Figure 1F). However, the effect of AZA on GSH levels was higher in BSO-sensitized cells compared with control cells, which suggested that the compounds had synergic effects in regulation of GSH levels.

In order to localize the compartment affected by GSH depletion, we studied the presence of oxidized proteins (by OxyBlot) in different cellular fractions (cytosol, mitochondria and nucleus) obtained from drug-treated cells (Figure 2A). The treatment with AZA (300 μmol/L) plus BSO (500 μmol/L) increased the oxidized proteins

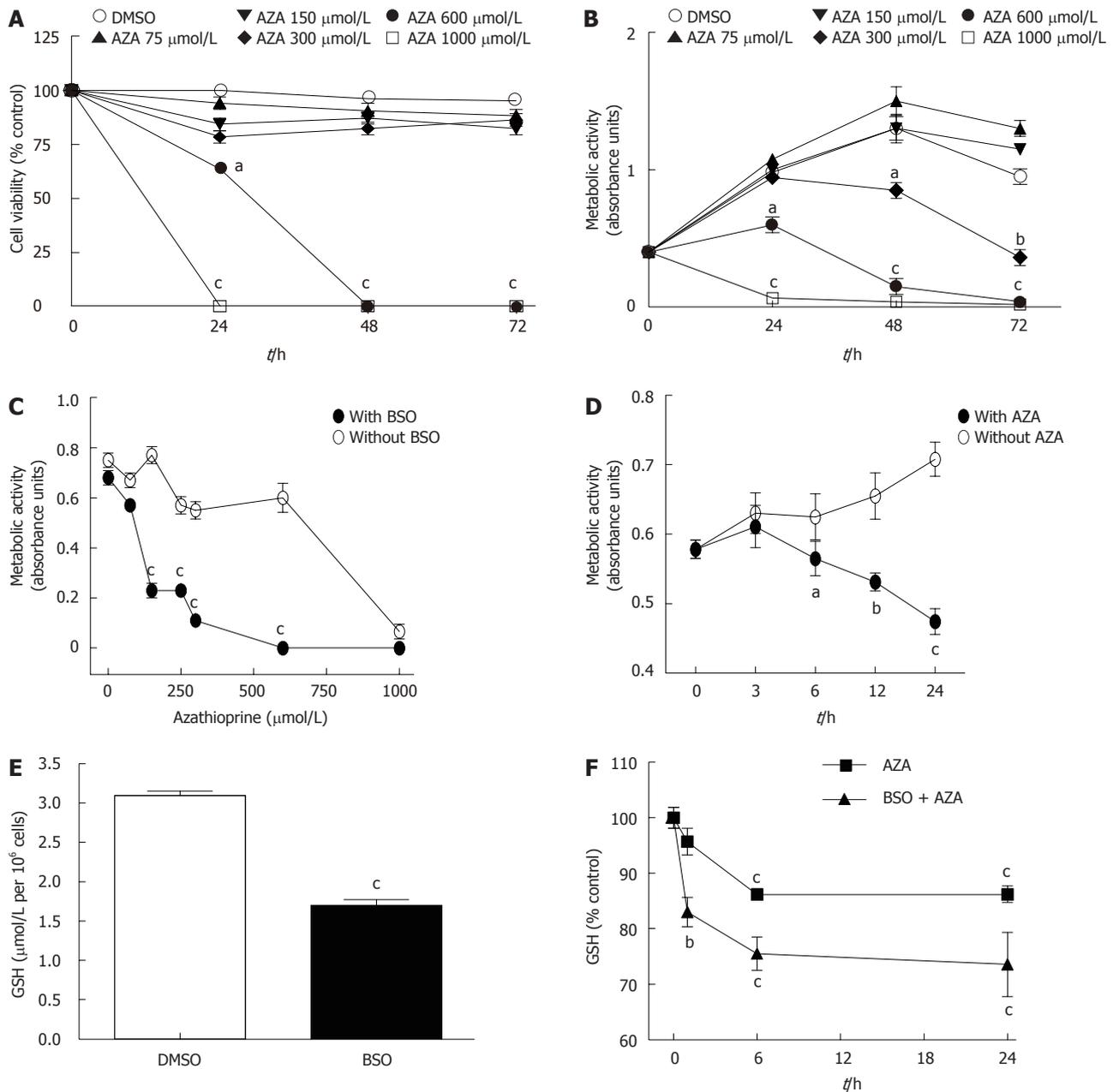


Figure 1 Effect of azathioprine treatment in HepG2 cells and sensitization of HepG2 cells by buthionine sulfoximine pretreatment. A: Cell viability (by trypan blue exclusion assay); B: Metabolic activity (by MTT assay) of HepG2 cells treated at different times with dimethyl sulfoxide (DMSO) or azathioprine (AZA) at different concentrations; C: Metabolic activity of cultured HepG2 cells pretreated for 24 h without or with buthionine sulfoximine (BSO) (500 $\mu\text{mol/L}$) and then cotreated with AZA at different concentrations for 24 h, or D with AZA (100 $\mu\text{mol/L}$) at different times; E: Glutathione (GSH) content of HepG2 cells pretreated for 24 h with DMSO or BSO (500 $\mu\text{mol/L}$); F: GSH content of HepG2 cells pretreated for 24 h with DMSO or BSO (500 $\mu\text{mol/L}$) and then cotreated with AZA (300 $\mu\text{mol/L}$) for different times. The significant differences with respect to control were statistically analyzed by analysis of variance with the Bonferroni post hoc test. ^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$.

in all fraction studied compared with cells treated with BSO (500 $\mu\text{mol/L}$). This effect was partially reversed by cotreatment with N-acetylcysteine (NAC, 1.5 mmol/L), a GSH replenisher. The levels of nucleoporin, PARP and cytochrome c were used as markers for the nucleus (nucleoporin and PARP) and mitochondria (cytochrome c). Interestingly, treatment with AZA plus BSO induced the depletion of nucleoporin and cytochrome c in the nucleus and in the mitochondria, respectively (Figure 2A). These effects were partially reversed by NAC.

Effect of AZA plus BSO combined on the metabolic activity of HCC cell lines and colon cancer lines

Treatment with AZA (300 $\mu\text{mol/L}$) plus BSO (500 $\mu\text{mol/L}$), decreased the metabolic activity in all cell lines (HepG2, Huh-7, Chang cells, LoVo, SW-480, RKO, SW-48) tested in comparison with control treatment (DMSO) (Figure 2B).

Effect of thiopurines plus BSO combined on the metabolic activity of HepG2 and colon cancer lines

Pretreatment with BSO (24 h) and subsequent treatment

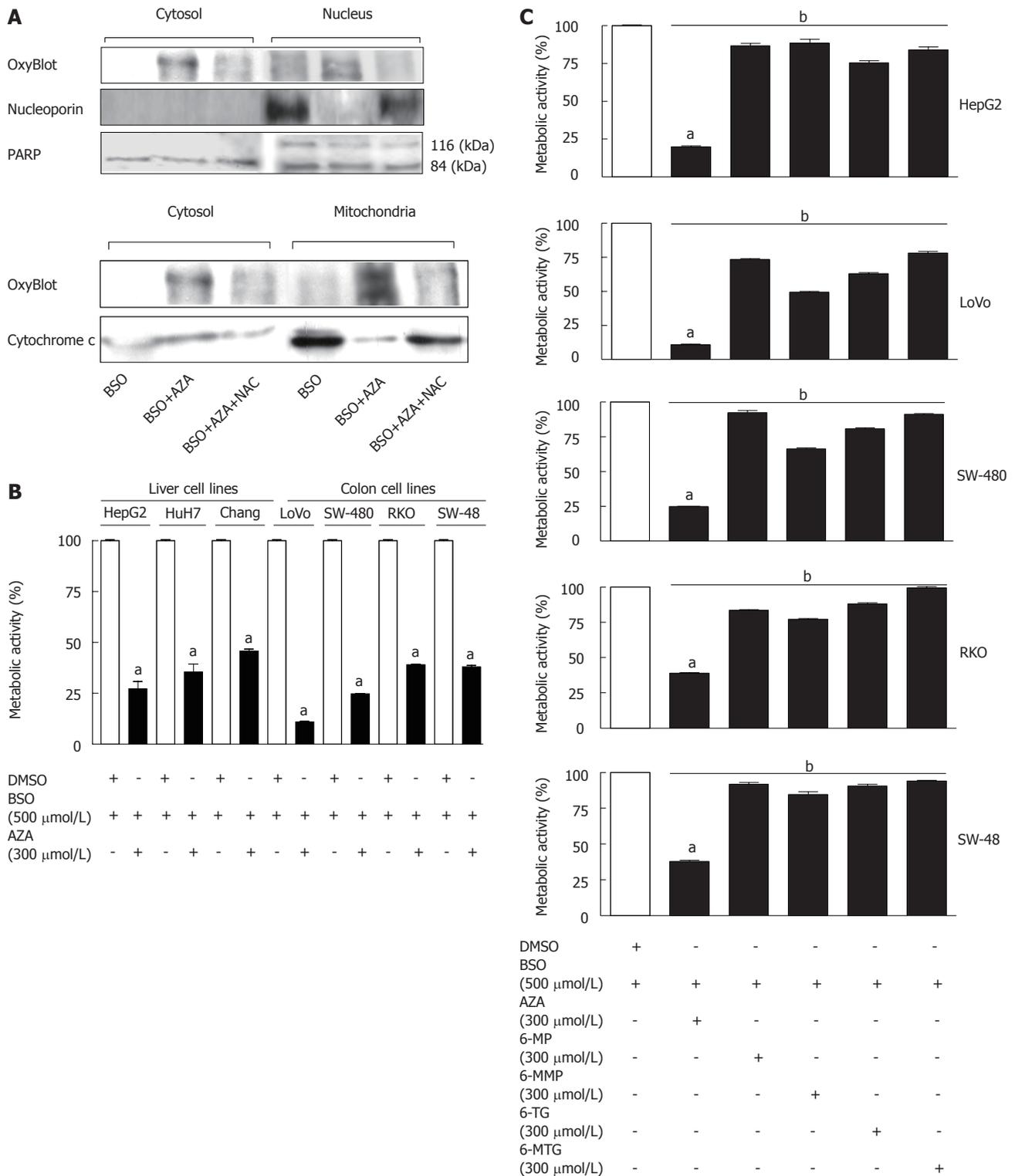


Figure 2 Effect of the treatment with thiopurines plus buthionine sulfoximine in cancerous cell lines. A: Western blotting of several proteins from enriched fractions of cytosol, nucleus and mitochondria obtained from HepG2 cells pretreated with buthionine sulfoximine (BSO) (500 μmol/L, 24 h), or pretreated with BSO (500 μmol/L, 24 h) and then cotreated with azathioprine (AZA) (300 μmol/L) for 6 h with or without N-acetylcysteine (NAC) (1.5 mmol/L); B: Metabolic activity (by MTT assay) in different cancer cell lines from liver and colon pretreated with BSO (24 h) and then cotreated with dimethyl sulfoxide (DMSO) or with AZA for 12 h; C: Metabolic activity (by MTT assay) in different cell lines pretreated with BSO (24 h) and then cotreated with AZA or with different thiopurines for 12 h. Significant differences with respect to control (DMSO plus BSO) were statistically analyzed by analysis of variance with the Bonferroni *post hoc* test. ^a*P* < 0.001, BSO plus AZA vs control. ^b*P* < 0.001, BSO plus AZA vs BSO plus different thiopurines.

with thiopurines (6-mercaptopurine, 6-methylmercaptopyrimidine, 6-thioguanine or 6-methylthioguanine) for

12 h caused a slight decrease in the metabolic activity of HepG2 cells with respect to control (cells treated with

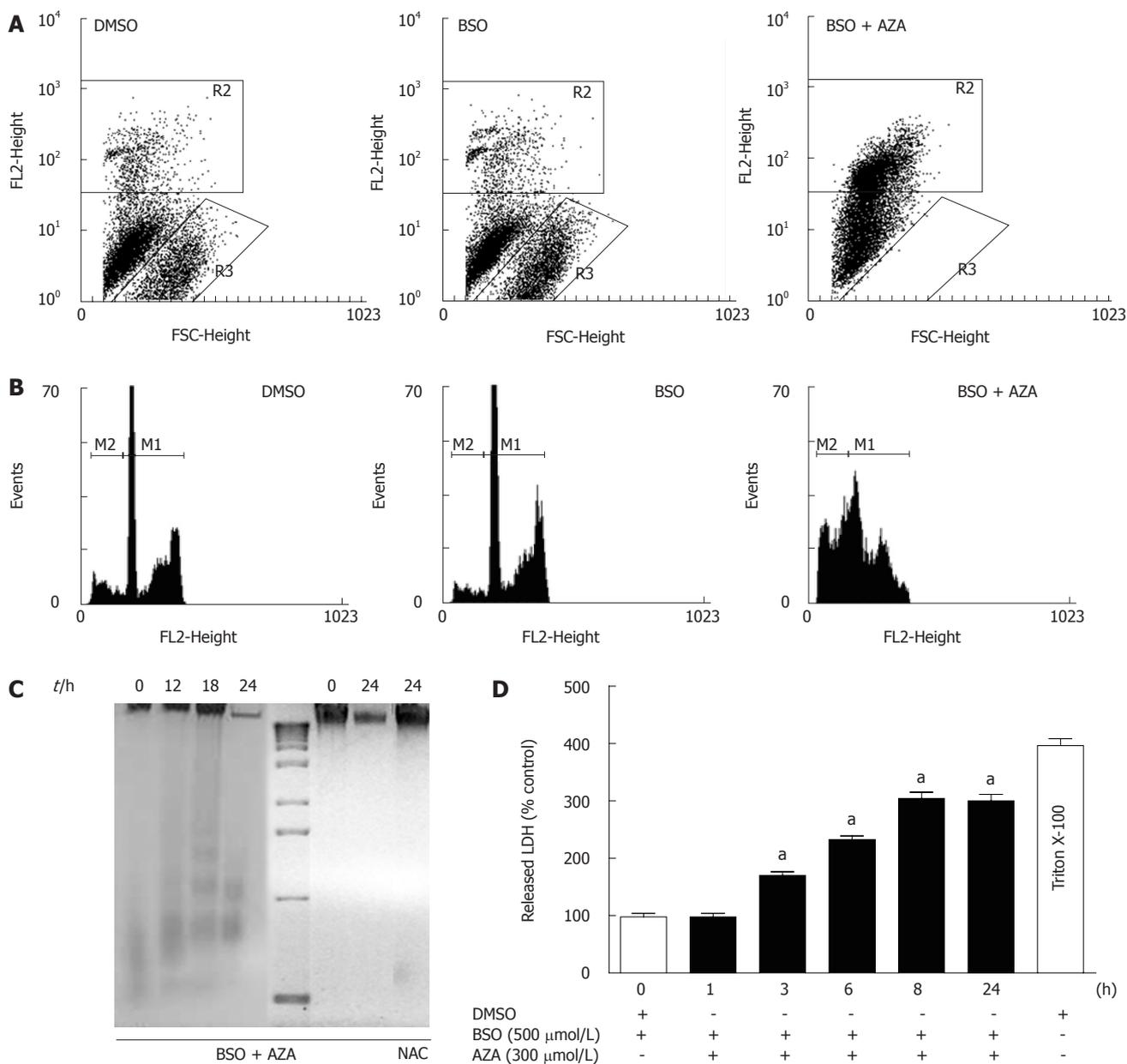


Figure 3 Effect of azathioprine plus buthionine sulfoximine treatment on cell viability, DNA fragmentation and lactate dehydrogenase release. A: Cell viability of HepG2 cells treated with dimethyl sulfoxide (DMSO) (24 h), buthionine sulfoximine (BSO) (500 $\mu\text{mol/L}$, 24 h) or pretreated with BSO (500 $\mu\text{mol/L}$, 24 h) and then cotreated with azathioprine (AZA) (300 $\mu\text{mol/L}$, 24 h); B: Study of cell cycle of HepG2 cells treated in the same conditions; C: DNA fragmentation of HepG2 cells pretreated with BSO (500 $\mu\text{mol/L}$, 24 h) and then cotreated with AZA (300 $\mu\text{mol/L}$) at different times with or without N-acetylcysteine (NAC) (1.5 mmol/L); D: Time course of lactate dehydrogenase (LDH) released by HepG2 cells pretreated with BSO (24 h) and then cotreated with AZA at different times. Triton X-100 (0.2%) used as control. Significant differences with respect to control (DMSO plus BSO) were statistically analyzed by analysis of variance with the Bonferroni *post hoc* test. ^a $P < 0.001$.

BSO) (Figure 2C); the effect of the preceding treatment with thiopurines on metabolic activity was very modest compared with the effect of AZA (Figure 2C). We observed significant differences between AZA and the other thiopurines studied. Subsequent studies were performed using AZA.

Effect of AZA plus BSO on necrosis and apoptosis parameters

Pretreatment of HepG2 cells for 24 h with BSO (500 $\mu\text{mol/L}$) and subsequent treatment with AZA (300 $\mu\text{mol/L}$)

for 24 h resulted in a significant loss of cell viability with respect to cells treated with BSO or with DMSO, as measured by staining of live cells with propidium iodine (Figure 3A). The R₃ area corresponded to viable cells and R₂ to non viable cells. Pretreatment of HepG2 cells with BSO (500 $\mu\text{mol/L}$) did not significantly change their viability compared with DMSO-treated cells (Figure 3A). To determine the mode of cell death, cell cycle analysis was performed by staining Triton X-100 permeabilized cells with propidium iodine followed by flow cytometry analysis (Figure 3B). In the presence of BSO

(500 $\mu\text{mol/L}$) and AZA (300 $\mu\text{mol/L}$), we observed a significant increase in the sub-G1 population (M2) with respect to cells treated with BSO or with DMSO alone. No significant differences were observed between cells treated with DMSO or with BSO (Figure 3B). These data suggested that the significant decrease in both metabolic activity and viability of cells treated with BSO and AZA was due in part to an increase in nuclear fragmentation characteristic of late apoptosis and early necrosis. To quantify the contribution of apoptosis and necrosis in cell death induced by BSO (500 $\mu\text{mol/L}$) plus AZA (300 $\mu\text{mol/L}$), we studied oligonucleosomal DNA fragmentation (Figure 3C) and LDH release (Figure 3D), respectively. The combined drug treatment induced a slight DNA laddering at 18 h treatment. After this period of time, treatment with BSO plus AZA induced extensive DNA degradation which was characteristic of necrosis. This degradation was prevented by the presence of NAC (1.5 mmol/L) (Figure 3C). Next we studied LDH release (Figure 3D). Our results showed that LDH release was statistically significant after 3 h combined drug treatment compared with control treatment. After 8 h treatment in the same conditions, we observed approximately 75% of necrotic cell death compared with control necrosis (HepG2 cells in the presence of Triton X-100) (Figure 3D). To obtain a new insight in the role of mitochondria in the cell death induced by the combination of BSO (500 $\mu\text{mol/L}$) and AZA (150 $\mu\text{mol/L}$ or 300 $\mu\text{mol/L}$), we evaluated: (1) annexin V bound to phosphatidylserine on the surface of Hep G2 cells; and (2) the mitochondrial functionality through JC-1 probe aggregation. Figure 4 shows that treatment of cells with BSO plus AZA: (1) increased the percentage of cells positive for annexin V (Q2 quadrant) in a dose-dependent manner: from 37% in control cells (DMSO), to 64% in cells treated with 500 $\mu\text{mol/L}$ BSO plus 150 $\mu\text{mol/L}$ AZA, then to 92% in cells treated with 500 $\mu\text{mol/L}$ BSO plus 300 $\mu\text{mol/L}$ AZA; and (2) decreased the percentage of cells with intact mitochondrial membrane potential, in a dose-dependent manner: from 85% in control cells (DMSO) to 68% in 500 $\mu\text{mol/L}$ BSO plus 150 $\mu\text{mol/L}$ AZA, then to 2% in 500 $\mu\text{mol/L}$ BSO plus 300 $\mu\text{mol/L}$ AZA. Annexin V staining was calculated as a percentage of R1 (cells with the mitochondrial membrane potential intact). R2 represented cells without an intact membrane potential mitochondrial. We obtained very similar proportions of cells stained with annexin V and JC-1 after treatment with DMSO (control cells) or with BSO (500 $\mu\text{mol/L}$) (data not shown).

It is very interesting to emphasize the recovery effect of NAC. Cells treated with BSO (500 $\mu\text{mol/L}$) and AZA (300 $\mu\text{mol/L}$) in the presence of NAC (1.5 mmol/L) showed a larger percentage of cells with intact mitochondrial membrane potential (68%) than cells treated with BSO and AZA in the absence of NAC (2%). However, a high percentage (82%) of annexin V-positive cells were observed in the NAC-treated cells, which suggest that they were at the beginning of apoptosis (Figure 4).

Effect of AZA plus BSO in cellular signaling

HepG2 cells were pretreated for 24 h with MEM or BSO (500 $\mu\text{mol/L}$), then were treated with or without SP600125 (a JNK specific inhibitor) or SB203580 (a p38 specific inhibitor), in the presence or in the absence of AZA (300 $\mu\text{mol/L}$) for 1 h (Figure 5A). In contrast to BSO, treatment with AZA induced p38 phosphorylation, which indicated enzyme activation. This effect increased in those cells treated with both AZA and BSO, and JNK1/JNK2 phosphorylation was also observed. The treatment of cells with SB203580 decreased both p38 and JNK1/JNK2 phosphorylation. SP600125 decreased JNK1/JNK2 phosphorylation and also p38 phosphorylation, which suggests a linked control mechanism between both pathways. On the other hand, NAC treatment inhibited both p38- and JNK1/JNK2-induced phosphorylation by AZA and BSO (Figure 5A).

In the next set of experiments (Figure 5B), we pretreated HepG2 cells for 24 h with BSO (500 $\mu\text{mol/L}$) and then with AZA (300 $\mu\text{mol/L}$) at different times (1, 3, 6, 8, 12 h), and we found that Bad, Bid and Bcl-2 remained constant, but Bax increased at 1 h and 3 h. There was general protein degradation at 24 h of treatment that affected Bad, Bid and β -tubulin (Figure 5B). Also we studied cytochrome c release from mitochondria. The maximum release of occurred at 3 h of AZA treatment and was attenuated by cotreatment with NAC (1.5 mmol/L) (Figure 6A). Next we examined the oxidation of proteins, with "OxyBlot kit", in cells pretreated with BSO (500 $\mu\text{mol/L}$) for 24 h and cotreated with AZA (300 $\mu\text{mol/L}$) at different times. The experiment showed oxidation of 53 kDa and 67 kDa bands at 6 h, but after this period of time, we observed decreased protein oxidation (Figure 6B). The protein oxidation was reversed by NAC (Figure 6D).

We studied the proteolysis of procaspase-3, GCS and PARP by Western blotting (Figure 6B). Pretreatment with BSO (500 $\mu\text{mol/L}$, 24 h) and then cotreatment with AZA (300 $\mu\text{mol/L}$) at different times, induced procaspase-3 dimerization (65 kDa band) but we did not observe fragmentation. The substrates of caspase-3, GCS and PARP, were fragmented with the combined treatment of BSO (500 $\mu\text{mol/L}$) plus AZA (300 $\mu\text{mol/L}$) (Figure 6B), however their proteolysis profiles did not correlate with those obtained in a classical model of caspase-3 activation, as observed for Hep G2 cells treated with actinomycin D (0.8 $\mu\text{mol/L}$) plus tumor necrosis factor- α (35 pmol/L) for 18 h (Figure 6C). We observed that NAC inhibited the proteolysis of procaspase-3, GCS and PARP induced by AZA plus BSO (Figure 6E). However, Z-VAD-mfk (a caspase-3 inhibitor) did not have any significant effect on proteolysis of procaspase-3, GCS and PARP (Figure 6E). The protective role of GSH in Hep G2 cell death induced by BSO plus AZA treatment and the absence of caspase-3 activation in this model is shown in Table 1.

Effect of AZA plus BSO in the development of the xenograft in nude mice

HepG2 cells were subcutaneously injected into nude mice,

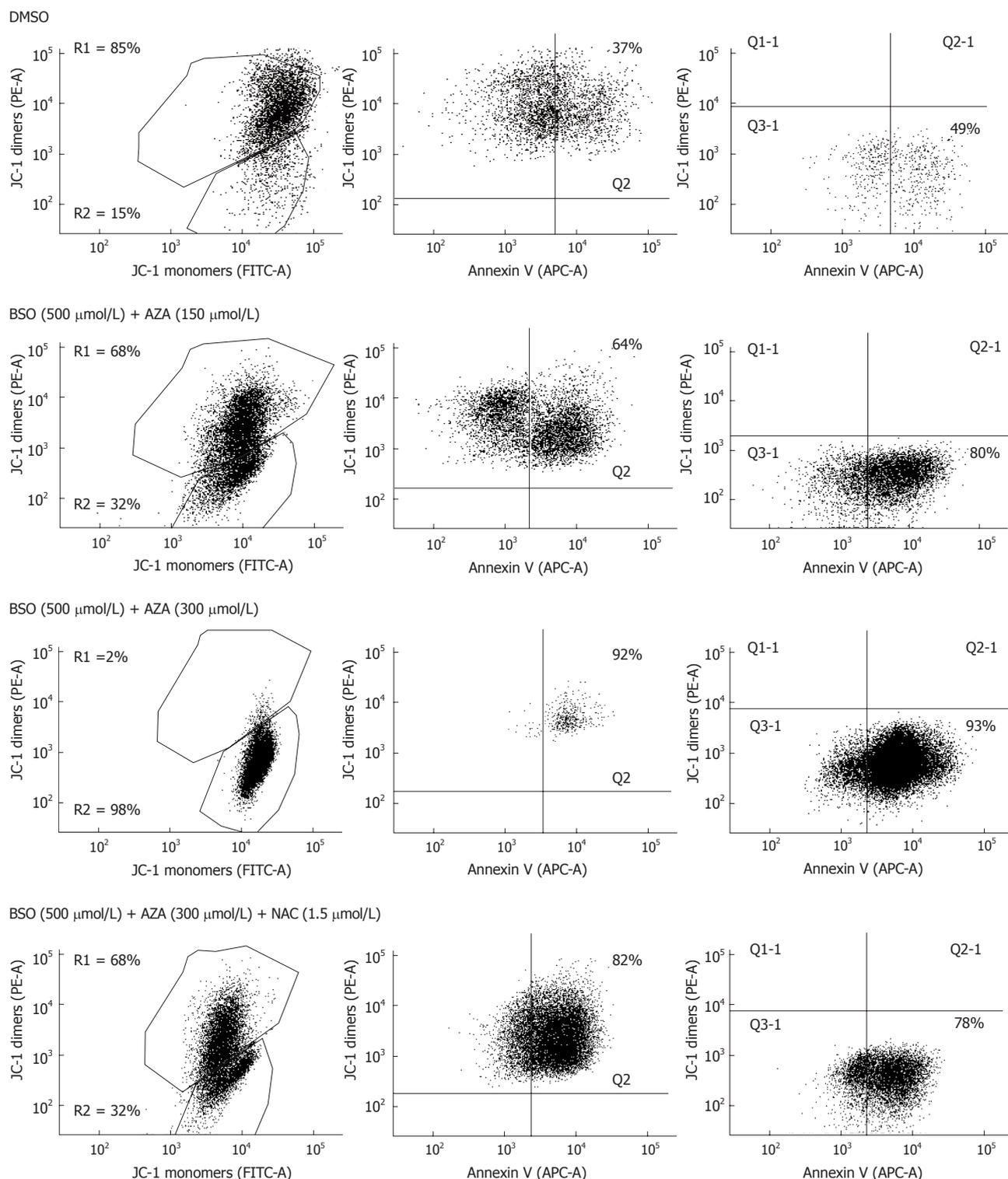


Figure 4 Analysis by flow cytometry of HepG2 cells apoptosis (annexin V) and of mitochondrial functionality (JC-1). Cells treated with dimethyl sulfoxide (DMSO) (24 h), or pretreated with buthionine sulfoximine (BSO) (24 h) and then cotreated with azathioprine (AZA) (24 h) in absence or presence of N-acetylcysteine (NAC). This experiment is representative of three others with similar results.

and the tumor size was measured every day. From the 7th day of BSO (90 mg/kg per day) plus AZA (6 mg/kg per day) treatment, the tumor volume was significantly decreased by a mean of 35% when compared with vehicle, DMSO (Figure 7A). These results showed no statistically significant differences ($P > 0.05$) between control (DMSO)

and BSO (90 mg/kg per day) treatment or between control (DMSO) and AZA (6 mg/kg per day) treatment (Figure 7A). GSH levels decreased in the tumors in BSO and AZA plus BSO groups compared with the corresponding control group (DMSO) (Figure 7C). Tumor cytolysis in the AZA plus BSO group was accompanied by a signifi-

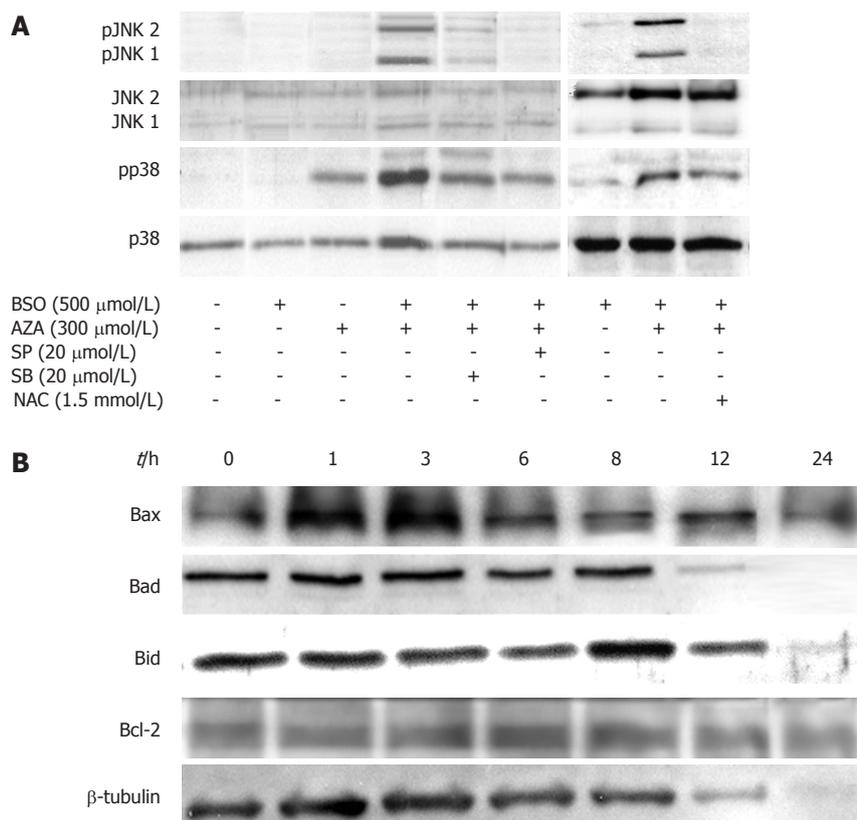


Figure 5 Effect of azathioprine plus buthionine sulfoximine treatment on cellular signaling. A: Stress activated kinases from HepG2 cells pretreated with buthionine sulfoximine (BSO) (24 h) and then cotreated with different combinations of azathioprine (AZA) with SP600125, SB203580 or N-acetylcysteine (NAC) for 1 h; B: Time course of mitochondrial regulatory proteins from HepG2 cells pretreated with BSO (500 $\mu\text{mol/L}$, 24 h) and then treated with AZA (300 $\mu\text{mol/L}$) at different times. This set of experiments is representative of three others with similar results.

Table 1 HepG2 cells metabolic activity and caspase-3 activity

	Metabolic activity (absorbance units)	Caspase-3 activity (% of CRL)			
		0 h	6 h	12 h	18 h
BSO/DMSO	0.833 \pm 0.016	100.00 \pm 6.62	-	-	-
BSO/AZA	0.544 \pm 0.046 ^a	-	95.87 \pm 8.56	92.79 \pm 6.09	-
BSO/AZA/NAC	0.933 \pm 0.059	-	93.29 \pm 4.03	98.87 \pm 2.64	-
BSO/AZA/Z-VAD-mfk	0.680 \pm 0.034 ^a	-	94.85 \pm 6.81	102.60 \pm 7.30	-
TNF- α (35 pmol/L)/ActD (0.8 $\mu\text{mol/L}$)	-	-	-	-	143.00 \pm 5.77 ^b
TNF- α (700 pmol/L)/ActD (0.8 $\mu\text{mol/L}$)	-	-	-	-	141.30 \pm 4.33 ^b

Cells were treated with buthionine sulfoximine (BSO) (500 $\mu\text{mol/L}$, 24 h), or pretreated with BSO (500 $\mu\text{mol/L}$, 24 h) and then cotreated for 12 h with: (1) azathioprine (AZA) (300 $\mu\text{mol/L}$), (2) AZA (300 $\mu\text{mol/L}$) plus N-acetylcysteine (NAC) (1.5 $\mu\text{mol/L}$) or (3) AZA (300 $\mu\text{mol/L}$) plus Z-VAD-mfk (50 $\mu\text{mol/L}$). tumor necrosis factor (TNF)- α plus actinomycin D (ActD) treatment for 18 h was used as a positive control (CRL). Significant differences with respect to control [(BSO plus dimethyl sulfoxide (DMSO))] were statistically analyzed by analysis of variance. ^a $P < 0.05$; ^b $P < 0.05$.

Table 2 Summary of enzyme levels in mice

Enzyme	DMSO	BSO	AZA	BSO/AZA
AST (IU/L)	91.07 \pm 8.36	62.79 \pm 3.89	124.90 \pm 10.56	154.30 \pm 19.75 ^a
ALT (IU/L)	31.50 \pm 9.12	22.36 \pm 2.85	36.17 \pm 5.44	42.29 \pm 9.04
CK (IU/L)	598.60 \pm 112.10	416.00 \pm 89.28	904.40 \pm 129.40	918.50 \pm 128.70

Enzyme activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatine kinase (CK) in plasma of nude mice transplanted with HepG2 cells at the end of the experiment (12 d of treatment in similar conditions as in Figure 7). Significant differences with respect to control animals [dimethyl sulfoxide (DMSO)] were statistically analyzed by analysis of variance. ^a $P < 0.001$. BSO: Buthionine sulfoximine; AZA: Azathioprine.

cant reduction in procaspase-3 compared with control (DMSO) (Figure 7D). Plasma creatine kinase, ALT and AST did not show marked changes in the AZA plus BSO group compared with control (DMSO) and with the other groups studied (Table 2). This implied that a combination of AZA plus BSO did not induce toxicity in the hosts.

DISCUSSION

New drug combinations are needed for treatment of patients with cancer, particularly those refractory to stan-

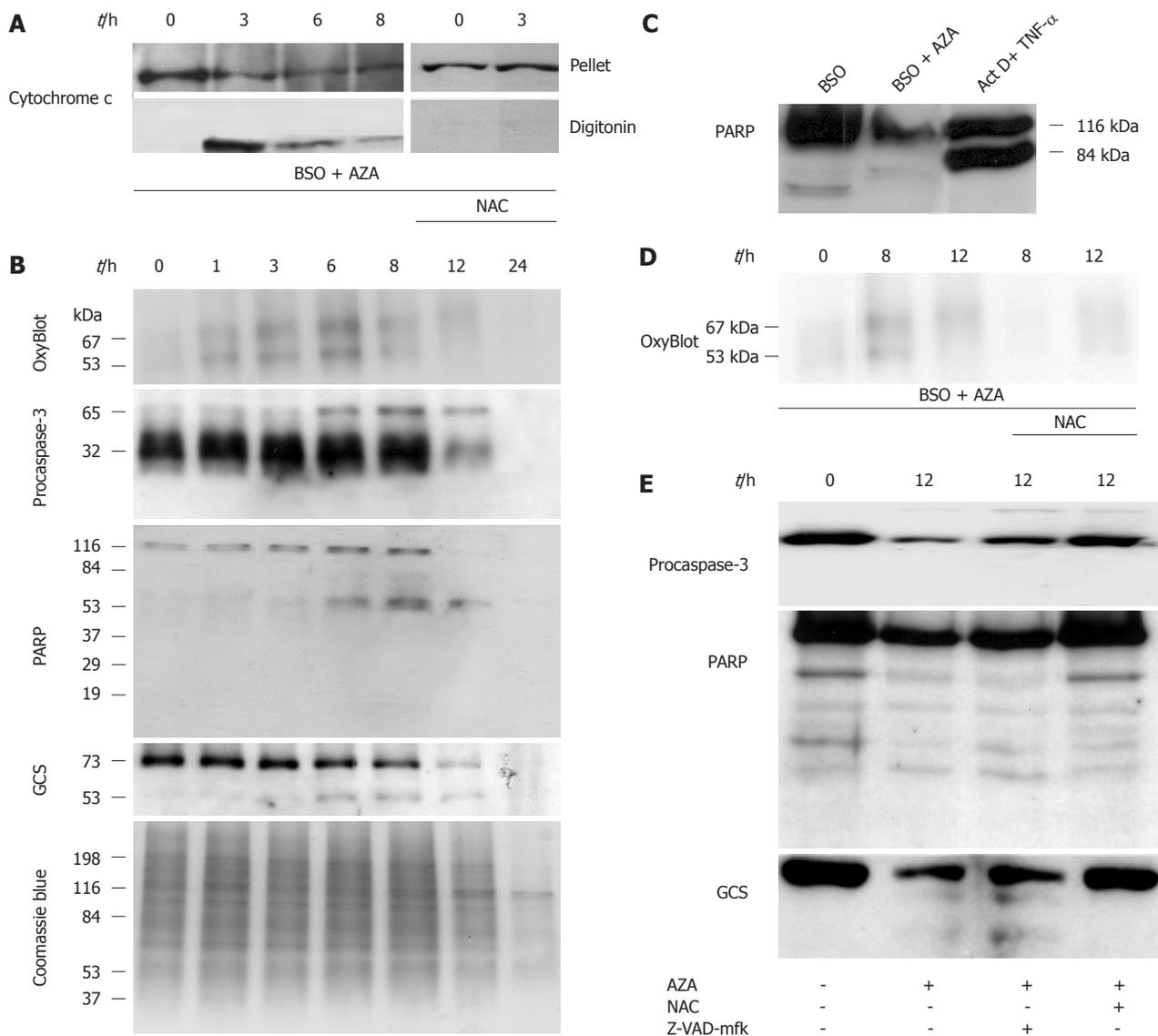


Figure 6 Effect of azathioprine plus buthionine sulfoximine treatment on biochemical markers of apoptosis. A: Time course of cytochrome c release from HepG2 cells pretreated with buthionine sulfoximine (BSO) (500 $\mu\text{mol/L}$) during 24 h and then cotreated with azathioprine (AZA) (300 $\mu\text{mol/L}$) at different times. N-acetylcysteine (NAC) (1.5 mmol/L) was added at the same time as AZA; B: Time course of apoptosis-related proteins in HepG2 cells pretreated with BSO (500 $\mu\text{mol/L}$) for 24 h and then cotreated with AZA (300 $\mu\text{mol/L}$) at different times; C: Western blotting of poly(ADP-ribose) polymerase (PARP) from HepG2 cells treated with BSO (500 $\mu\text{mol/L}$, 24 h), or pretreated with BSO (500 $\mu\text{mol/L}$, 24 h) and then cotreated with AZA (300 $\mu\text{mol/L}$, 12 h). HepG2 cells treated with actinomycin D (0.8 $\mu\text{mol/L}$) plus tumor necrosis factor (TNF)- α (35 pmol/L) for 18 h were used as positive control; D: Time course of the oxidized proteins from HepG2 cells treated in similar conditions as in panel A; E: Time course of apoptosis-related proteins from HepG2 cells treated in similar conditions as in panel A. NAC (1.5 mmol/L) or Z-VAD-mfk (50 $\mu\text{mol/L}$) were added at the same time as AZA. This set of experiments is representative of three others with similar results.

dard therapy. **Unresectable HCC is treated by TACE^[3,13]**, in which tumor necrosis is induced by hypoxia and by classic (doxorubicin, epirubicin, mitomycin c, cisplatin or 5-fluorouracil) or new (zinostatin stimalamer) drugs^[13]. Our preclinical results suggest that a new combination of old drugs could be useful in locoregional treatment of HCC. The AZA plus BSO combination offers the advantage of a new mechanism of action to the previous one used in TACE therapy, as we showed in the present paper. Moreover, our results revealed that AZA plus BSO may have a new spectrum of activity in comparison with the parent molecules, 6-mercaptopurine and 6-thioguanine. The localized application of AZA (6 mg/kg per day) and BSO (90 mg/kg per day)

produced a decrease in the volume of the HepG2 tumor xenograft that was not achieved by AZA or BSO alone. The doses used in the nude mouse model are comparable to those used for AZA in liver transplantation^[14] and in clinical trials of BSO^[7]. The therapeutic effect was produced with only minor changes in biochemical parameters used in the evaluation of the drug safety profile. Our results suggested that the AZA plus BSO combination induced necroptosis by JNK activation. Necroptosis is a caspase-independent regulated cell death, that results in morphological features resembling necrosis. Our data suggested that AZA and BSO can induce HepG2 cell death by depletion of the different pools of GSH, which could explain the synergistic effect of the compounds on

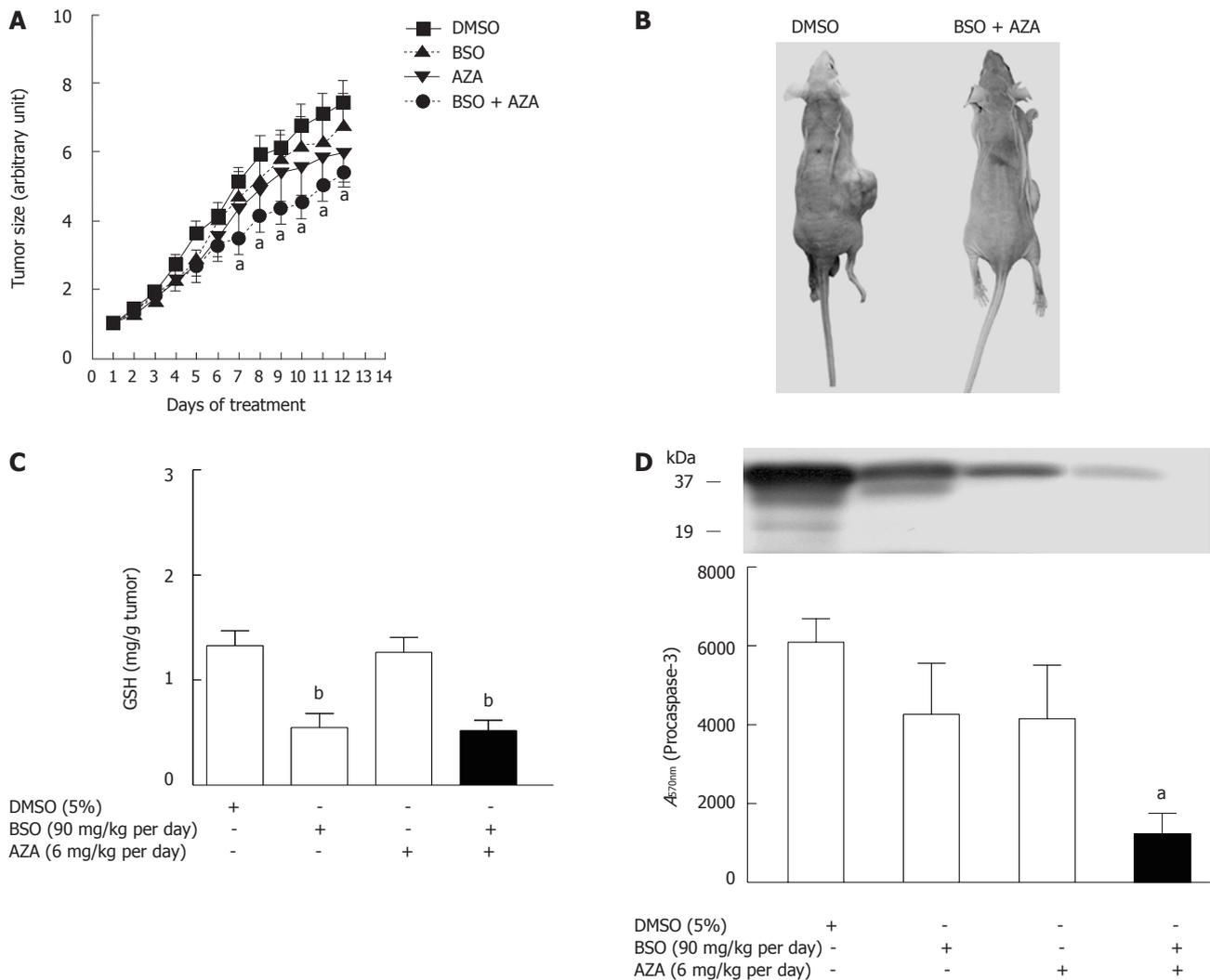


Figure 7 Effect of azathioprine plus buthionine sulfoximine treatment on tumor volume of nude mice. A: Inhibition of tumor growth by buthionine sulfoximine (BSO) (90 mg/kg per day) plus azathioprine (AZA) (6 mg/kg per day), AZA (6 mg/kg per day) or BSO (90 mg/kg per day). HepG2 cells (107) were implanted subcutaneously into nude mice as described in Materials and Methods; B: Representative animals of the groups treated with dimethyl sulfoxide (DMSO) or BSO plus AZA after 12 d of treatment. Levels of glutathione (GSH) (C) and of procaspase-3 (D) in the tumors of nude mice after 12 d of treatment with the corresponding drugs. There were eight mice in each group. Data points represent the mean \pm SE. ^a*P* < 0.05; ^b*P* < 0.01.

cell cytotoxicity. This suggestion is based on: (1) AZA decreases GSH levels in pretreated cells with BSO; (2) AZA kinetics on GSH depletion is more rapid and profound in cells pretreated with BSO than in control cells; (3) AZA is able to activate p38 by a GSH-dependent mechanism, while BSO is not; (4) AZA is able to stimulate JNK activity in cells GSH-depleted by BSO; this last effect is completely reversed by NAC; (5) AZA is able to stimulate the oxidation of cytosolic, nuclear and mitochondrial proteins by a GSH-dependent mechanism as demonstrated by the reversal by NAC; and (6) AZA treatment depletes both nucleoporin and cytochrome c in cells pretreated by BSO; these effects are reversed by NAC. From kinetic studies, we suggest that GSH depletion induced by AZA plus BSO stimulates JNK, Bax translocation and subsequent deregulation of mitochondria and activation of a cytotoxic cascade in HepG2 cells. The mitochondrial crisis is demonstrated by cytochrome c release and an increase in oxidized proteins. Classically, a link has been

observed between mitochondria and caspase-3 activation^[15]. Our results demonstrated the proteolysis of procaspase-3, PARP, γ -glutamylcysteine synthetase, the loss of membrane integrity, the release of LDH and DNA degradation after treatment with AZA plus BSO. Several of these cellular and biochemical changes are characteristic of apoptosis and of necrosis. We observed (Figure 8) at short times (0 to 6 h) of drug treatment, rapid regulatory changes associated with GSH levels, such as JNK activation and Bax translocation, which are followed by mitochondrial cytochrome c release and protein oxidation, all parameters classical hallmarks of apoptosis. However, cytochrome c release did not lead to caspase-3 activation and subsequent PARP proteolysis in 89 kDa fragments, as observed in control experiments performed in the presence of tumor necrosis factor- α and actinomycin-D. In addition, caspase-3 activity did not increase in the presence of BSO plus AZA. The absence of caspase-3 activation is corroborated using Z-VAD-mfk, because this caspase-3 in-

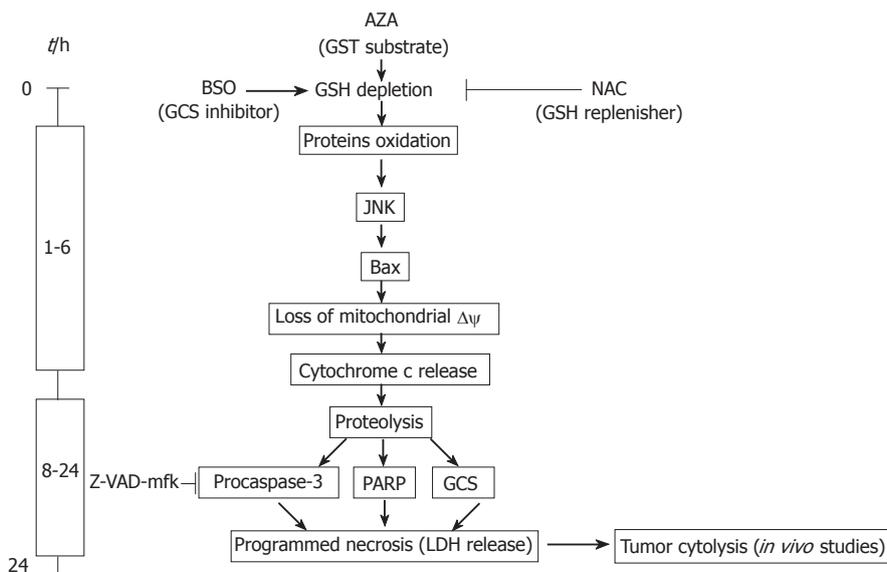


Figure 8 Time course of the events in our molecular model. AZA: Azathioprine; GST: Glutathione-S-transferase; GSH: Glutathione; GCS: γ -glutamylcysteine synthetase; BSO: Buthionine sulfoximine; NAC: N-acetylcysteine; PARP: Poly (ADP-ribose) polymerase; LDH: Lactate dehydrogenase.

hibitor did not protect against AZA plus BSO-induced cytotoxicity. These data exclude the involvement of caspase-3 in the cell death induced by the combination of both compounds. We observed at longer periods (6 to 24 h) of treatment with both compounds (Figure 8), a generalized protein degradation which affected structural (β -tubulin), regulatory (Bad, Bid, procaspase-3), metabolic (GCS) and DNA binding (PARP) proteins. Interestingly, Bcl-2 levels did not change with AZA plus BSO treatment which rules out apoptosis as the mechanism of cell death. Recently, a novel form of cell death called necroptosis or programmed necrosis has been proposed^[15], which like apoptosis, can be executed by a regulated mechanism. The first steps are common or similar to apoptosis but the inactivation of caspases causes a shift from apoptosis to mixed cell death (necrotic/apoptotic) or to full-blown necrosis^[15]. Our results suggest a role for GSH in the shift between apoptosis and necrosis induced by AZA plus BSO, because the addition of NAC to the drug combination reversed the majority of the cytotoxic effects induced by AZA plus BSO (JNK activation, cytochrome c release, protein oxidation, extensive proteolysis, mitochondrial membrane depolarization, and DNA fragmentation), but it did not inhibit annexin V-staining, which is characteristic of the early step of apoptosis. Recently, the importance of GSH has been shown in the induction of cell death by regulation of the cascade GST-JNK. The JNK activity is inhibited by binding with GST and, in this molecular model, a decrease in GSH causes oxidation of Cys47 and Cys101 in the GST protein, which in turn induces JNK release and subsequent activation^[16]. Our results suggest a similar model of activation of JNK by AZA plus BSO. In contrast, JNK activation in HepG2 cells by staurosporin, H₂O₂, etoposide or ultraviolet light induces classical apoptosis^[17]. In some instances, it may be desirable to trigger necrotic cell death, for example in hepatocarcinoma treatment, necrosis is the target of all

effective locoregional therapies^[3]. Our results provide important insights for the comprehensive understanding of the mechanism cell death induced by AZA and BSO in HepG2 cells and in other cancerous cells from the liver (HuH7, Chang cells) and from the colon (LoVo, SW-48, RKO, SW-48). To be considered as a therapeutic agent, the administered doses of AZA plus BSO must be compatible with those used in clinical practice; in this respect we observed that the doses of both compounds locally injected in the xenograft were able to reduce the tumor volume without any serious side effects. This combined therapy has an additional advantage, in that the presence of NAC can protect major organs from unexpected damage. The effectiveness of BSO in the treatment of solid tumors has been observed previously in combination with arsenic trioxide^[18], sodium borocaptate^[19] or melphalan^[6]. Interestingly, in this last paper BSO was administered efficiently by infusion into the hepatic artery, as in TACE. Our results provide experimental evidence to support the clinical use of the combined treatment of AZA and BSO in liver cancer.

ACKNOWLEDGMENTS

The authors thank Dr. Lasuncion and Dr. Roperro for kindly providing some cell lines. We thank Unidad de Cultivos Celulares and Centro de Experimentación Animal for their technical help.

COMMENTS

Background

The incidence of human liver carcinoma is increasing and is the fifth most common cancer in the world. Unfortunately, the disease is often diagnosed too late. For unresectable liver carcinoma, an effective treatment is transarterial chemoembolization (TACE). One way to progress with this encouraging treatment is to find a synergistic combination of classical drugs.

Research frontiers

One of the problems in treating hepatocarcinoma is chemoresistance, thus the majority of systemic therapies have been disappointing. Researchers for years have observed that elevation of intracellular glutathione (GSH) has been associated with resistance to irradiation and to chemotherapy and correspondingly the diminution of GSH levels is associated with sensitization to both types of therapy. Buthionine sulfoximine (BSO) is a competitive inhibitor of γ -glutamylcysteine synthetase, the rate-limiting step in the biosynthetic pathway of GSH production, and reduces their levels. In this study, the authors demonstrate that azathioprine (AZA) plus BSO could induce: (1) cell death of several cancer lines *in vitro* by activation of JNK; and (2) HepG2 cell death in a xenograft model.

Innovations and breakthroughs

The results point out the role of GSH in the synergism observed by the combination of AZA plus BSO, because the addition of N-acetylcysteine (NAC) reversed JNK activation, cytochrome c release, protein oxidation, extensive proteolysis, mitochondrial membrane depolarization and DNA fragmentation observed in the presence of AZA plus BSO. However, NAC was not able to inhibit annexin V staining which is characteristic of the early step of apoptosis. The modulation of the shift between apoptosis and necrosis induced by AZA plus BSO, in the presence or in absence, respectively, of NAC may be important in overcoming the chemoresistance observed during treatment of liver tumors.

Applications

The present findings may provide: (1) a new drug combination for the treatment of liver neoplasms; (2) identification of molecular targets to inhibit chemoresistance of cancer. These two pathways could be promising in the treatment of liver cancer.

Terminology

AZA is an immunosuppressant for clinical use belonging to the family of the thiopurines. It is used in organ transplantation and treatment of autoimmune diseases. BSO is an inhibitor of glutathione synthesis that is being evaluated clinically as an adjuvant in the treatment of cancer.

Peer review

The paper is of interest and deserves consideration for publication.

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S- Editor Tian L L- Editor Cant MR E- Editor Zhang DN

Conscious or unconscious: The impact of sedation choice on colon adenoma detection

Mark Metwally, Nicholas Agresti, William B Hale, Victor Ciofoaia, Ryan O'Connor, Michael B Wallace, Jonathan Fine, Yun Wang, Seth A Gross

Mark Metwally, Nicholas Agresti, William B Hale, Victor Ciofoaia, Ryan O'Connor, Jonathan Fine, Seth A Gross, Department of Gastroenterology, Norwalk Hospital, 34 Stevens St. Norwalk, CT 06850, United States

Michael B Wallace, Department of Gastroenterology, Mayo Clinic, 4500 San Pablo Road, FL 32224, United States

Yun Wang, Department of Statistics, Yale University, 333 Cedar Street, New Haven, CT 06510, USA 34 Stevens St. Norwalk, CT 06850, United States

Author contributions: Metwally M, Gross SA, Hale WB and Agresti N designed the research; Metwally M, Agresti N, Ciofoaia V and O'Connor R collected data; Metwally M, Hale WB, Gross SA, Wang Y and Wallace MB analyzed data; Metwally M, Hale WB, Fine J and Gross SA wrote the paper.

Correspondence to: Mark Metwally, MD, Department of Gastroenterology, Norwalk Hospital, 34 Stevens St. Norwalk, CT 06850, United States. mark.metwally@gmail.com

Telephone: +1-203-249-1416 Fax: +1-203-855-3589

Received: January 20, 2011 Revised: March 29, 2011

Accepted: April 5, 2011

Published online: September 14, 2011

Abstract

AIM: To determine if anesthesiologist-monitored use of propofol results in improved detection of adenomas when compared with routine conscious sedation.

METHODS: This retrospective study was conducted at two separate hospital-based endoscopy units where approximately 12 000 endoscopic procedures are performed annually, with one endoscopy unit exclusively using anesthesiologist-monitored propofol. Three thousand two hundred and fifty-two patients underwent initial screening or surveillance colonoscopies. Our primary end point was the adenoma detection rate, defined as the number of patients in whom at least one adenoma was found, associated with the type of sedation.

RESULTS: Three thousand two hundred and fifty-two

outpatient colonoscopies were performed by five selected endoscopists. At least one adenoma was detected in 27.6% of patients (95% CI = 26.0-29.1) with no difference in the detection rate between the anesthesiologist-propofol and group and the gastroenterologist-midazolam/fentanyl group (28.1% vs 27.1%, $P = 0.53$).

CONCLUSION: The type of sedation used during colonoscopy does not affect the number of patients in whom adenomatous polyps are detected.

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Key words: Sedation; Colonoscopy; Adenoma

Peer reviewer: John Marshall, MD, Professor of Medicine, Division of Gastroenterology, University of Missouri School of Medicine, Columbia, MO 65201, United States

Metwally M, Agresti N, Hale WB, Ciofoaia V, O'Connor R, Wallace MB, Fine J, Wang Y, Gross SA. Conscious or unconscious: The impact of sedation choice on colon adenoma detection. *World J Gastroenterol* 2011; 17(34): 3912-3915 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i34/3912.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i34.3912>

INTRODUCTION

Colonoscopy is recognized as the gold standard examination in screening for colon polyps. Adenomas can be precursors to colon cancer and their removal by polypectomy prevents the development of cancer in many instances^[1,2]. However, adenomas may be missed in up to 24% of exams according to studies employing same day, tandem colonoscopies^[3,4]. Among the factors shown to affect polyp detection rates are: quality of preparation, withdrawal time, image enhancements (high definition, narrow band imaging), increased visualization of the

colonic mucosa with the Third Eye Retroscope and even the time of day that the exam is performed^[5-9].

Two methods of sedation are commonly used for colonoscopy exams: conscious sedation with gastroenterologist-monitored use of fentanyl and midazolam, and modified anesthesia with anesthesiologist-monitored use of propofol. Advantages of the latter method include a deeper level of sedation with less patient movement and awareness. Also, with anesthesiologist controlled propofol sedation, the endoscopist is freed from making decisions regarding the level of sedation and, therefore, can fully focus on the examination. A 5-year review of the Clinical Outcomes Research Initiative database showed that more large (> 9 mm) adenomas were detected during average risk screening colonoscopy exams using deep sedation with propofol than with moderate conscious sedation^[10]. Given that the smaller adenomas comprise most of the ones missed on colonoscopies, we determined in this study whether anesthesiologist-monitored use of propofol was associated with increased adenoma detection rate, regardless of size, compared to nurse-gastroenterologist monitored fentanyl and midazolam. Such a finding would help justify the higher cost of monitored use of propofol.

MATERIALS AND METHODS

Study design

This retrospective study was conducted at two separate hospital-based endoscopy units where approximately 12 000 endoscopic procedures are performed annually, with one endoscopy unit exclusively using anesthesiologist-monitored propofol. Two CRNAs staff the unit, each using the same method of administering propofol with an induction bolus followed by smaller bolus doses as required. The second unit serves both inpatients and outpatients. In this unit, gastroenterologist-controlled fentanyl and midazolam is the predominant method of sedation. Initial sedation with both medications is supplemented by small doses (0.5 to 1.0 mg midazolam or 25 mcg fentanyl) of either or both agents at the discretion of the gastroenterologist. We studied the adenoma detection rates of five experienced gastroenterologists who had each performed at least 10 000 colonoscopy exams and worked in both endoscopy units. All exams were performed using standard definition white light colonoscopes from the CF-Q160AL and CF-180AL series (Olympus America, Inc.) with standard definition flat screen monitors. The study was approved by the Norwalk Hospital Institutional Review Board.

Study sample

The study population consisted of consecutive outpatients undergoing initial screening or surveillance colonoscopy by one of the five endoscopists between January 1, 2008 and May 31, 2009. Patients were included if they met standard guidelines for screening and surveillance colonoscopy^[11]. Patients were excluded if they had a personal history of inflammatory bowel disease, a familial

history of a polyposis syndrome (familial adenomatous polyposis, hereditary non-polyposis colon cancer, juvenile polyposis, *etc.*) or had an inadequate bowel preparation.

Patients were assigned to the endoscopy units using either propofol or midazolam/fentanyl groups based on the day of the week. Each endoscopist spent the same day each week in the anesthesiologist-propofol unit. We recorded using the electronic medical record patient demographics including: age, (segmented into 5 groups: 20-49 years, 50-64 years, 65-74 years, 75-84 years and 85 years and older), gender, ethnicity, the indication for colonoscopy (screening or surveillance), the total number of adenomas detected and the number of adenomas detected in each patient.

Outcomes

Our primary end point was the adenoma detection rate, defined as the number of patients in whom at least one adenoma was found, associated with the type of sedation.

Statistical analysis

We conducted bivariate analyses to describe the differences in patient characteristics and adenoma detection between the use of anesthesiologist-propofol and gastroenterologist-midazolam/fentanyl sedation across all 5 endoscopists. Chi-square test was used for comparing categorical variables and *t*-test for comparing continuous variables. The hierarchical generalized linear model (HGLM) approach was used to assess the differences in adenoma detection between the two types of sedation by modeling the log-odds of adenoma detection as a function of patient demographics and the use of propofol. To determine the influence of various patient characteristics on the adenoma detection and incidence rates, we also fitted an HGLM without adjusting for those patient characteristics. All of the HGLM were constructed with a random endoscopist-specific effect, which accounts for a within-endoscopist correlation of the observed outcomes and separates within-endoscopist variation from between-endoscopist variation. The 95% CI was also calculated for each estimate obtained from the models and all statistical testing was 2-sided at a significance level of 0.05. All analysis was done using STATA version 10.0 (STATA Corporation, College Station, TX).

RESULTS

Patient characteristics

The study population consisted of 3252 outpatients having colonoscopy performed by one of the five selected endoscopists. Individual procedural volumes among the endoscopists ranged from 396 to 938 over the study period. The mean age was 61 years, predominately being 87.6% Caucasian (Table 1) and 53% male. The group receiving propofol was nine months older than the group receiving midazolam/fentanyl ($P = 0.037$). There were no statistical differences between the two sedation groups in terms of gender, ethnicity or indications for colonoscopy.

Table 1 Patient characteristics *n* (%)

Characteristic	Propofol		Midazolam/Fentanyl		<i>P</i> value
	<i>n</i> = 1456		<i>n</i> = 1796		
Mean age	61.0	10.3	60.2	9.9	0.037
Female	669	45.9	860	47.8	0.285
White	1291	88.7	1556	86.7	0.08
Surveillance	398	27.3	525	29.3	0.227

Table 2 Physician adenoma detection rates

Physician	<i>n</i>	Propofol		<i>n</i>	Midazolam/Fentanyl		<i>P</i> value	Total cases
		Adenoma (≥ 1)	(%)		Adenoma (> 1)	(%)		
A	452	135	31.3	506	160	31.6	0.903	938
B	327	116	35.5	442	154	34.8	0.856	769
C	278	82	29.3	382	100	25.9	0.255	660
D	267	37	13.9	222	26	11.7	0.459	489
E	157	39	24.8	239	47	19.7	0.222	396
Overall	1456	409	28.1	1796	487	27.1	0.547	3252

Table 3 Hierarchical generalized linear model predicting adenoma detection

Model	Odds ratio	Adenoma detection 95% CI	<i>P</i> value
Unadjusted for patient characteristics and physician			
Versed (reference)	1.00		
Propofol	1.09	0.93-1.28	0.267
Adjusted for patient characteristics and physician			
Versed (reference)	1.00		
Propofol	1.07	0.91-1.26	0.402

Adenoma detection rates

Overall, at least one adenoma was detected in 27.6% of patients (95% CI: 26.0-29.1) with no difference noted in the detection rate between the anesthesiologist-propofol group and the gastroenterologist-midazolam/fentanyl group (28.1% *vs* 27.1%, *P* = 0.53). Although there was substantial variation in overall adenoma detection rates among the individual endoscopists (ranging from 35.1% to 12.9%), the type of sedation used was not associated with differences in rates of adenoma detection (Table 2).

Because patient assignment to either the propofol or midazolam/fentanyl sedation group was not randomized, HGLM was used to determine whether any of the patient characteristics (age group, ethnicity, gender or indication for colonoscopy) contributed to the observed adenoma detection rates. Using midazolam/fentanyl sedation as the reference standard, the odds ratio for the detection of adenomas does not differ when propofol is used and the results are adjusted for patient and physician characteristics (Table 3). Using the model, no difference in adenoma detection rates was identified when each of the five patient age groups were examined individually.

DISCUSSION

This study shows that anesthesiologist-monitored propofol sedation was not associated with an overall significant difference in adenoma detection rate compared to gastroenterologist-monitored sedation with midazolam and fentanyl. Adjustments for patient characteristics and variation in endoscopist detection rates also failed to show an overall advantage for the detection of adenomas associated with propofol sedation.

Clear benefits of the use of propofol do exist. Anesthesiologist-monitored propofol sedation allows colonoscopy to be performed more efficiently with a more rapid onset of sedation and shorter patient recovery times. This type of sedation also frees the endoscopist from making decisions regarding the level of sedation and patient monitoring, allowing full concentration on the endoscopic task at hand. A 5-year review of the Clinical Outcomes Research Initiative database showed that more large (> 9 mm) polyps were detected during average risk screening colonoscopy exams using deep sedation with propofol than with moderate conscious sedation^[10]. In this study, 20% more polyps were detected and multivariate analysis found no factors other than propofol sedation to account for the difference. In contrast, our study examined the detection of potentially pre-cancerous adenomatous polyps of any size, and did not find a benefit to the use of propofol. Both studies were retrospective in nature.

Polyp detection rates vary with the time spent examining the colonic mucosa during withdrawal of the instrument, the quality of the preparation and optical enhancements such as high definition scopes and monitors and narrow band imaging. In our study, procedural technique and daily patient volume were the same for each endoscopist in both the anesthesiologist-propofol and gastroenterologist - midazolam/fentanyl endoscopy units. All preparations were graded good to excellent and the exams

were performed with a mix of Olympus CF-Q160AL and CF-180AL series instruments, the latter with narrow band imaging capability. High definition instruments were not in use in our units at the time of the study.

Our arrangement with 2 endoscopy suites, one providing exclusively anesthesiologist-monitored propofol and the other providing predominantly gastroenterologist-controlled fentanyl and midazolam, provided a convenient, albeit non randomized method to compare whether type of sedation was associated with adenoma detection rates. The fact that each physician worked in the propofol unit on the same day each week introduced a potential confounding variable, however, we are not aware of any literature correlating adenoma detection rates with the day of the week the procedure is performed.

While retrospective, this study represents the 'real world' practice of colonoscopy with experienced clinicians performing both screening and surveillance exams on patients who are receiving either anesthesiologist or operator controlled sedation. Our data show that the detection rate of adenomatous polyps of all sizes is not increased by the use of anesthesiologist monitored propofol sedation compared to gastroenterologist monitored sedation with midazolam/fentanyl. While the use of propofol based anesthesia is certainly associated with increased patient satisfaction, the detection rate of adenomatous colon polyps is not enhanced.

COMMENTS

Background

Colonoscopy is the gold standard examination in screening for colon polyps. Adenomas can be precursors to colon cancer and their removal by polypectomy prevents the development of cancer in many instances. Yet it has been shown that adenomas may be missed up to 24% of the time.

Research frontiers

Many factors have been shown to affect polyp detection rates including: quality or preparation, withdrawal time, image enhancement, polyp size, increased visualization with Third Eye Retroscope and time of day. However, few studies have shown any adenoma detection rate differences in the type of sedation used.

Innovations and breakthroughs

This is one of the first studies of its kind to investigate whether or not anesthesiologist monitored use of propofol would affect the adenoma detection rate when compared to gastroenterologist-monitored use of fentanyl and midazolam. The data suggest that anesthesia monitored propofol sedation was not associated an overall significant difference in adenoma detection rate compared to gastroenterologist monitored sedation with midazolam and fentanyl.

Applications

The data suggest that the detection rate of adenomatous polyps of all sizes is not increased by the use of anesthesiologist monitored propofol sedation compared to gastroenterologist monitored sedation with midazolam/fentanyl. While the use of propofol based anesthesia is certainly associated with increased patient satisfaction, the detection rate of adenomatous colon polyps is not enhanced. This study could have future financial implications given the cost of anesthesiologist monitored propofol sedation.

Terminology

Propofol is a short-acting, intravenously administered hypnotic agent. Its uses

include the induction and maintenance of general anesthesia, sedation for mechanically ventilated adults, and procedural sedation. Midazolam is a short-acting drug in the benzodiazepine class that is used for treatment of acute seizures, moderate to severe insomnia, and for inducing sedation and amnesia before medical procedures. Fentanyl is a strong agonist on the μ -opioid receptors and is commonly used before procedures as an anesthetic in combination with a benzodiazepine.

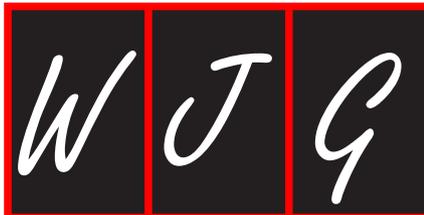
Peer review

This paper is well written and covers important topics and findings.

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S- Editor Tian L L- Editor Rutherford A E- Editor Li JY



Pediatric functional constipation treatment with *Bifidobacterium*-containing yogurt: A crossover, double-blind, controlled trial

Paula VP Guerra, Luiza N Lima, Tassia C Souza, Vanessa Mazochi, Francisco J Penna, Andreia M Silva, Jacques R Nicoli, Elizabet V Guimarães

Paula VP Guerra, Luiza N Lima, Francisco J Penna, Elizabet V Guimarães, Departamento de Pediatria, Faculdade de Medicina, Universidade Federal de Minas Gerais, 30130-100 Belo Horizonte, MG, Brazil

Tassia C Souza, Jacques R Nicoli, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, 32270-901 Belo Horizonte, MG, Brazil

Vanessa Mazochi, Andreia M Silva, Universidade Federal de São João del Rei, Campus de Sete Lagoas, 35701-970 São João del Rei, MG, Brazil

Author contributions: Guerra PVP, Lima LN, Souza TC, Mazochi V and Silva AM designed and performed the research; Penna FJ, Nicoli JR and Guimarães EV designed the research, analyzed the data and wrote the paper.

Supported by Grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico and Fundação de Amparo à Pesquisa do Estado de Minas Gerais

Correspondence to: Jacques R Nicoli, PhD, Full Professor, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, 32270-901 Belo Horizonte, MG, Brazil. jnicoli@icb.ufmg.br

Telephone: +55-31-34092757 Fax: +55-31-34092730

Received: February 11, 2011 Revised: March 29, 2011

Accepted: April 5, 2011

Published online: September 14, 2011

Abstract

AIM: To evaluate the treatment of pediatric functional chronic intestinal constipation (FCIC) with a probiotic goat yogurt.

METHODS: A crossover double-blind formula-controlled trial was carried out on 59 students (age range: 5-15 years) of a public school in Belo Horizonte, MG, Brazil, presenting a FCIC diagnostic, according to Roma III criteria. The students were randomized in two groups to receive a goat yogurt supplemented with 10^9 colony forming unit/mL *Bifidobacterium longum* (*B.*

longum) (probiotic) daily or only the yogurt for a period of 5 wk (formula). Afterwards, the groups were intercrossed for another 5 wk. Defecation frequency, stool consistency and abdominal and defecation pain were assessed.

RESULTS: Both treatment groups demonstrated improvement in defecation frequency compared to baseline. However, the group treated with probiotic showed most significant improvement in the first phase of the study. An inversion was observed after crossing over, resulting in a reduction in stool frequency when this group was treated by formula. Probiotic and formula improved stool consistency in the first phase of treatment, but the improvement obtained with probiotic was significantly higher ($P = 0.03$). In the second phase of treatment, the group initially treated with probiotic showed worsening stool consistency when using formula. However, the difference was not significant. A significant improvement in abdominal pain and defecation pain was observed with both probiotic and formula in the first phase of treatment, but again the improvement was more significant for the group treated with *B. longum* during phase I ($P < 0.05$). When all data of the crossover study were analyzed, significant differences were observed between probiotic yogurt and yogurt only for defecation frequency ($P = 0.012$), defecation pain ($P = 0.046$) and abdominal pain ($P = 0.015$).

CONCLUSION: An improvement in defecation frequency and abdominal pain was observed using both supplemented and non-supplemented yogurt, but an additional improvement with *B. longum* supplementation was obtained.

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Key words: Functional chronic constipation; Probiotic; *Bifidobacterium longum*; Yogurt; Adolescents; Children

Peer reviewer: Mario Guslandi, Professor, Department of Gastroenterology, S: Raffaele University Hospital, S: Raffaele University Hospital via Olgettina 60, Milano 20132, Italy

Guerra PVP, Lima LN, Souza TC, Mazochi V, Penna FJ, Silva AM, Nicoli JR, Guimarães EV. Pediatric functional constipation treatment with *Bifidobacterium*-containing yogurt: A crossover, double-blind, controlled trial. *World J Gastroenterol* 2011; 17(34): 3916-3921 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i34/3916.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i34.3916>

INTRODUCTION

The worldwide prevalence of childhood constipation in the general population ranges from 0.7% to 29.6%^[1], and the wide range indicates differences in definition and selection of patients. This functional defecation disorder is characterized by infrequent defecation less than three times per week, frequent episodes of fecal incontinence, the periodic passage of large and painful stools which clog the toilet, and retentive posturing. Upon physical examination a palpable fecal mass is often found in the abdomen and the rectum. Accompanying symptoms may include irritability, decreased appetite, and/or early satiety. In the vast majority of cases (90% to 95%), no underlying organic cause is found and functional constipation is diagnosed^[2].

The standard treatment consists of disimpaction and the administration of laxatives to achieve a normal bowel habit of passing a soft stool without pain. Even though the traditional treatment is well established and safe, for many patients it does not provide a satisfying improvement, prompting interest in other therapeutic strategies^[3].

Probiotics are increasingly being used as an alternative in the management of constipation. Probiotics are defined as live microorganisms which when administered in adequate amounts confer a benefit on the host health^[4]. In a recent review, the efficacy and safety of probiotic supplementation for the treatment of constipation was evaluated^[5]. Studying 5 randomized controlled trials, with a total of 377 subjects (three trials with adults and two trials with children), the data suggests a favorable effect of some strains of *Lactobacillus*, *Bifidobacterium* and *Escherichia coli* (*E. coli*). Only one of the randomized controlled trials described the ineffectiveness of *Lactobacillus rhamnosus* Goldin and Gorbach as an adjunct to lactulose for the treatment of constipation in children^[6]. The authors of the review concluded that until more data are available, the use of probiotics for the treatment of constipation should be considered investigational. More recently, *Lactobacillus reuteri* administered in infants with chronic constipation had a positive effect on frequency of bowel movements, but not on stool consistency^[7] and the intake of mixed probiotic strains [*Lactobacillus plantarum*, *Bifidobacterium breve*, *Bifidobacterium animalis* subspecies *lactis* (*B. animalis* var. *lactis*)] was able to relieve evacuation disorders and hard stools in healthy adults^[8].

In the present study, the ingestion of goat yogurt containing a *Bifidobacterium longum* (*B. longum*) strain was evaluated for the treatment of functional chronic intestinal constipation (FCIC) in children and adolescents.

MATERIALS AND METHODS

Subjects and eligibility criteria

Children aged 5-15 years and with FCIC, referred to a public school in the central area of the city of Belo Horizonte, Minas Gerais, Brazil, were eligible for study entry. Constipation was characterized according to Rome III criteria as presenting at least two out of six of the following symptoms for two or more months: two or fewer defecations per week; at least one episode of fecal incontinence per week; history of retentive posturing or excessive volitional stool retention; history of painful or hard bowel movements; presence of a large fecal mass in the rectum; history of wide diameter stools that may obstruct the toilet^[2]. Exclusion criteria were the use of any oral laxative < 4 wk before intake, metabolic disease, a history of gastrointestinal surgery and fecal incontinence. Patients with fecal incontinence were excluded in order to make the sample more homogeneous in relation to disease severity. The follow-up protocol included defecation frequency, stool consistency, and abdominal and defecation pains recorded daily by the adolescents or parents. All children older than 12 years and/or parents gave informed consent. The study was approved by the Ethical Committee in Research of the Universidade Federal de Minas Gerais (COEP/UFMG, number ETIC0506/08).

Yogurt and bacterium

The *B. longum* strain used in the trial was isolated from the feces of a healthy child and identified by Multiplex Polymerase Chain Reaction. This strain was selected as a candidate for probiotic use based on technological (aerotolerance and high growth rate) and beneficial (wide antagonistic spectrum against pathogenic indicators, few antimicrobial resistance) criteria. The bacterium was grown in de Man, Rogosa and Sharp broth (Difco, Sparks, United States) for 48 h at 37 °C in an anaerobic chamber (Forma Scientific Company, Marietta, United States) containing N₂ 85%, H₂ 10% and CO₂ 5%. After growth, the culture was concentrated by centrifugation and resuspended in peptone sterile water. An aliquot of 1 mL of the concentrated bacterium suspension was added to 9 mL of a commercial goat yogurt (Capril Jacomé, Contagem, Brazil) to obtain a final concentration of 10⁹ colony forming unit (CFU)/mL. The control formula was prepared by addition of 1 mL of peptoned water to 9 mL of goat yogurt. The goat yogurt contained the two classical yogurt starters, *Lactobacillus delbrueckii* subspecies *bulgaricus* and *Streptococcus thermophilus* from the YF-L812 commercial culture (DVS - Christian Hansen Laboratory, Horsholm, Denmark). Both yogurts were maintained at 4 °C until use and for a maximum of one week. During this period, the *Bifidobacterium* cells remained viable at 10⁹ CFU/mL levels.

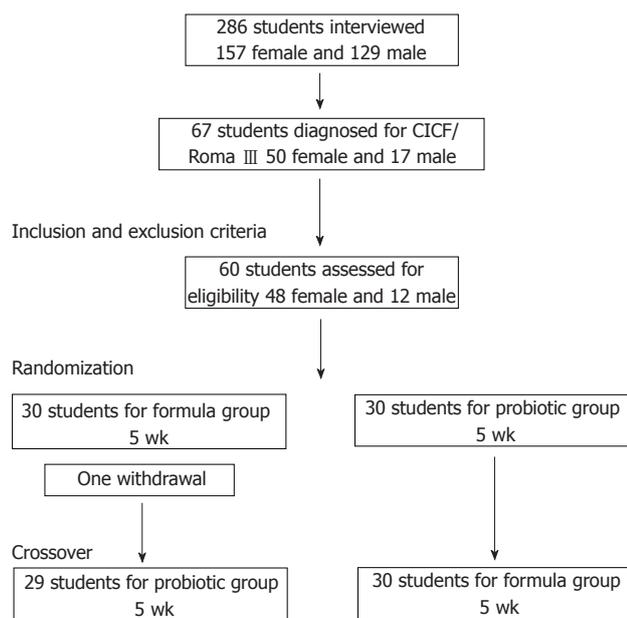


Figure 1 Participant flow diagram.

Study intervention

This study was carried out using a crossover double-blind, formula-controlled design with two parallel groups. In order to determine the sample size, a preliminary trial was done with 15 children to evaluate the average and variance of the difference in defecation frequency between formula and probiotic groups. To guarantee that the sample size calculation considered the crossover design, an equation for the comparison between averages of difference^[9] was used considering a significance level set at 0.05, a power of 80%, a difference average of 0.75 and a variance difference of 3.15. Under the assumptions made here, the smallest sample size was 22 for each group. The students were randomized in two groups to receive 1 mL of goat yogurt supplemented with 10⁹ CFU/mL *B. longum* (probiotic) daily or the same dose of goat yogurt daily for a period of 5 wk (formula). Afterwards, the groups were crossed over to alternate intervention for another 5 wk. Defecation frequency, stool consistency and abdominal or defecation pain were assessed at the first (A1), third (A2) and fifth week (A3) before crossing over, and the first (B1), third (B2) and fifth week (B3) after crossing over. The stool consistency was characterized using the Bristol Stool Scale^[10]. To describe feces consistency, the subjects and/or their parents received instructions with a stool illustration and explanation in advance for the purpose of objectively selecting the stool form. The two products, goat yogurt with or without *B. longum* were identical in weight, color, smell, taste and package. All doctors and children involved were unaware of the treatment administered. Children were instructed to maintain their ordinary dietary habits, but were asked to avoid consuming other fermented dairy products or yogurts during the study. The allocation sequence and randomization list were computer-generated using the Epi Info Program.

Table 1 Characteristics of subjects at baseline

	Formula (n = 29)	Probiotic (n = 30)
Female/Male	23/6	24/6
Age (yr)		
5 to 7	6	12
8 to 9	7	5
10 to 12	12	11
13 to 15	4	2
Previous treatment for intestinal constipation		
Yes	5	3
No	24	27
Defecation frequency		
≤ 2 times/wk	19	17
3-6 times/wk	7	13
7 or more times/wk	3	0
Stool consistency Bristol scale		
1	9	3
2	7	13
3	11	13
4	2	1
5	0	0
Defecation pain (> 1/wk)		
Yes	26	20
No	3	10
Abdominal pain (> 1/wk)		
Yes	25	26
No	4	4

Statistical analysis

Pearson exact test and Wilcoxon test were used to compare the variables (defecation frequency, stool consistency and abdominal and defecation pain) between the two groups. Differences in the variable distributions at each moment of each sequence of intervention were used for these analyses. The data were analyzed using the software Statistical Package for Social Sciences (SPSS 15.0). Normality was evaluated by Shapiro Wilk test. All tests were two sided, and *P* < 0.05 was considered statistically significant. All analyses were performed on an intention-to-treat basis.

RESULTS

The participant flow diagram (Figure 1) shows that among 286 students interviewed, 67 (23.4%) were diagnosed with FCIC following the Roma III criteria. Seven of them were excluded based on the exclusion criteria, and the remaining students were randomized to receive the probiotic or formula treatment. After the beginning of the trial only one parental withdrawal occurred in the formula group. There was no adverse effect due to the interventions in the present study protocol.

Table 1 summarizes the subjects' baseline demographic and clinical characteristics. The two groups were comparable in regard to age, sex, and baseline features of constipation. More female subjects than male were present in the two groups and at a similar frequency (79.3% and 80.0% in formula and probiotic groups, respectively)

Figure 2A shows and compares the evolution of the two groups for hard stool consistency (Bristol scale < 4) during the trial. An improvement was observed with both

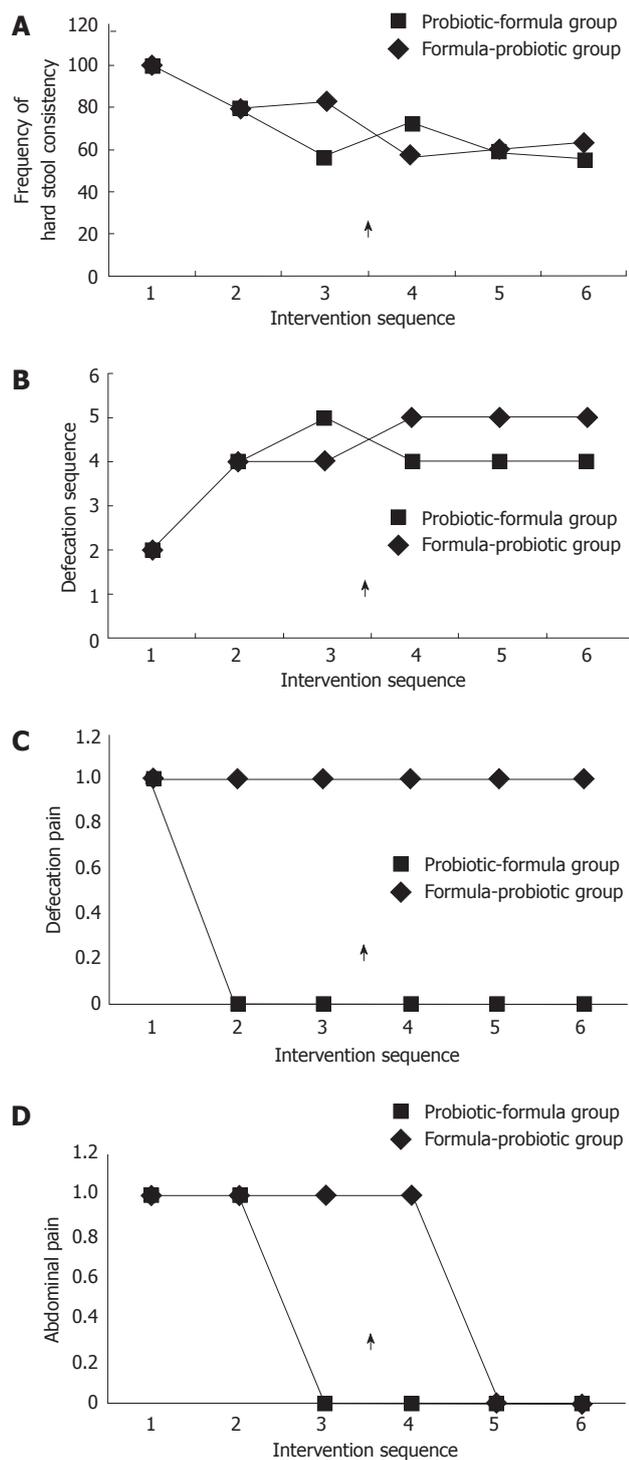


Figure 2 Evolution of hard stool consistency (Bristol scale < 4) (A), defecation frequency (B), defecation pain (C) and abdominal pain (D) during the intervention sequence. 1: A1; 2: A2; 3: A3; 4: B1; 5: B2; 6: B3. Arrow shows the moment of inversion in the intervention sequence.

treatments as compared to baseline. This improvement was greater in the probiotic group during the first part of the intervention when a significant difference ($P = 0.03$) was observed between the two groups after 5 wk of treatment (A3 phase). After crossing over, an inversion was observed, but this was not statistically significant.

Figure 2B shows and compares the evolution of the two groups for defecation frequency during the trial. A

significant improvement in defecation frequency was also noted for both groups when compared to baseline with a tendency to a slight additional improvement at the end of the first intervention when *B. longum* was supplemented, and an inversion after crossing over.

Figure 2C shows and compares the evolution of defecation pain in the two groups during the trial. An improvement was observed for both treatments in relation to the baseline, but with a better evolution for the probiotic group. However, a significant difference ($P = 0.009$) between formula and probiotic was observed only for phase B1, and contrarily to Figures 1 and 2 an inversion was not observed after crossing over.

Figure 2D shows and compares the evolution of abdominal pain in the two groups during the trial. When the symptomatology was compared before and after the intervention, a significant improvement was noted for both groups as compared to baseline, but again with better results for the probiotic group. However, at the end of the second intervention after crossing over, the symptomatology was similar for the two groups.

When all data of the crossover study were analyzed, significant differences were observed between probiotic yogurt and yogurt only for defecation frequency ($P = 0.012$), defecation pain ($P = 0.046$) and abdominal pain ($P = 0.015$).

DISCUSSION

The prevalence of FCIC observed in the present study (23.4%) was similar to the data cited in the literature^[1]. The predominance of FCIC in female subjects (about 80%) was also described in the literature^[1].

Within the first week of intervention, a significant improvement in all constipation symptoms was observed in both treatment groups (yogurt or yogurt plus *B. longum*) when compared to the baseline. However, when the yogurt was supplemented with the probiotic, further improvement was obtained when compared to the yogurt only. Yogurt is generally considered to alleviate gastrointestinal conditions such as constipation and diarrhea^[11]. However, regarding the effect of yogurt alone on constipation, few reports are available in the literature, and the results reported are contradictory. Additionally, in most of the clinical trials comparing the effect of probiotic yogurt with control yogurt, the starter lactic acid bacteria are heat-killed in the second situation, which does not correspond to the reality. In the few studies where viability of the starter strains was maintained in the control yogurt, improvement of constipation symptoms was observed in both probiotic and control groups with an increment in the first one^[12].

There are several hypotheses to explain how probiotics might have therapeutic potential for the treatment of constipation. Firstly, quite old and well known observations showed that the absence of gut microbiota in germ-free animals result in abnormal characteristics of the intestinal morphology and function such as increased transit time of contents, altered myenteric neurons, impaired intestinal muscle function and decreased intestinal

mass^[13,14]. Interestingly, the mono-association of germ-free animals with *Lactobacillus acidophilus* or *Bifidobacterium bifidum* reduced the migrating myoelectric complex period and accelerated the small intestinal transit. Inversely, some *E. coli* strains presented an inverse effect when mono-associated in gnotobiotic animals^[14]. Short-chain fatty acids (SCFAs), main metabolic products derived from the fermentative activity of the gut microbiota, have a direct influence on intestinal motility through the Gpr41 receptor^[15]. In colonized Gpr41 knockout mice, an increased intestinal transit rate was associated with a reduced expression of peptide YY, an enteroendocrine cell-derived hormone that normally inhibits gut motility^[16]. Secondly, there are some data suggesting differences in the intestinal microbiota of healthy individuals and patients with chronic constipation^[17,18]. The main features were an increased number of clostridia and enterobacteria, and a decrease in bifidobacteria and lactobacilli. These differences have an influence on the metabolic profile of the gut environment, and particularly on SCFA pattern^[19]. However, a key question is if this dysbiosis is a secondary manifestation of constipation, or is a factor contributing to constipation. Another set of data favoring the microbiota influence describes the higher defecation frequency and softer stool consistency in breast-fed than in formula-fed infants in the first four months of life, which can be due to the higher fecal levels of bifidobacteria in breast-fed infants^[20]. Thirdly, studies involving the administration of *Bifidobacterium animalis* subspecies *lactis* DN-173010 have shown improved colonic transit times, both in a healthy population^[21] and in constipated patients^[22]. Another study showed that the intake of probiotic (*Lactobacillus helveticus* and *B. longum*) can modify the gut microbial ecology and metabolic profiles^[23]. Finally, in a study using a guinea-pig isolated tissue model, results showed that cytoplasmatic fraction of probiotic bacteria (*Lactobacillus*, *Bifidobacterium*) stimulated the contraction of the ileum segment and induced proximal colon relaxation^[24].

In conclusion, an improvement in constipation symptoms was observed using both supplemented and non-supplemented yogurt. An additional improvement with *B. longum* supplementation was suggested in the present intercrossed double-blind formula-controlled study.

ACKNOWLEDGMENTS

The authors acknowledge the staff of the Instituto de Educação, Belo Horizonte, Brazil, and all of the families and children who participated in the present study.

COMMENTS

Background

The worldwide prevalence of childhood constipation in the general population ranges from 0.7% to 29.6%, and the standard treatment that consists of disimpaction and administration of laxatives does not provide satisfying improvement in 40% of the children. Development of new therapeutic strategies is necessary to treat these patients more effectively, and probiotics are increasingly being used as one of such alternatives in the management of constipation.

Research frontiers

There is growing interest in the use of probiotics in organic and functional gastrointestinal disorders. A limited number of studies have been published about the effects of probiotics on constipation in children, and encouraging results have been obtained. But so far there is no hard evidence to recommend this use in children, and a general consensus in the scientific literature recommends more clinical trials. In the present intercrossed double-blind formula-controlled study, an additional improvement in constipation symptoms was observed using yogurt supplemented with *Bifidobacterium longum* when compared to non-supplemented yogurt.

Innovations and breakthroughs

Recent reports have highlighted that the administration of probiotics could be recommended as an adjunctive therapy of chronic constipation. It is well known that the indigenous digestive microbiota affects intestinal motility and accelerates small intestinal transit. Among the components of this microbial ecosystem, bacteria of the *Bifidobacterium* genus seems to be involved in the phenomenon and therefore are microorganisms of choice for use as probiotics.

Applications

The results of the present clinical trial suggest that the ingestion of a goat yogurt supplement with a *Bifidobacterium* strain may represent an alternative strategy in the treatment of pediatric functional constipation.

Peer review

A clearcut, well conducted trial showing how the addition of a specific probiotic agent can improve intestinal symptoms in children and adolescent with constipation.

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S- Editor Tian L L- Editor O'Neill M E- Editor Xiong L

Sorafenib inhibits growth and metastasis of hepatocellular carcinoma by blocking STAT3

Fang-Ming Gu, Quan-Lin Li, Qiang Gao, Jia-Hao Jiang, Xiao-Yong Huang, Jin-Feng Pan, Jia Fan, Jian Zhou

Fang-Ming Gu, Quan-Lin Li, Qiang Gao, Jia-Hao Jiang, Xiao-Yong Huang, Jin-Feng Pan, Jia Fan, Jian Zhou, Liver Cancer Institute, Zhongshan Hospital and Shanghai Medical School, Fudan University, Shanghai 200032, China

Author contributions: Gu FM, Li QL and Gao Q contributed equally to this work; Gu FM and Li QL performed the experiments and interpretation of the data and statistical analysis; Zhou J and Gao Q contributed to the conception and design of the study; Gu FM, Gao Q, Li QL and Zhou J wrote the manuscript; Jiang JH, Huang XY, Pan JF and Fan J made substantial contribution to the design and conception of the study and interpretation of data; all authors read and approved the final manuscript.

Supported by Grants from the China 863 Project, No. 2007A-A02Z479; the National Natural Science Foundation of China, No. 30972949 and 30901432; Shanghai Rising-Star Program, No. 10QA1401300; and Research Fund for the Doctoral Program of Higher Education of China, No. 20090071120026

Correspondence to: Jian Zhou, MD, PhD, Liver Cancer Institute, Zhongshan Hospital and Shanghai Medical School, Fudan University, 180 Feng Lin Road, Shanghai, 200032, China. zhou.jian@zs-hospital.sh.cn

Telephone: +86-21-64037181 Fax: +86-21-64037181

Received: February 1, 2011 Revised: April 12, 2011

Accepted: April 19, 2011

Published online: September 14, 2011

Abstract

AIM: To investigate the inhibitory role and the underlying mechanisms of sorafenib on signal transducer and activator of transcription 3 (STAT3) activity in hepatocellular carcinoma (HCC).

METHODS: Human and rat HCC cell lines were treated with sorafenib. Proliferation and STAT3 dephosphorylation were assessed. Potential molecular mechanisms of STAT3 pathway inhibition by sorafenib were evaluated. *In vivo* antitumor action and STAT3 inhibition were investigated in an immunocompetent orthotopic rat HCC model.

RESULTS: Sorafenib decreased STAT3 phosphorylation

at the tyrosine and serine residues (Y705 and S727), but did not affect Janus kinase 2 (JAK2) and phosphatase shatterproof 2 (SHP2), which is associated with growth inhibition in HCC cells. Dephosphorylation of S727 was associated with attenuated extracellular signal-regulated kinase (ERK) phosphorylation, similar to the effects of a mitogen-activated protein kinase (MEK) inhibitor U0126, suggesting that sorafenib induced S727 dephosphorylation by inhibiting MEK/ERK signaling. Meanwhile, sorafenib could also inhibit Akt phosphorylation, and both the phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002 and Akt knockdown resulted in Y705 dephosphorylation, indicating that Y705 dephosphorylation by sorafenib was mediated by inhibiting the PI3K/Akt pathway. Finally, in the rat HCC model, sorafenib significantly inhibited STAT3 activity, reducing tumor growth and metastasis.

CONCLUSION: Sorafenib inhibits growth and metastasis of HCC in part by blocking the MEK/ERK/STAT3 and PI3K/Akt/STAT3 signaling pathways, but independent of JAK2 and SHP2 activation.

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Key words: Hepatocellular carcinoma; Sorafenib; Signal transducer and activator of transcription 3; Extracellular signal regulated kinase; Akt

Peer reviewers: Masahiro Arai, MD, PhD, Department of Gastroenterology, Toshiba General Hospital, 6-3-22 Higashi-ooi, Shinagawa-ku, Tokyo 140-8522, Japan; Giammarco Fava, MD, Department of Gastroenterology, Università Politecnica delle Marche, Ancona, via Gervasoni 12, 60129 Ancona, Italy; Jian Wu, Associate Professor of Medicine, Internal Medicine/Transplant Research Program, University of California, Davis Medical Center, 4635 2nd Ave. Suite 1001, Sacramento CA 95817, United States

Gu FM, Li QL, Gao Q, Jiang JH, Huang XY, Pan JF, Fan J, Zhou J. Sorafenib inhibits growth and metastasis of hepatocellular carcinoma by blocking STAT3. *World J Gastroenterol* 2011; 17(34): 3922-3932 Available from: URL: <http://www.wjgnet.com>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most frequent cause of cancer-related death globally. Although prognosis of patients with HCC has increased in recent decades, long-term survival remains unsatisfactory because of the high rate of recurrence and metastasis^[1]. Advances in treating this disease are likely to develop from a better understanding of its biology and behavior, which are affected by multiple molecular pathways controlled by transcription factors^[2,3].

Signal transducer and activator of transcription 3 (STAT3) plays a critical role in transcriptional regulation of genes that are involved in tumor cell proliferation, survival and invasion^[4]. Constitutive activation of STAT3 is observed in 72.4% of human HCC^[5] and in a wide variety of other cancer types^[6,7]. Overexpression of constitutively activated forms of STAT3 induces the formation of foci *in vitro* and tumors in mouse models. Moreover, inhibiting STAT3 function *via* RNA knockdown, peptide inhibition, and expression of dominant-negative forms in cancer cells leads to a decrease in tumor progression^[8]. Our group has also reported that knockdown of STAT3 with antisense oligonucleotides inhibits tumor growth and metastasis in a mouse xenograft model of HCC^[9]. In human HCC tissues, constitutive activation of STAT3 is a significant predictor of overall survival^[5]. Thus, targeting of STAT3 activation may prove to be an effective approach to controlling HCC.

Sorafenib (Nexavar, BAY 43-9006) is a multikinase inhibitor that has shown anti-tumor activity against a wide variety of cancers including HCC^[10,11]. Sorafenib blocks tumor cell proliferation and angiogenesis by targeting the Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway and receptor tyrosine kinases (RTKs), such as vascular endothelial cell growth factor receptor (VEGFR)-2, VEGFR-3, platelet-derived growth factor receptor- β , fms-like tyrosine kinase receptor-3 (FLT3), RET, and c-KIT^[10,11]. Recently, sorafenib has been shown to suppress tumor growth by decreasing STAT3 phosphorylation in a group of human malignancies^[12-15]. In HCC, sorafenib has also been suggested to overcome TRAIL resistance through the inhibition of STAT3^[16]. However, thus far, the exact molecular mechanisms by which sorafenib inhibited STAT3 have not been fully elucidated.

Here, we found that sorafenib decreased STAT3 phosphorylation at both tyrosine and serine residues (Y705 and S727), which were independent of Janus kinase 2 (JAK2) and phosphatase shatterproof 2 (SHP2) activity. We further demonstrated that inhibition of the phosphatidylinositol-3-kinase (PI3K)/Akt and MEK/ERK path-

ways was responsible for Y705 and S727 dephosphorylation, respectively, by sorafenib. Consistent with these findings, sorafenib markedly inhibited STAT3 dephosphorylation, suppressed tumor growth and metastasis in an immunocompetent orthotopic rat HCC model.

MATERIALS AND METHODS

Reagents and cell lines

Sorafenib (BAY 43-9006, Bayer Pharmaceutical Corporation) was dissolved in sterile dimethyl sulfoxide (DMSO) for *in vitro* experiments, and in Cremophor EL (Sigma) and 95% ethanol (50:50) for *in vivo* experiments. DMSO was added to cultures at 0.1% (v/v) final concentration as a vehicle control. Primary antibodies, STAT3 and phosphorylated STAT3 (p-STAT3; Y705 and S727); Akt and phosphorylated Akt (p-Akt; S473); JAK2 and phosphorylated JAK2 (p-JAK2; Y1007/1008); ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2; T202/Y204); SHP2 and phosphorylated SHP2 (p-SHP2; Y580) were purchased from Cell Signaling Technology. Cyclin D1 was from Abcam. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Millipore. Human or rat Akt small interfering RNA (siRNA), control siRNA, were obtained from Shanghai GenePharma Co. (Shanghai, PR China). LY294002, a PI3K inhibitor, and U0126, a MEK inhibitor, were purchased from Cell Signaling Technology.

Two human HCC cell lines, HCCLM3^[17,18] and HepG2 (ATCC), and a rat HCC cell line, Morris hepatoma 3924A (MH) cells (German Cancer Research Center Tumor Collection)^[19], were maintained in high-glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin. Cell lines were cultured at 37 °C in a humidified incubator in 5% CO₂.

MTT assay

The effect of sorafenib on HCC cell growth was determined with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay. Cells were seeded into 96-well flat-bottom plates (1×10^3 /well) and cultured for 24, 48 or 72 h in medium supplemented with sorafenib (0, 0.05, 0.1, 1, 5, 10 or 20 μ mol/L; 6 wells/dose), and each experiment was repeated at least three times. After sorafenib treatment, cells were incubated with MTT (20 μ L/well) at 37 °C for 4 h, and then 200 μ L DMSO was added. The absorbance of individual wells was determined at 570 nm.

Western blotting analysis

Western blot analysis was performed as previously described^[20]. Briefly, total cell lysates were prepared, and proteins were separated by SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes. The membranes were washed, blocked, and incubated with the specific primary antihuman antibodies against p-STAT3-Y705 (1:1000), p-STAT3-S727 (1:1000), STAT3

(1:1000), p-Akt (1:1000), Akt (1:1000), p-ERK1/2 (1:1000), ERK1/2 (1:1000), p-JAK2 (1:1000), JAK2 (1:1000), p-SHP2 (1:1000), SHP2 (1:1000), cyclin D1 (1:1000), or GAPDH (1:5000), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence assay (Pierce-Thermo Scientific).

Quantitative reverse transcription-polymerase chain reaction

Cells were plated and treated with 10 $\mu\text{mol/L}$ sorafenib. The cells were harvested after 24 h, and total RNA was extracted with Trizol Reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was reverse transcribed with RevertAid™ first-strand cDNA synthesis kit (Fermentas). Human and rat STAT3 mRNA levels were determined by qPCR using SYBR Premix Ex Taq (TaKaRa, Dalian, China) and normalized to human and rat β -actin respectively, using the following primers: β -actin (human) forward, 5'-CAA CTG GGA CGA CAT GGA GAA AAT-3' and reverse, 5'-CCA GAG GCG TAC AGG GAT AGC AC-3'; β -actin (rat) forward, 5'-TCC ACC CGC GAG TAC AAC CTT CTT-3' and reverse, 5'-GGC CCG GGG AGC ATC GTC-3'; STAT3 (human) forward, 5'-CCC CCG CAC TTT AGA TTC AT-3' and 5'-GGT AGG CGC CTC AGT CGT AT-3'; STAT3 (rat) forward, 5'-GGT GAT GAG TTT CCG AGT GTG TCT GA-3' and reverse, 5'-AAA GCG CCT GCG CCT GCG ATA AAG TTC T-3'. Relative gene expression was calculated with the $2^{-\Delta\Delta C_t}$ method.

Akt silencing by siRNA

Akt siRNA and negative control mismatch sequences were transfected into HepG2 and MH cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. The following sense and anti-sense siRNA strands were used: Akt (human) GUG CCA UGA UCU GUA UUU ATT (sense), UAA AUA CAG AUC AUG GCA CTT (anti-sense); Akt (rat) GCU CAG AUG AUC ACC AUC ATT (sense), UGA UGG UGA UCA UCU GAG CTT (anti-sense). After 72 h, cells were lysed, and protein was analyzed by Western blotting.

Animal experiments

Male ACI rats (Harlan Inc., Indianapolis, IN, United States; 200-220 g) were maintained in laminar-flow cabinets under specific pathogen-free conditions and a 12-h dark-light cycle. The animals were cared for and handled according to recommendations of the NIH guidelines for care and use of laboratory animals. The Shanghai Medical Experimental Animal Care Committee approved the experimental protocol. Intrahepatic tumor implantation with Morris Hepatoma fragments was performed under aseptic conditions as previously described^[21].

The rats were randomly assigned to 3 groups ($n = 10$ per group): vehicle control, sorafenib early treatment, and sorafenib late treatment. Sorafenib treatment groups were given 30 mg/kg sorafenib in 500 μL carrier solu-

tion once daily by gavage. The dose of sorafenib was based on doses commonly used in murine models^[11,22]. Treatment was started on days 5 and 17 after tumor implantation in the sorafenib early and late treatment groups, respectively. On day 17, the HCC xenografts reached approximately 700 mm^3 , which was demonstrated in our preliminary experiment. Control rats received an equal volume of carrier solution by gavage. The rats were sacrificed at day 38 after tumor implantation. At necropsy, tumor volume was calculated as $V = \pi/6 \times \text{length} \times \text{width} \times \text{height}$. Lung and lymph node metastasis, as well as peritoneal seeding, were denoted as the visually positive tumor nodules. For Western blot analysis, tumor tissues were homogenized in tumor lysis buffer. For immunohistochemical staining, tumors were fixed in paraformaldehyde for 24 h and embedded in paraffin for sectioning.

Immunohistochemistry

Tissue sections (4 μm) were stained with hematoxylin and eosin for histologic analysis and with the specific primary antihuman antibodies against p-STAT3-Y705, p-STAT3-S727, p-Akt, p-ERK and cyclin D1 for immunohistochemistry. Briefly, after microwave antigen retrieval, tissues were incubated with primary antibodies overnight at 4 °C, followed by 30-min incubation with the secondary antibody. The reaction was visualized with diaminobenzidine, and tissues were counterstained with hematoxylin.

The tissue sections were viewed at $\times 200$ magnification and images were captured. Five fields per section were analyzed, excluding peripheral connective tissue and necrotic regions. Scoring for p-STAT3-Y705 and p-STAT3-S727 was assigned based on both staining proportion and intensity as previously described^[5]. For expression intensity of p-Akt, p-ERK and cyclin D1, the integrated absorbance and the area in a photograph were measured using Image-Pro Plus v6.0 software (Media Cybernetics, Inc.). A uniform setting of color segmentation was loaded for counting the integrated absorbance of all the pictures, and the mean p-Akt, p-ERK or cyclin D1 density was calculated as the product of the integrated absorbance/total area.

Detection and quantitation of apoptosis

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method was based on the specific binding of terminal deoxynucleotidyl transferase to the 3'-OH ends of DNA, ensuring the synthesis of a polydeoxynucleotide polymer. For this purpose, the In Situ Cell Death Detection kit-Peroxidase (Roche) was used according to the manufacturer's directions.

Capture of the photographs and measurement of positive staining density were performed as described previously^[11,22]. Briefly, the tissue sections were viewed at $\times 200$ magnification and images were captured. The Apoptosis Index was determined by counting at least 1000 cells in 5 randomly selected fields, using Image-Pro Plus v6.0

Table 1 Sorafenib suppresses tumor growth and metastasis *in vivo* *n* (%)

Groups (<i>n</i> = 10)	Tumor volume (mm ³)	Lung metastasis	Lymph node metastasis	Peritoneal seeding	Ascites
Vehicle	12795.71 ± 2980.56	9/10 (90)	7/10 (70)	3/10 (30)	4/10 (40)
Sorafenib (early)	351.26 ± 97.58 ^a	0/10 (0) ^b	0/10 (0) ^b	0/10 (0) ^c	0/10 (0) ^c
Sorafenib (late)	2248.33 ± 971.68 ^a	4/10 (40) ^b	0/10 (0) ^b	0/10 (0) ^c	0/10 (0) ^c

^a*P* < 0.001, Student *t* test (*vs* vehicle group); ^b*P* < 0.05, Fisher's exact test (*vs* vehicle group); ^c*P* < 0.05, Fisher's exact test (*vs* vehicle group).

software (Media Cybernetics, Silver Spring, MD).

Statistical analysis

Statistical analysis was performed with SPSS 16.0 software (SPSS, Chicago, IL). Measurement values were expressed as mean ± SD. The Student *t*-test and Fisher's exact test were used as appropriate. Two-tailed *P* < 0.05 were considered significant.

RESULTS

Sorafenib inhibits tumor growth *in vitro*

To determine the growth inhibition effect of sorafenib in HCC, HCC cell lines HCCLM3, HepG2 and MH were incubated for 24, 48 and 72 h with sorafenib (0.01–20 μmol/L). As shown in Figure 1A, sorafenib inhibited tumor cell growth in both a time- and dose- dependent manner.

Sorafenib inhibits STAT3 phosphorylation independent of JAK2

Given that STAT3 is constitutively activated in HCC, we next evaluated whether sorafenib could induce HCC growth arrest by inhibiting STAT3. We found that sorafenib inhibited phosphorylation of STAT3 at both Y705 and S727 in a dose-dependent manner. Furthermore, inhibition was evident as early as 2 h after treatment and lasted for 24 h (Figure 1B and 1C) in all HCC cell lines, which corresponded with sorafenib-induced growth inhibition as assessed by MTT assay.

JAK2 is considered as one of the most common activators of STAT3. We therefore determined the effects of sorafenib on JAK2. Western blot analysis showed that JAK2 phosphorylation was not reduced during the 24-h sorafenib treatment (Figure 1B and 1C). In addition, total STAT3 protein and mRNA expression levels also remained unchanged during the 24-h treatment (*P* > 0.05 for all; Figure 1C and 1D).

Sorafenib-induced S727 dephosphorylation is dependent on MEK/ERK

Because 24 h treatment with 10 μmol/L sorafenib significantly inhibited S727 and ERK phosphorylation in HCC lines, we evaluated the effect of the selective MEK inhibitor U0126 in HepG2 and MH cells to determine whether MEK was involved in this effect (Figure 2A). Dephosphorylation of S727, not Y705, was associated with attenuated ERK phosphorylation by U0126, suggesting that sorafenib induced S727 dephosphorylation (but not Y705), at least partly, through inhibiting MEK/

ERK phosphorylation.

Sorafenib-induced Y705 dephosphorylation is dependent on PI3K/Akt

A 2-h treatment with the PI3K inhibitor, LY294002, significantly inhibited phosphorylation of Y705, but not S727, in a dose-dependent manner (Figure 2B). This inhibitory effect lasted for 24 h after treatment (Figure 2B), which was in parallel with results of sorafenib treatment. Similarly, we found that knockdown of Akt expression and activation by siRNA also significantly inhibited phosphorylation of Y705, but not S727, in HepG2 and MH cell lines (Figure 2C), suggesting that the PI3K/Akt may be responsible for Y705 phosphorylation in HCC. We further found that sorafenib could inhibit Akt activation in HCC cell lines, mainly at lower concentrations (Figure 2D). Collectively, these results suggested that sorafenib could downregulate Y705 phosphorylation in part by blocking the PI3K/Akt pathway.

Apart from PI3K/Akt, some other effectors, such as SHP2^[23], may also take an active part in Y705 dephosphorylation. Here, we also determined the effects of sorafenib on SHP2. Western blot analysis showed that SHP2 phosphorylation was not affected during the 24 h sorafenib treatment (Figure 2E). Cyclin D1 is an important target gene of STAT3. Our data also showed that the expression of cyclin D1 decreased in HCC cells treated with sorafenib (Figure 2E).

Sorafenib inhibits HCC tumor growth and metastasis *in vivo*

Various manifestations exhibited by our immunocompetent rat model of HCC including local growth, regional invasion, spontaneous metastasis to lungs, lymph nodes, and peritoneal seeding (Figure 3A), as well as molecular signatures like constitutively active JAK2, SHP2, STAT3, Akt and ERK, are similar to those observed in HCC patients.

As shown in Table 1, sorafenib significantly reduced tumor volume (mean tumor volume: vehicle control group, 12795.71 ± 2980.56 mm³; sorafenib late treatment group, 2248.33 ± 971.68 mm³; *P* < 0.001). Furthermore, the primary tumor volume was even lower in the sorafenib early treatment group (mean tumor volume: 351.26 ± 97.58 mm³; *P* < 0.001). Sorafenib treatment (early and late) also suppressed lung and lymph node metastasis compared with the vehicle control (*P* < 0.05; Figure 3A and 3B). Sorafenib treatment reduced peritoneal seeding and bloody ascites, but these differences

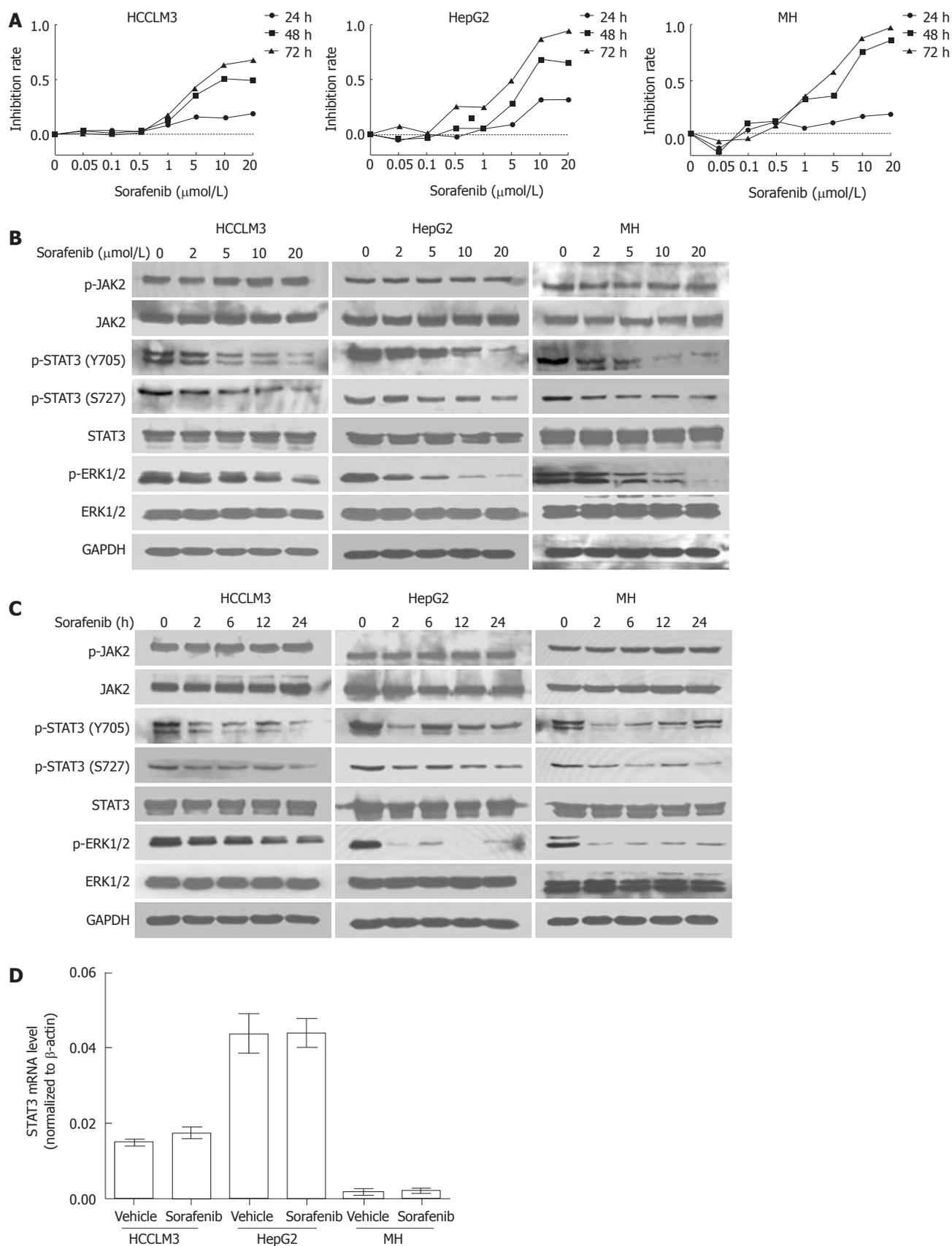


Figure 1 Inhibition of signal transducer and activator of transcription 3 signaling by sorafenib is associated with reduced cell proliferation. A: Sorafenib inhibited cell proliferation in a time and dose-dependent manner, as assessed by the MTT assay; B: Sorafenib inhibited phosphorylation of signal transducer and activator of transcription 3 (STAT3) and extracellular signal-regulated kinase 1/2, but not janus kinase 2 (JAK2), in a dose-dependent manner. Hepatocellular carcinoma (HCC) cells were exposed to sorafenib for 2 h, and proteins were analyzed by Western blot; C: Sorafenib durably inhibited phosphorylation of STAT3 and ERK1/2 but not JAK2. HCC cells were treated with 10 μmol/L sorafenib for different durations, and cell lysates were analyzed by Western blotting; D: Sorafenib did not affect STAT3 mRNA levels in HCC cell lines ($P > 0.05$ for all). After 24-h sorafenib treatment (10 μmol/L), STAT3 mRNA levels were analyzed by qRT-PCR.

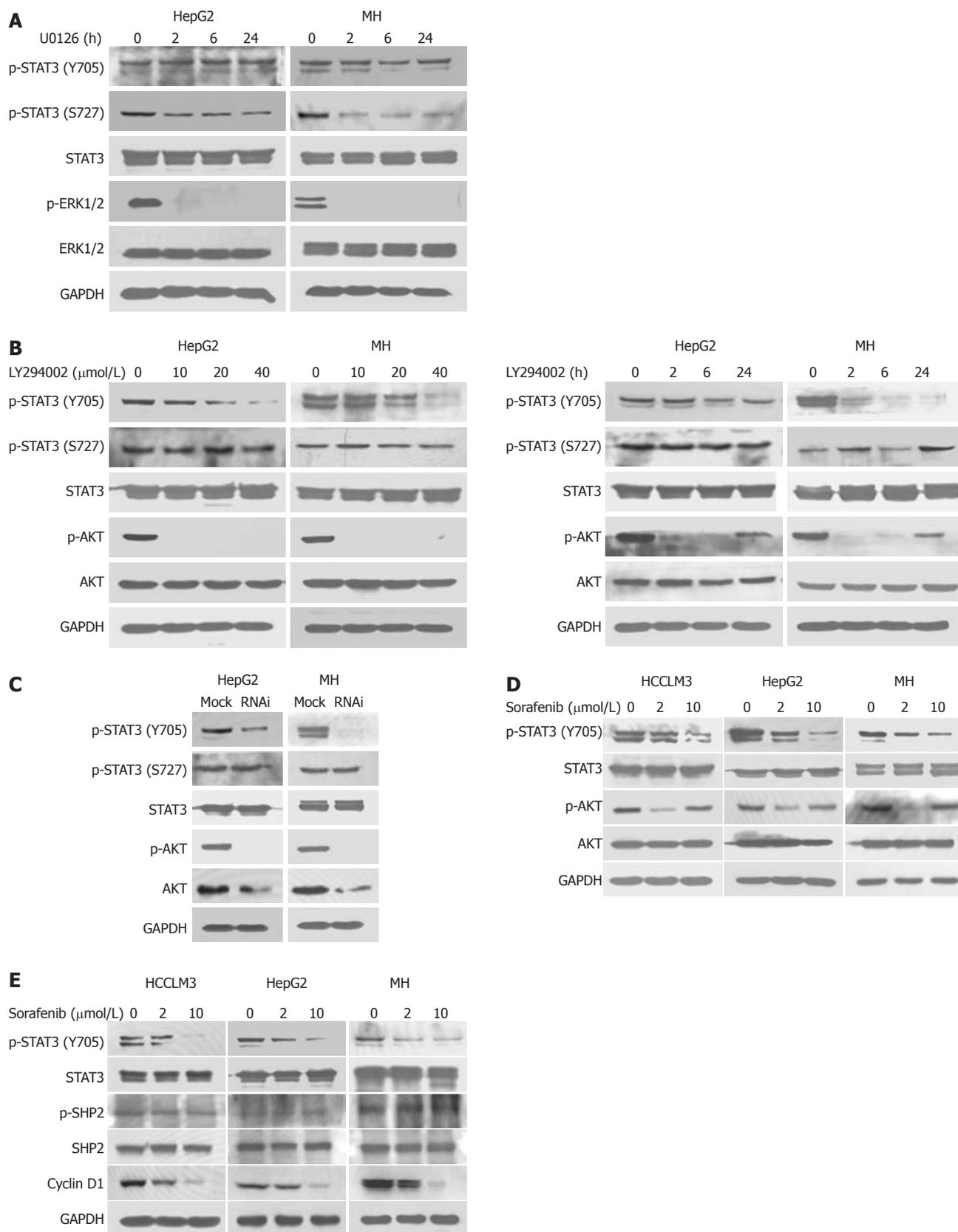
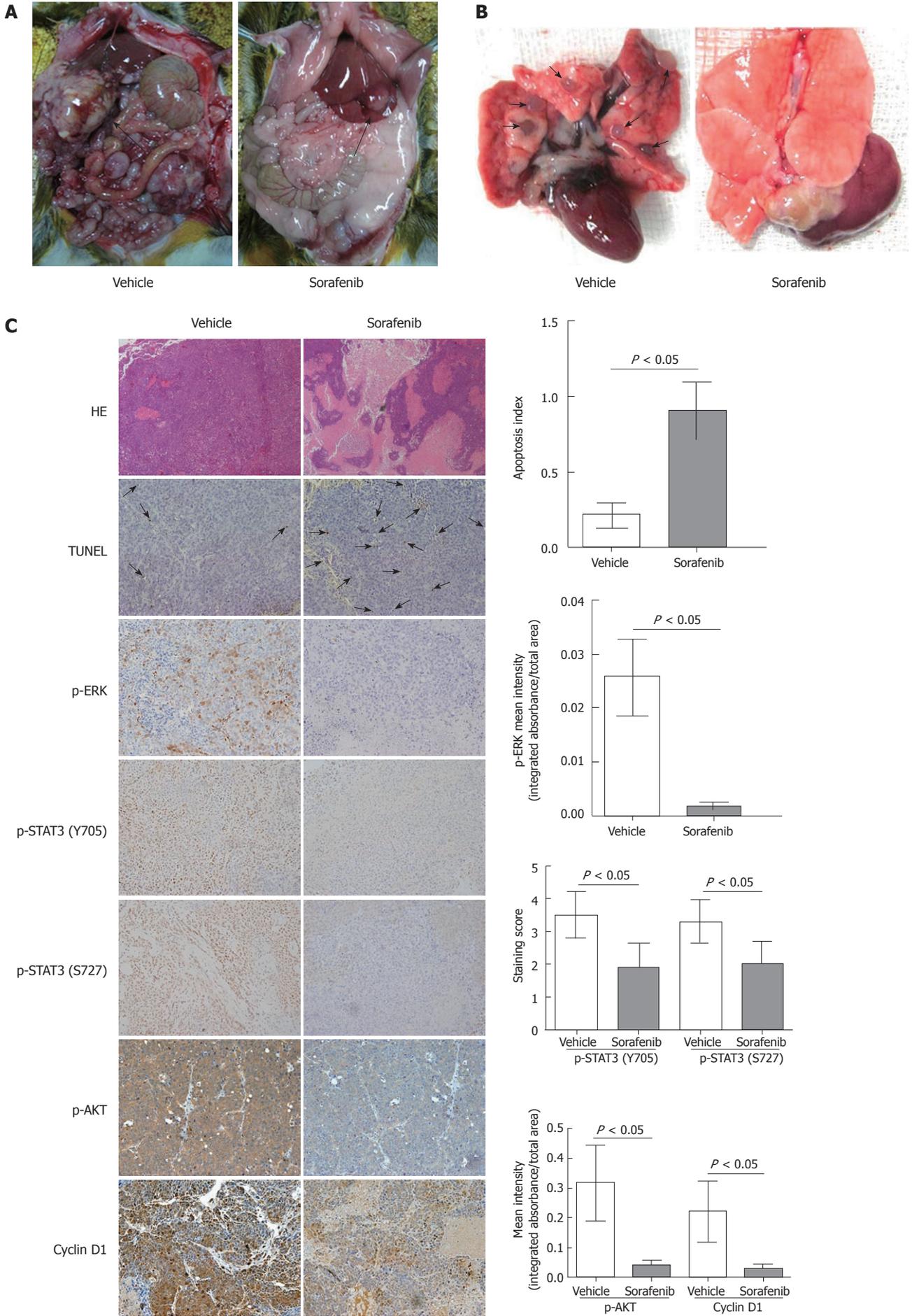


Figure 2 Inhibition of signal transducer and activator of transcription 3 signaling in hepatocellular carcinoma. A: U0126 (20 $\mu\text{mol/L}$) durably inhibited phosphorylation of signal transducer and activator of transcription 3 (STAT3) (S727) in HepG2 and Morris hepatoma (MH) cell lines; B: A 2-h exposure to LY294002 inhibited phosphorylation of STAT3 (Y705) in HepG2 and MH cells in a dose-dependent manner (left). LY294002 (20 $\mu\text{mol/L}$) durably inhibited phosphorylation of STAT3 (Y705) in HepG2 and MH cells (right); C: Akt silencing by siRNA inhibited phosphorylation of STAT3 (Y705, but not S727) in HepG2 and MH cells; D: A 2-h exposure to sorafenib inhibited phosphorylation of Akt, mainly at low concentration (2 $\mu\text{mol/L}$) in HCCLM3, HepG2, and MH cells; E: Sorafenib promoted Y705 dephosphorylation and obviously reduced the expression levels of cyclin D1 regardless of shatterproof 2 (SHP2).



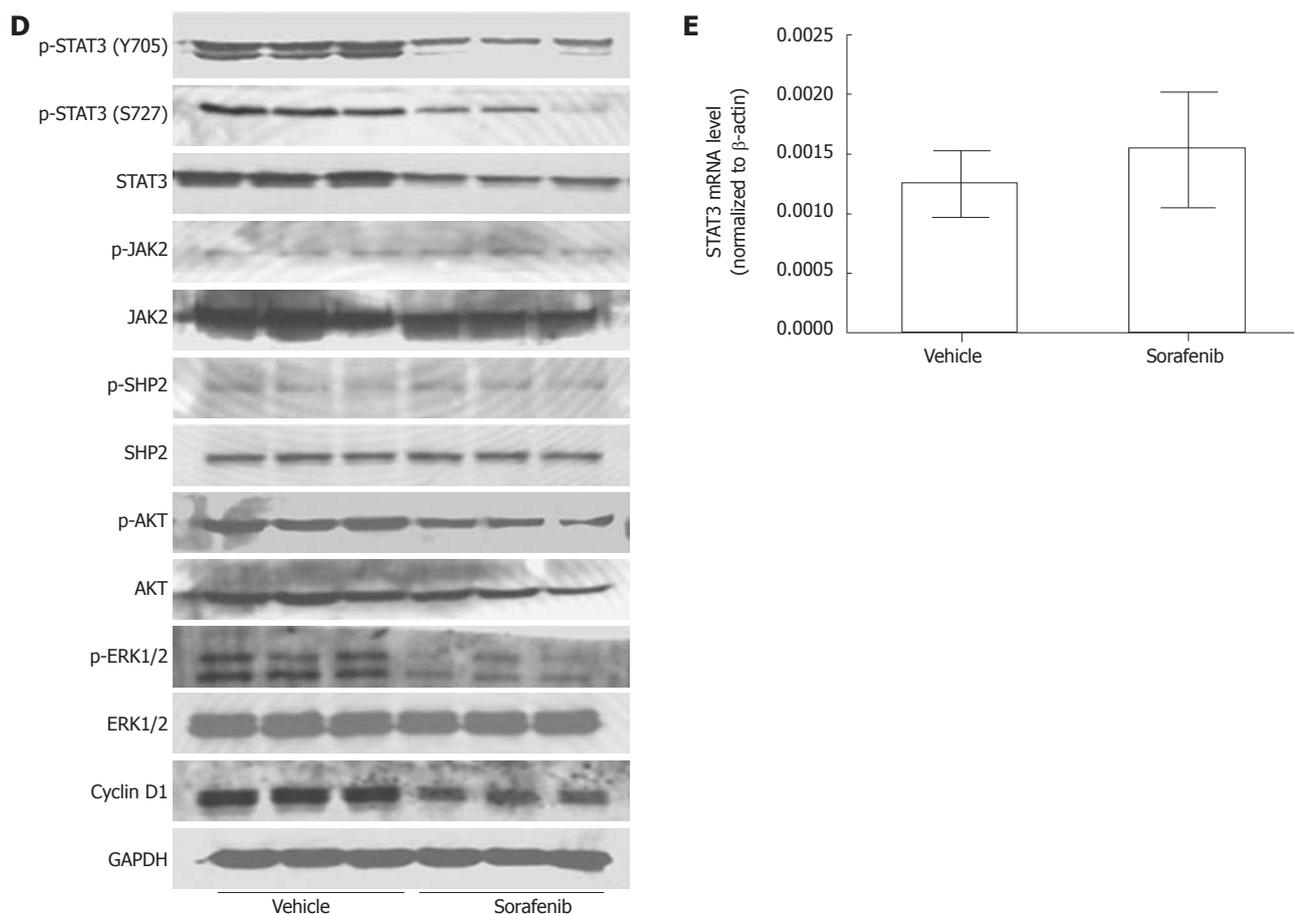


Figure 3 Inhibition of tumor growth and metastasis by sorafenib *in vivo*. Rats with morris hepatoma (MH) tumors were treated with vehicle or sorafenib (30 mg/kg per day) starting on day 17 after tumor implantation. Tumors were removed on day 38 following implantation; A: Tumor growth and abdominal lymph node metastasis were reduced by sorafenib treatment (arrows); B: Sorafenib treatment reduced lung metastasis (arrows); C: Significant tumor necrosis in the sorafenib-treated group was visualized by hematoxylin-eosin staining (magnification, $\times 40$). As shown by TUNEL (arrows), sorafenib also induced tumor cell apoptosis significantly (apoptosis index, 0.217 ± 0.825 vs 0.909 ± 0.189 ; $P < 0.05$; magnification, $\times 200$). Immunohistochemical analysis showed that the expression levels of cyclin D1 and phosphorylation of signal transducer and activator of transcription 3 (STAT3) (Y705 and S727), Akt, and extracellular signal-regulated kinase (ERK) were significantly reduced by sorafenib treatment ($P < 0.05$ for all; magnification, $\times 200$); D: Sorafenib reduced phosphorylation of STAT3, Akt, and ERK, but not Janus kinase 2 and shatterproof 2. Sorafenib also reduced the expression levels of cyclin D1 significantly; E: Sorafenib did not affect STAT3 mRNA levels in rat tumor tissues ($P > 0.05$).

were not significant ($P > 0.05$). Obvious weight loss or death (data not shown) was not observed in sorafenib-treated rats, suggesting that sorafenib was well tolerated and effective in this rat HCC model.

Significant tumor necrosis in the sorafenib treatment group was visualized by hematoxylin-eosin staining (Figure 3C). As shown by TUNEL, sorafenib also induced tumor cell apoptosis significantly (apoptosis index, 0.217 ± 0.825 vs 0.909 ± 0.189 ; $P < 0.05$; Figure 3C). Immunohistochemistry confirmed that phosphorylation of STAT3 (Y705 and S727), Akt and ERK was much lower in the sorafenib treatment group than in the vehicle control ($P < 0.05$ for all; Figure 3C). Western blot analysis also indicated that the dose of sorafenib used in this study was sufficient to inhibit phosphorylation of Akt, ERK and STAT3 in tumors (Figure 3D), which was consistent with its antitumor effects. As with *in vitro* results, sorafenib *in vivo* treatment did not reduce the STAT3 mRNA level ($P > 0.05$; Figure 3E), nor JAK2 and SHP2 phosphorylation (Figure 3D). In addition, expression of STAT3-related cyclin D1 was reduced in the sorafenib

late treatment group ($P < 0.05$; Figure 3C and 3D).

DISCUSSION

STAT3 is constitutively active in most tumor cells and not in normal cells, and hence represents an attractive molecular target^[24]. Here, we showed that sorafenib inhibited STAT3 phosphorylation: (1) at S727 through the MEK/ERK signaling pathway; (2) at Y705 by blocking PI3K/Akt signaling pathways; and (3) independent of JAK and SHP2, thereby inhibiting HCC tumor growth *in vitro* and *in vivo*. Although some studies have reported that sorafenib inhibits STAT3 signaling in some cancers including HCC^[12-16], to the best of our knowledge, this is the first report demonstrating the full inhibition of STAT3 activity on 2 phosphorylating residues by sorafenib acting on distinct underlying mechanisms in HCC (Figure 4).

Phosphorylation of STAT3 at Y705 enables its dimerization, nuclear translocation, DNA binding, and gene transcription^[25], whereas phosphorylation of an-

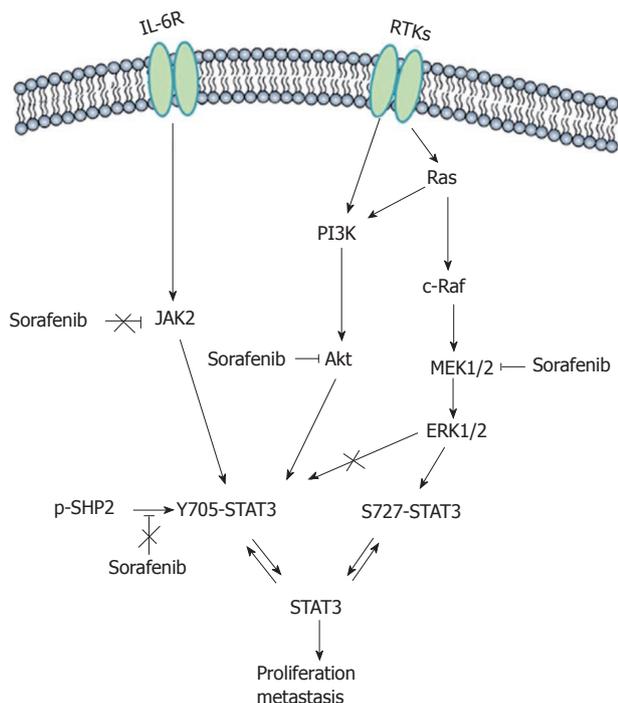


Figure 4 Signaling pathways involved in sorafenib-induced inhibition of signal transducer and activator of transcription 3 phosphorylation. In hepatocellular carcinoma, extracellular signal-regulated kinase-related pathways regulate signal transducer and activator of transcription 3 (STAT3) phosphorylation at S727, and phosphatidylinositol-3-kinase (PI3K)/Akt can be responsible for phosphorylation at Y705. Thus, sorafenib inhibits STAT3 phosphorylation in part through blockade of the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK)/STAT3 and PI3K/Akt/STAT3 signaling pathways, regardless of the Janus kinase 2 and phosphatase shatterproof 2.

other conserved STAT3 residue, S727, enhances STAT3 transcriptional activity^[26]. Cooperation of tyrosine and serine phosphorylation is necessary for the full activation of STAT3^[27]. We found that STAT3 was constitutively phosphorylated at Y705 and S727 in HCC and sorafenib inhibited phosphorylation of the both sites among HCC cells that exhibited different molecular or genetic characteristics. The inhibition was evident as early as 2 h after treatment and lasted for 24 h, suggesting the actions of sorafenib are relatively rapid and prolonged. Furthermore, we identified the potential mechanisms involved in blocking STAT3 signaling.

First, we observed that sorafenib induced STAT3 dephosphorylation at S727 was accompanied by simultaneously blocked MEK/ERK signaling. Similar results were observed with the MEK inhibitor U0126, indicating that ERK-related pathways participate in the regulation of STAT3 in HCC. We reasoned that sorafenib-induced S727 dephosphorylation may be mediated through the MEK/ERK/STAT3 pathway. However, the phosphorylation of both S727 and Y705 were inhibited by sorafenib, whereas U0126 produced no obvious effects on Y705. Therefore, yet unidentified signaling pathways may be involved in sorafenib-induced Y705 dephosphorylation of STAT3.

To clarify this point, we exposed HCC cells to both

the PI3K inhibitor LY294002 and Akt knockdown, and found these treatments resulted in Y705 dephosphorylation. We thus inferred that PI3K/Akt pathway was involved in Y705 dephosphorylation in HCC. Furthermore, we found sorafenib could also inhibit Akt phosphorylation, suggesting that sorafenib could down-regulate Y705 phosphorylation in part by blocking the PI3K/Akt pathway. Given that the PI3K/Akt pathway is an important downstream effector of RTKs^[10], and that RTKs are the major well established targets of sorafenib, we propose that RTK inhibition by sorafenib may be responsible for Akt dephosphorylation in HCC. However, to some extent, Akt dephosphorylation was not correlated well with the inhibition of Y705 phosphorylation by sorafenib. Some other mechanisms, besides PI3K/Akt, may also be involved in Y705 dephosphorylation by sorafenib that need further investigation.

In addition, as a first attempt to elucidate the STAT3 signaling pathways involved in sorafenib-treated HCC cells, we examined the effect of sorafenib on JAK2 and SHP2 activation. JAK2 is a typical non-RTK involved in interleukin-6 intracellular signaling that activates STAT3^[28]. SHP2, a cytoplasmic tyrosine phosphatase, has been recently shown to operate in sorafenib induced STAT3 dephosphorylation in cholangiocarcinoma^[29]. However, both sorafenib-treated and untreated HCC cells contained constitutively activated JAK2 and SHP2, indicating that sorafenib promoted STAT3 dephosphorylation regardless of JAK2 and SHP2. Meanwhile, we did not find that total STAT3 protein expression and mRNA levels were affected.

Because host immune responses play a critical role in hepatocarcinogenesis, invasion and dissemination^[30,31], we chose an immunocompetent model for our *in vivo* studies. In this model, sorafenib increased tumor necrosis, induced tumor cell apoptosis and decreased tumor growth, as compared with control treatment. Importantly, consistent with *in vitro* results, *in vivo* sorafenib treatment significantly inhibited STAT3 activity at both Y705 and S727 with concomitant dephosphorylation of Akt and ERK, while dephosphorylation of JAK2 and SHP2 was not observed. In addition, the expression levels of cyclin D1, an important target gene of STAT3, were also reduced accordant with STAT3 inhibition both *in vitro* and *in vivo*.

In conclusion, we demonstrated that sorafenib is capable of inhibiting HCC growth and metastasis by suppressing STAT3 activity. PI3K/Akt and MEK/ERK signaling pathways may be collectively involved in inhibiting STAT3 activity, independent of SHP2 and JAK2. Our findings not only elucidate an additional potential molecular target of sorafenib, but also provide a rational basis for the development of combination strategies to maximize the HCC response.

COMMENTS

Background

Recent studies have shown that signal transducer and activator of transcription 3 (STAT3) is constitutively activated in hepatocellular carcinoma (HCC) and

may be an attractive molecular target. Sorafenib is a multikinase inhibitor that has shown efficacy against advanced HCC. This study investigated the molecular mechanisms of STAT3 inhibition by sorafenib in HCC.

Research frontiers

STAT3 plays a critical role in transcriptional regulation of genes that are involved in tumor cell proliferation, survival and invasion, thus targeting of STAT3 activation may prove to be an effective approach to controlling HCC. Recently, sorafenib has been shown to suppress tumor growth by decreasing STAT3 phosphorylation in a group of human malignancies including HCC. However, thus far, the exact molecular mechanisms by which sorafenib inhibited STAT3 were not fully elucidated.

Innovations and breakthroughs

Although some studies have reported that sorafenib inhibits STAT3 signaling in some cancers including HCC, to the best of our knowledge, this is the first report demonstrating that sorafenib inhibits growth and metastasis of HCC by the full inhibition of STAT3 activity on 2 phosphorylating residues involved in the distinct underlying mechanism of HCC.

Applications

The findings not only elucidate an additional potential molecular target of sorafenib, but also provide a rational basis for the development of combination strategies to maximize the HCC response.

Terminology

STAT3, an important transcription factor, plays a critical role in transcriptional regulation of genes that are involved in tumor cell proliferation, survival and invasion. STAT3 is constitutively activated in diverse cancer cell types and has been shown to be an essential signaling molecule in tumorigenesis through its transcriptional activity on tumor-associated genes.

Peer review

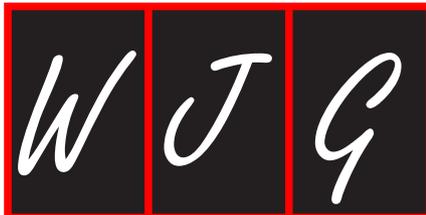
In this manuscript, the authors showed the mechanisms of the suppression of HCC growth by sorafenib *in vivo* and *in vitro*. The study is organized well, the results are interesting and the manuscript is written properly.

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S- Editor Tian L L- Editor Cant MR E- Editor Li JY



Prognostic significance of erythropoietin and erythropoietin receptor in gastric adenocarcinoma

Lin Wang, Hai-Gang Li, Zhong-Sheng Xia, Jian-Ming Wen, Jun Lv

Lin Wang, Hai-Gang Li, Department of Pathology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510120, Guangdong Province, China

Zhong-Sheng Xia, Department of Gastroenterology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510120, Guangdong Province, China

Jian-Ming Wen, Department of Pathology, the First Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

Jun Lv, Department of Nephrology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510120, Guangdong Province, China

Author contributions: Wang L designed the study and wrote the manuscript; Li HG performed the majority of experiments; Xia ZS, Wen JM and Lv J were involved in editing the manuscript.

Correspondence to: Lin Wang, MD, Department of Pathology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510120, Guangdong Province, China. gzdwanlin@126.com

Telephone: +86-20-81332590 Fax: +86-20-81332471

Received: February 9, 2011 Revised: April 20, 2011

Accepted: April 27, 2011

Published online: September 14, 2011

Abstract

AIM: To investigate the expression of Erythropoietin (Epo) and its receptor (EpoR) in gastric adenocarcinoma (GAC) and the correlation with angiogenesis and clinicopathological features.

METHODS: The expressions of Epo, EpoR and vascular endothelial growth factor (VEGF), as well as microvessel density were evaluated in 172 GAC biopsies by immunohistochemical staining. The correlations between these parameters and patient's clinicopathological features were analyzed statistically.

RESULTS: The proportion of Epo and EpoR alterations in GAC was higher than that in adjacent normal mucosa ($P = 0.035$ and 0.030). Epo high-expression was associated with EpoR high-expression, Lauren type, extensive

lymph node metastasis and advanced stage of GAC ($P = 0.018$, 0.018 , 0.004 and 0), while EpoR expression was linked with older age, World Health Organization type, extensive lymph node metastasis and advanced stage ($P = 0.001$, 0.013 , 0.008 and 0.001). VEGF high expression was significantly correlated with EpoR low-expression, Lauren type, extensive lymph node metastasis and advanced stage ($P = 0.001$, 0.001 , 0.001 and 0.007). The expression of Epo or EpoR was associated with microvessel density ($P = 0.004$ and 0.046). On multivariate analysis, only lymph node metastasis, abnormal Epo expression and tumor nodes metastases stage were independently associated with survival. In addition, a strong association with the immunohistochemical expression of EpoR and the angiogenic protein, VEGF, was noted.

CONCLUSION: Increased expression of Epo and EpoR may play a significant role in the carcinogenesis, angiogenesis and progression of GAC. Epo may be an independent prognostic factor.

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Key words: Erythropoietin; Erythropoietin receptor; Gastric adenocarcinoma; Immunohistochemistry; Prognosis

Peer reviewer: Guida Portela-Gomes, MD, PhD, Associate Professor in Experimental Pathology and Assistant Professor in Gastroenterology, Department of Gastroenterology, Faculty of Medicine, University of Lisbon, Rua Domingos Sequeira-128, Estoril 2765-525, Portugal

Wang L, Li HG, Xia ZS, Wen JM, Lv J. Prognostic significance of erythropoietin and erythropoietin receptor in gastric adenocarcinoma. *World J Gastroenterol* 2011; 17(34): 3933-3940 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i34/3933.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i34.3933>

INTRODUCTION

Gastric adenocarcinoma (GAC) is one of the common-

est fatal malignancies in the world. The incidence varies considerably between geographical areas, with a higher incidence in China and other Asian countries than in Western Europe and the United States^[1]. Patients with tumors limited to the mucosa and submucosa have an excellent prognosis, with a 5-year survival rate of over 90% after surgery^[2]. In contrast, the prognosis for patients with advanced cancers is less predictable and generally poorer. At present, therapeutic decisions are based on clinical-pathological parameters, including age, tumor nodes metastases (TNM) stage and histological grade. Although useful, these factors often fail to differentiate more aggressive tumor types from less aggressive types^[3]. As a result, there is an urgent need to find special markers, which are closely related to biologic characteristics, outcome of gastric adenocarcinoma and performance of antigen-specific therapeutic targeting strategy.

Erythropoietin (Epo) is a low-molecular-weight glycoprotein hormonal stimulator of erythropoiesis produced in the fetal liver and subsequently in the adult kidney^[4]. Epo exerts its action through its specific receptor (EpoR), a member of the cytokine receptor superfamily, which is mainly expressed on erythroid colony-forming units^[5]. The presence of an autocrine-paracrine Epo-EpoR system in tumors and the possible effects of Epo on the tumor microenvironment and angiogenesis are consistent with a complex biology for Epo-EpoR signaling in cancer.

Epo is a pleiotropic cytokine that exerts diverse biological effects in many non-hematopoietic tissues. There is increasing evidence suggesting a wider biological role for Epo/EpoR unrelated to erythropoiesis. Angiogenesis, the process by which new blood vessels arise from pre-existing vessels, has been shown to be one of the extra-hematopoietic functions of Epo^[6]. The precise role of Epo in angiogenesis has not been clarified, although many critical functions of Epo have been reported. Endothelial cells from some sources express EpoR^[7]. Moreover, Epo induces endothelial cell proliferation and migration^[7-9] and has been shown to stimulate angiogenesis in rat aortic rings *in vitro*^[10]. The expression of EpoR in tumor vascular endothelium suggests that Epo may affect the tumor microenvironment, perhaps by stimulating tumor angiogenesis^[6]. In addition, Epo was considered to inhibit endothelial cell apoptosis induced by high glucose^[11]. A clinical trial demonstrated that EpoR level correlated with angiogenesis and progression of patients with gastric carcinoma, and that Epo might have a trophic effect on the vasculature of the gastrointestinal tract^[12].

The angiogenic potential of Epo was found to be similar to that of vascular endothelial growth factor (VEGF) when stimulating human adult myocardial endothelial cells^[13]. VEGFs, as the main regulators in angiogenesis, have chemotactic effects and promote the division of cells. Many studies have identified that the expression levels of VEGFs in malignant tumor were strongly correlated with their malignant grading, mi-

crovessel density and prognosis^[14].

In the present study, we investigated the expression of Epo and EpoR in human gastric adenocarcinomas and assessed their possible association with various histopathological features, microvascular density and expression of the *VEGF* gene. Moreover, the association between Epo/EpoR expression and prognosis was evaluated.

MATERIALS AND METHODS

Patient data

Tumor specimens were obtained from 172 patients (108 males and 64 females; mean age 55.9 years; range 24 to 82 years) who underwent surgery for gastric adenocarcinomas from November 2005 through April 2008. None had received prior chemotherapy or radiotherapy. All patients provided written informed consent.

Clinical and pathological records and slides were available for all cases. HE-stained slides of gastric adenocarcinomas were reviewed and one block with tumor and adjacent normal mucosa (ANM) tissue was selected for immunohistochemical staining. Histopathological examination indicated that 64 GAC samples were intestinal type according to Lauren-type classification, 70 were diffusal type and 38 were mixed type, respectively. According to World Health Organization (WHO) histological classification, 142 patients were diagnosed as tubular type, 12 patients were diagnosed as mucinous type, 4 patients were diagnosed as papillary type, and 14 patients were diagnosed as signet ring cell type. According to TNM classification, there were 10 cases at stage I, 18 at stage II, 80 at stage III and 64 at stage IV, respectively.

Processing of specimens and immunohistochemistry

Sections (4 μ m) of tissue blocks were transferred to an adhesive-coated slide. A 3-step immunoperoxidase technique using streptavidin-peroxidase (S-P) was employed for Epo, EpoR and VEGF detection. All the sections were routinely deparaffinized and rehydrated, then the sections were rinsed in phosphate-buffered saline (PBS, pH = 7.4), and were subsequently treated for antigen retrieval (10 min, microwave oven, 800 W, citrate buffer, pH = 6.0). After cooling at room temperature for 20 min, the sections were rinsed in PBS, and then immersed in 3% H₂O₂ for 15 min to block the endogenous enzymes. Thereafter, the sections were incubated with normal goat serum at 37 °C for 15 min to block non-specific antibodies. The primary antibodies were a polyclonal goat antiserum for Epo (polyclonal, N-19, Santa Cruz, United States), a polyclonal rabbit antiserum for EpoR (polyclonal, H-194, Santa Cruz, United States), monoclonal rabbit antiserum for VEGF (monoclonal, ZA-0509, Zhongshan, China) and a mouse monoclonal antibody against the human endothelial cell marker CD34 (monoclonal, ZM-0046, Zhongshan, China), which were diluted at 1:50 dilution. They were used for overnight incubation at 40 °C. The sections were then rinsed in PBS and incubated with biotinylated secondary

antibodies (SP kit, Zhongshan, China) and rinsed in PBS again. After interaction with streptavidin-HRP (SP kit, Zhongshan, China) and then rinsed in PBS, the sections were visualized by reaction with 3, 3'-diaminobenzidine and counterstained with hematoxylin. For both antibodies, adequate positive controls of kidney and liver were used according to the manufacturer's recommendations, and normal goat serum and PBS substituting the primary antibody were used as negative controls.

Scoring of the results

The immunostaining results were evaluated and scored independently by two pathologists without knowledge of the clinical data of patients. Antibody staining results were scored according to the percentage of cytoplasmic positive cells as follows: (-), < 10%; (+), 11%-20%; (++) , > 21%. Only epithelial labeling was scored. Epo, EpoR and VEGF high-expression was defined as > 20% tumor cells with positive staining, whereas low-expression was < 20%.

Microvessel density

The counting of microvessels in GAC was evaluated by a previously reported method^[15]. Briefly, intratumoral microvessel density (IMD) was observed in areas of most intense neovascularization or hotspots in tumor by light microscopy. After determining the area of highest neovascularization, single microvessels were manually counted on a 200 × field by two different observers without knowledge of patient outcome. Any brown-stained endothelial cell or cell cluster clearly separated from adjacent microvessels was considered as a single, countable microvessel.

Statistical analysis

All statistical analyses were performed with SPSS 13.0 software for Windows. The chi-square test was used to assess Epo, EpoR and VEGF expression with clinicopathological characteristics. Univariate analysis by Student's *t* test was used to assess protein expression in relation to angiogenesis of GAC. The survival curve of patients was determined using the Kaplan-Meier method and Cox regression, and statistical evaluation was performed using the log rank test. *P* < 0.05 was considered statistically significant.

RESULTS

Expression of Epo, EpoR in GAC biopsies, and their correlation

Epo and EpoR expression were strong on the cell membrane and in the cytoplasm of GAC cells (Figure 1A and B). However, the signals were weak in ANM cells. Of the sections with ANM, only 12 (6.98%) of 172 cases were stained positively with Epo antibodies and 15 (8.72%) were stained positively with EpoR antibodies. 74.4% (128/172) of carcinomas stained positively for Epo, and 60.5% (104/172) of carcinomas stained positively for EpoR. A stronger positivity was found in GAC than in ANM for both Epo and EpoR ($\chi^2 = 4.434$, *P* =

Table 1 Correlation between expression of erythropoietin, erythropoietin receptor and vascular endothelial growth factor

EpoR	Epo		<i>P</i> value	VEGF		<i>P</i> value
	Low expression	High expression		Low expression	High expression	
0	24	44	0.018	22	46	0.001
1	20	84		60	44	

EpoR: Erythropoietin receptor; Epo: Erythropoietin; VEGF: Vascular endothelial growth factor.

0.035 and $\chi^2 = 4.719$, *P* = 0.030, respectively).

Epo and EpoR were detected in concomitant expression in the same portion of tumor cell on serial sections. Epo expression was found to be closely related to the expression of EpoR in tumor (Table 1).

Epo, EpoR and VEGF expression in correlation with clinicopathological parameters in GAC

Epo high-expression (74.4%, 128/172) was significantly correlated with Lauren type, extensive lymph node metastasis and advanced stage of GAC. However, no significant correlation between Epo high-expression and older age, gender or WHO type was observed. High-expression of EpoR (60.5%, 104/172) was found to have a significant positive correlation with older age, WHO type, extensive lymph node metastasis and advanced stage. No significant correlation between EpoR high-expression and gender or Lauren type was observed (Table 2).

Positive VEGF expression was found in 52.3% of (90/172) tumor tissues (Figure 1C). VEGF high-expression was significantly correlated with Lauren type, extensive lymph node metastasis and advanced stage of GAC. However, no significant correlation between VEGF high-expression and gender or WHO type was observed (Table 2). VEGF and EpoR were detected in concomitant expression in the same portion of tumor cell on serial sections. VEGF expression was found to be closely related to the expression of EpoR in tumor (Table 1).

Relationship of Epo, EpoR and VEGF expression with microvessel density

Microvessels in GAC, indicated by CD34 immunostaining, were observed to be scattered in the tumor cell nests (Figure 1D), and were scored as IMD. The correlation between IMD and protein expression in GAC is shown in Figure 2. Mean IMD value was 31.62 ± 14.01 and 31.57 ± 14.01 in the cases with high Epo or EpoR expression, respectively, which were significantly higher than those in the cases with low Epo or EpoR expression (24.82 ± 11.74 and 27.29 ± 13.06 , *P*-values were 0.004 and 0.046, respectively). We examined the relationship between other clinicopathological characteristics and IMD in tumors; however, no significant correlations were found. Therefore, angiogenesis in GAC seemed to be independent of gender, age, WHO type, lymph node metastasis, TNM stage and VEGF expression in patients.

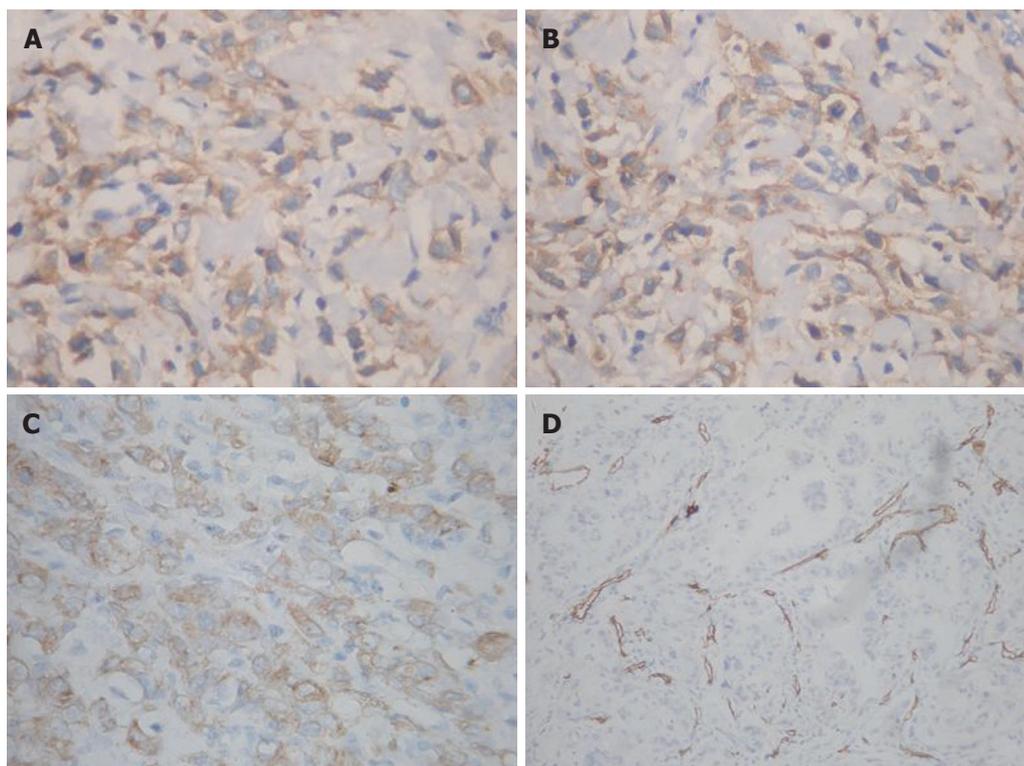


Figure 1 Erythropoietin, erythropoietin receptor, vascular endothelial growth factor and CD34 expression in biopsies from gastric adenocarcinoma patients. A, B: Erythropoietin and erythropoietin receptor positive immunostaining signals were localized in the cytoplasm of tumor cells with a scattered distribution pattern in most cases; C: Vascular endothelial growth factor immunoreactivity was detected in the cytoplasm of gastric adenocarcinoma. Original magnification: $\times 200$; D: Microvessels in tumor were highlighted by staining endothelial cells for anti-CD34. Any brown-staining endothelial cell or cell cluster that was clearly separated from adjacent microvessels was considered as a single, countable microvessel. Original magnification: $\times 100$.

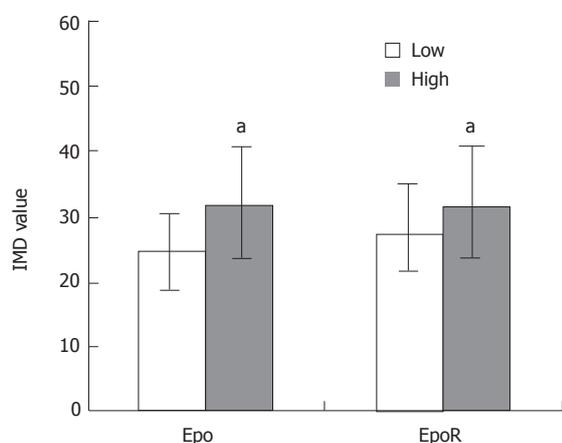


Figure 2 Correlation between intratumoral microvessel density and protein expression in gastric adenocarcinoma. The mean intratumoral microvessel density in the high-expressed erythropoietin (Epo) and erythropoietin receptor (EpoR) cases was significantly increased compared to that of low-expressed cases ($^aP < 0.05$). IMD: Intratumoral microvessel density.

Association of Epo, EpoR and VEGF expression with survival of GAC patients

In this study, 150 cases had adequate follow-up data for the final analysis, whereas 22 cases were excluded from survival analysis because the patients were lost to follow-up. The 150 cases were followed up for 1 to 56 mo (mean 27.4 mo), and 98 patients (65.3%) died of tumor during this period.

Univariate survival analysis demonstrated that patients with high Epo immunoreactivity, had a significantly worse overall survival compared to patients with low Epo immunoreactivity (log rank test: $P = 0.0167$, Figure 3B). Median survival time of patients with low Epo expression was 45 mo, which was much longer than patients with high positivity whose median survival time was 14 mo.

Patient survival was also associated with lymph node metastasis and advanced clinical stage ($P = 0.000$ and $P = 0.0006$, Figure 3A, 3C). The difference in survival rate was not significant between the GAC patients with different EpoR expressions ($P = 1.0000$). When patients were stratified according to tumor high-expression of both Epo and EpoR (84 cases), the difference in survival rate was not significant between the patients with both high-expression of Epo and EpoR compared with patients with low-expression of both Epo and EpoR or single protein high-expression ($P = 0.5306$). In addition, the difference in survival rate was not significant between the patients with different VEGF expression status in tumor cells ($P = 0.9756$).

On multivariate analysis, only lymph node metastasis (hazard ratio, 1.672; 95% confidence interval, 1.2199-2.2916, $P = 0.0014$), abnormal Epo expression of GAC tumor cells (hazard ratio, 1.6517; 95% confidence interval, 0.9979-2.7338, $P = 0.0509$) and TNM stage (hazard ratio, 1.4292; 95% confidence interval, 1.0403-1.9636, $P = 0.0276$)

Table 2 Correlation between protein expression and clinicopathological parameters of gastric adenocarcinoma patients (χ^2 test)

	Case (n = 172)	Epo			EpoR			VEGF		
		Low	High	P value	Low	High	P value	Low	High	P value
Age group				0.557			0.001			0.115
< 60	107	29	78		53	54		46	61	
≥ 60	65	15	50		15	50		36	29	
Gender				0.19			0.822			0.428
Male	108	24	84		42	66		54	54	
Female	64	20	44		26	38		28	36	
Lauren type				0.018			0.475			0.001
Intestinal	64	22	42		28	36		42	22	
Diffusal	70	10	60		28	42		28	42	
Mixed	38	12	26		12	26		12	26	
WHO type				0.07			0.013			0.734
Tubular	142	40	102		64	78		70	72	
Mucinous	12	2	10		2	10		4	8	
Papillary	4	2	2		0	4		2	2	
Signet-ring cell	14	0	14		2	12		6	8	
Lymph node involvement				0.004			0.008			0.011
No	34	10	24		16	18		20	14	
1	42	18	24		8	34		26	16	
> 1	96	16	80		44	52		36	60	
TNM stage				0			0.041			0.007
I	10	4	6		4	6		2	8	
II	18	8	10		6	12		14	4	
III	80	28	52		24	56		32	48	
IV	64	4	60		34	30		34	30	
VEGF expression				0.298			0.001			
Low	82	18	64		22	60				
High	90	26	64		46	44				

EpoR: Erythropoietin receptor; Epo: Erythropoietin; VEGF: Vascular endothelial growth factor; WHO: World Health Organization.

Table 3 Multivariate analysis (cox regression model)

Variable	B	SE	Wald	df	Sig	R
LN metastasis	0.514	0.161	10.212	1	0.001	0.096
Epo	0.502	0.257	3.810	1	0.051	0.045
TNM	0.357	0.162	4.856	1	0.028	0.056

Epo: Erythropoietin; LN: Lymph node.

were independently associated with survival (Table 3).

DISCUSSION

Epo is used to manage anemia in cancer patients^[16,17]. Patients treated with recombinant human Epo not only have increased levels of hemoglobin, but their performance status also improves significantly, and they enjoy a significantly enhanced quality of life^[17]. The expression of the Epo-EpoR system in tumor vascular endothelium suggests that this system may affect the tumor microenvironment, perhaps by stimulating tumor angiogenesis^[6,18,19]. In this study, we found a correlation between the expression of Epo or EpoR and CD34 in vascular endothelial cells using immunohistochemistry. Compared with that in cases with low Epo or EpoR expression, microvessel density in tumor in the cases with high Epo or EpoR expression was significantly higher. These results suggested that the Epo-EpoR system is an important factor in gastric adenocarcinoma angiogenesis. The possible effects of Epo/EpoR

on tumor angiogenesis are consistent with the complex biology of Epo-EpoR signaling in cancer. Thus, the potential role of the Epo-EpoR system in angiogenesis may be considered as a subsidiary of its possible function in improving overall tissue oxygenation^[11].

Epo and EpoR were detected in solid tumors of the brain^[20], breast^[21,22], kidney^[23], female genital tract^[24], squamous cell carcinoma of the uterine cervix and tongue^[25,26], and were implicated in tumor growth, invasion and metastasis. In the present study, we clearly demonstrated the presence of Epo and EpoR proteins in a series of GAC by immunohistochemistry. Epo and EpoR were highly expressed in GAC as compared to ANM. These results suggest that Epo and EpoR may be involved in the pathogenesis of GAC.

Nakamatsu *et al*^[27] demonstrated that Epo was not detectable in normal or cirrhotic liver tissues without tumors using radioimmunoassay, while immunoreactive EpoR was detectable in the endothelium of intervening vessels of all hepatic tumors using immunohistochemistry. The reason for selective expression of EpoR in the tumor vessels is unclear. The immature nature of tumor vessels compared with mature hepatic vessels may be related to the selective expression of EpoR. In the present study, Epo expression was significantly associated with Lauren type, while EpoR expression was significantly associated with WHO type. These results may suggest that the signaling pathway of Epo is different from that of EpoR in GAC. The most interesting finding of this

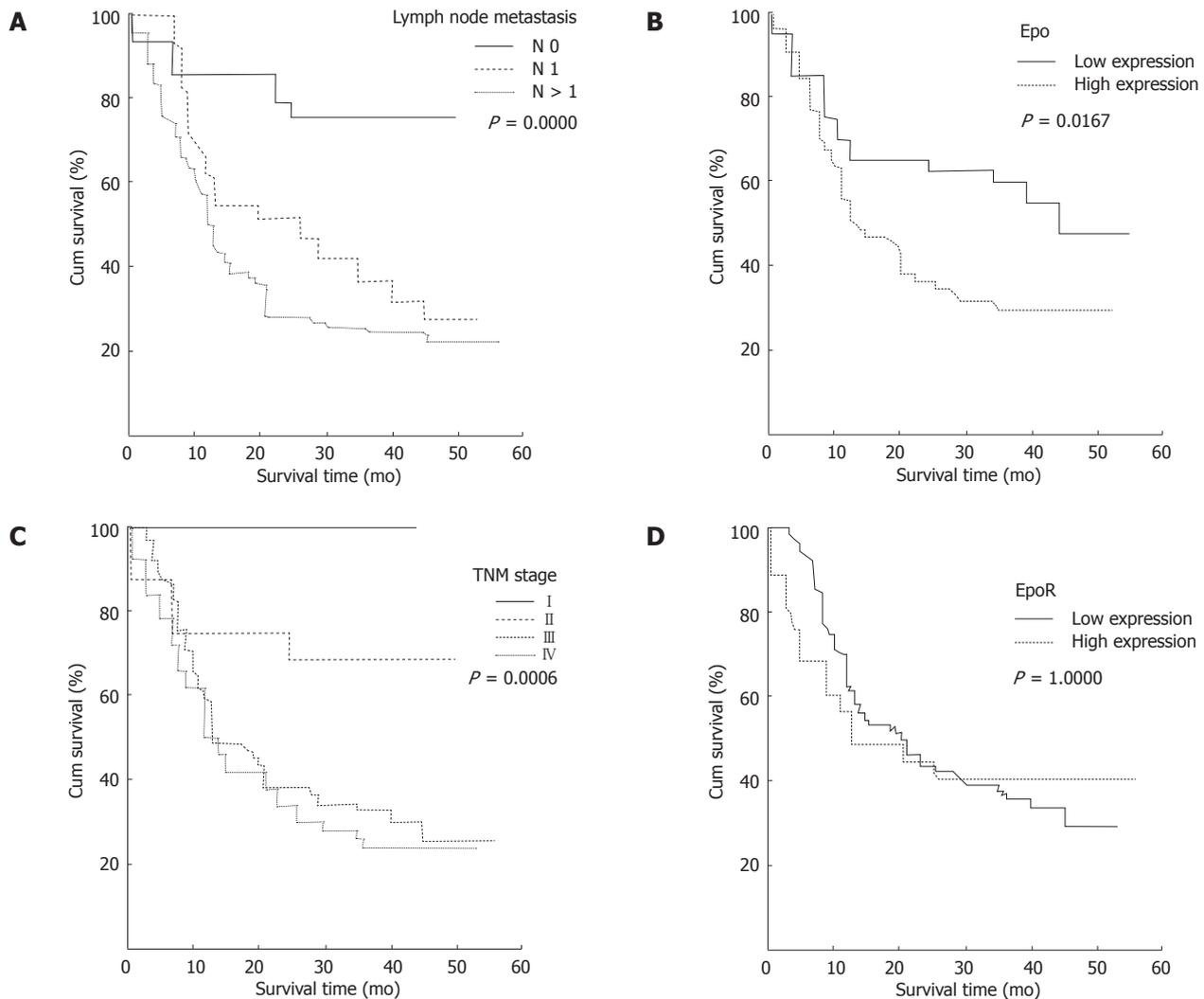


Figure 3 Kaplan-Meier survival analyses of gastric adenocarcinoma patients. A: Kaplan-Meier curve showing that patients with lymph node metastasis ($N \geq 1$) have a lower survival rate than those without lymph node metastasis (N0); B: A significant difference in survival rate was found between gastric adenocarcinoma (GAC) patients with high and low erythropoietin expression in tumor; C: A significant difference in survival rate was found between different clinical stages; D: No significant difference in survival rate was found between GAC patients with high and low erythropoietin receptor expression in tumor.

study is the association between EpoR expression and age. EpoR expression was higher in older patients than in younger patients. The reason for such selective expression in GAC is not clear.

Phenotypic traits of malignant tumor may be caused by microenvironmental selection pressure during carcinogenesis. Hypoxia can drive a tumor towards more malignant phenotypes^[28], such as invasion and migration of tumor cells, which may be the basis for lymph node metastasis and distant metastasis. Our previous study demonstrated that increased levels of Epo and EpoR promote invasiveness and lymph node metastases of human tongue squamous cell carcinoma^[26]. In the current study, the observed relationship between high Epo or EpoR expression and lymph node metastasis as well as advanced tumor stage indicated that Epo or EpoR might be possible mediators that contribute to the extensive lymph node metastasis and accelerated progression of GAC.

In the Epo-EpoR system, both pathways were important in executing their multifunctions. The co-expression

of Epo and EpoR in tumor cells suggests the involvement of an autocrine Epo-EpoR signaling loop. However, in the survival analysis, although high Epo expression in tumor cells was closely associated with lower survival rate of patients with GAC, high EpoR expression was not. Moreover, on multivariate analysis of prognostic factors in GAC, only high Epo expression emerged as an independent factor which influenced the prognosis of GAC patients. These results indicate that the tumor-cell-derived Epo-EpoR system may contribute, in part, to metastasis of lymph node and progression of GAC through an autocrine pathway. Epo is an independent prognostic factor in predicting the prognosis of GAC.

Epo is a hypoxia-inducible stimulator of erythropoiesis. Acting *via* its receptor (EpoR), Epo up-regulates bcl-2 and inhibits apoptosis of erythroid cells, and then, rescues neurons from hypoxic damage^[29]. Although we did not detect expression and regulation of bcl-2 in GAC tumor cells, it is reasonable to believe that only abnormal Epo expression, including absent and endocytosed

expression resulting in dysfunction of Epo complexes, plays important roles in metastasis and progression of GAC. Yasuda *et al.*^[30] blocked Epo signaling in xenografts of stomach choriocarcinoma in nude mice by ip injections of EpoR antagonist, and found inhibition of angiogenesis and tumor cells, and destruction of tumor masses. The mechanism of Epo and EpoR protein over-expression in GAC merits further investigation to establish these proteins as therapeutic targets.

The expression, regulation and biological significance of the Epo/EpoR system and VEGF in malignant tumor are complicated. It is well known that VEGF induced by hypoxia might mediate hypoxia-initiated angiogenesis^[31], while hypoxia could rapidly activate hypoxia inducible factor-3 α (HIF-3 α) gene expression^[32]. Under hypoxic stimulation, HIF-1 has been shown to activate the transcription of Epo^[30]. Epo and EpoR gene expressions are under the direct control of hypoxia through stabilization of the HIF1 α transcription factor that binds to the hypoxia-responsive element of the *Epo* gene^[33]. Nakano *et al.*^[34] demonstrated the important roles of the vascular EpoR system, including induction of postischemic angiogenesis, secretion of VEGF from ischemic muscle and BM-derived cells, and enhancement of VEGFR-2 in ischemic tissue. These results suggested that EpoR might be important for VEGF secretion and angiogenesis.

In the current study, VEGF expression was significantly correlated with Lauren type, lymph node metastasis, TNM stage and EpoR reactivity. To the best of our knowledge, this is the first study to demonstrate a tight relationship between EpoR and VEGF expression in GAC. High EpoR reactivity was linked with low VEGF expression, possibly as a result of an intracellular signal overflow. Thus, we infer a rivalry action existing between EpoR and VEGF when combining with the receptor on tumor surface.

COMMENTS

Background

Gastric adenocarcinoma is one of the commonest fatal malignancies worldwide especially in China and other Asian countries. The prognosis for patients with advanced cancers is generally poor. There is an urgent need to find special markers closely related to tumor outcome and therapy.

Research frontiers

Erythropoietin (Epo) and its specific receptor (EpoR) are members of the cytokine receptor superfamily. The possible effects of Epo on tumor microenvironment and angiogenesis are consistent with the complex biology of Epo-EpoR signaling in cancer. However, the precise role of Epo-EpoR in gastric adenocarcinoma is still not clear. In this study, the authors demonstrate that the Epo-EpoR system is an important factor in gastric adenocarcinoma angiogenesis.

Innovations and breakthroughs

Epo and the EpoR have been implicated in some solid tumors. However, there are very few data in the medical literature regarding the role of Epo and the EpoR in the carcinogenesis or progression of gastric adenocarcinoma. This study demonstrates that Epo and EpoR are over-expressed in gastric adenocarcinoma. Furthermore, the authors suggest that Epo or EpoR might be possible mediators that contribute to the extensive lymph node metastasis and accelerated progression of gastric adenocarcinoma.

Applications

By understanding the important roles of the Epo-EpoR system in metastasis

and progression of gastric adenocarcinoma, this study may represent a future strategy for therapeutic intervention in the treatment of patients with gastric adenocarcinoma.

Terminology

Epo is a pleiotropic cytokine that exerts diverse biological effects in many non-hematopoietic tissues such as stimulating tumor angiogenesis. Epo exerts its action through EpoR.

Peer review

The authors examined the expression of Epo and EpoR in gastric adenocarcinoma and the correlation with angiogenesis and clinicopathological features. It revealed that Epo and EpoR system provides advantage to the carcinogenesis, angiogenesis, and malignant progression of gastric adenocarcinoma. The results are interesting and indicate that Epo might be an independent prognostic factor in gastric adenocarcinoma.

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S- Editor Sun H L- Editor Webster JR E- Editor Li JY

Interleukin-10 gene polymorphisms and hepatocellular carcinoma susceptibility: A meta-analysis

Yong-Gang Wei, Fei Liu, Bo Li, Xi Chen, Yu Ma, Lv-Nan Yan, Tian-Fu Wen, Ming-Qing Xu, Wen-Tao Wang, Jia-Yin Yang

Yong-Gang Wei, Fei Liu, Bo Li, Xi Chen, Yu Ma, Lv-Nan Yan, Tian-Fu Wen, Ming-Qing Xu, Wen-Tao Wang, Jia-Yin Yang, Department of Liver and Vascular Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: Wei YG and Liu F designed the study, collected and analyzed the data and wrote the manuscript; Li B collected and analyzed the data and wrote the manuscript; Chen X and Ma Y collected and analyzed the data; Yan LN analyzed the data and contributed to the discussion; Wen TF and Xu MQ revised the manuscript; Wang WT and Yang JY contributed to the discussion; Wei YG and Liu F contributed equally to this work.

Supported by The National Natural Science foundation of China, No. 30901720

Correspondence to: Bo Li, MD, Department of Liver and Vascular Surgery, West China Hospital, Sichuan University, 37 Guo Xue Road, Chengdu 610041, Sichuan Province, China. cdlibo688@163.com

Telephone: +86-28-85422476 Fax: +86-28-85423724

Received: December 23, 2010 Revised: April 19, 2011

Accepted: April 26, 2011

Published online: September 14, 2011

Abstract

AIM: To assess the association between *Interleukin-10* (IL-10) gene IL-10-1082 (G/A), IL-10-592(C/A), IL-10-819 (T/C) polymorphisms and hepatocellular carcinoma (HCC) susceptibility.

METHODS: Two investigators independently searched the Medline, Embase, China National Knowledge Infrastructure, and Chinese Biomedicine Database. Summary odds ratios (ORs) and 95% confidence intervals (95% CIs) for IL-10 polymorphisms and HCC were calculated in a fixed-effects model (the Mantel-Haenszel method) and a random-effects model (the DerSimonian and Laird method) when appropriate.

RESULTS: This meta-analysis included seven eligible

studies, which included 1012 HCC cases and 2308 controls. Overall, IL-10-1082 G/A polymorphism was not associated with the risk of HCC (AA vs AG + GG, OR = 1.11, 95% CI = 0.90-1.37). When stratifying for ethnicity, the results were similar (Asian, OR = 1.12, 95% CI = 0.87-1.44; non-Asian, OR = 1.10, 95% CI = 0.75-1.60). In the overall analysis, the IL-10 polymorphism at position -592 (C/A) was identified as a genetic risk factor for HCC among Asians; patients carrying the IL-10-592*C allele had an increased risk of HCC (OR = 1.29, 95% CI = 1.12-1.49). No association was observed between the IL-10-819 T/C polymorphism and HCC susceptibility (TT vs TC + CC, OR = 1.02, 95% CI = 0.79-1.32).

CONCLUSION: This meta-analysis suggests that IL-10-592 A/C polymorphism may be associated with HCC among Asians. IL-10-1082 G/A and IL-10-819 T/C polymorphisms were not detected to be related to the risk for HCC.

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Key words: Hepatocellular carcinoma; Interleukin-10; Gene polymorphism; Meta-analysis

Peer reviewer: Dario Conte, Professor, GI Unit - IRCCS Osp. Maggiore, Chair of Gastroenterology, Via F. Sforza, 35, Milano 20122, Italy

Wei YG, Fei Liu, Li B, Chen X, Ma Y, Yan LN, Wen TF, Xu MQ, Wang WT, Yang JY. *Interleukin-10* gene polymorphisms and hepatocellular carcinoma susceptibility: A meta-analysis. *World J Gastroenterol* 2011; 17(34): 3941-3947 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i34/3941.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i34.3941>

INTRODUCTION

Hepatocellular carcinoma (HCC), which is the fifth most common cancer and the third leading cause of cancer-

related death worldwide, is a global health problem^[1,2]. The estimated annual number of cases exceeds 500 000, with a mean annual incidence of around 3%-4%^[3]. Patients with HCC have a poor prognosis, with a five-year survival rate of 5% in developing countries in 2002^[1] because of the lack of effective therapy in most patients^[4]. Aetiologically, carcinogenesis of HCC is a complex, multistep and multifactor process, in which many factors are implicated. As we know, chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is the most well-established environmental risk factor for HCC worldwide. However, only a fraction of HBsAg carriers eventually develop HCC and only 2.5% of HCV infected individuals develop HCC later in life^[5]. The exact mechanism of hepatocarcinogenesis is still incompletely understood, and the risk factors for HCC still need to be further elucidated.

Interleukin-10 (IL-10), whose encoding gene is located on chromosome 1 (1q31-1q32), is an immunoregulatory cytokine produced by Th2 cells, monocytes/macrophages, and regulatory T cells. It plays an anti-inflammatory action by inhibiting the synthesis of cytokines such as IL-1 α , IL-1 β , IL-6, IL-8, IL-12 and tumor necrosis factor- α (TNF- α) in activated macrophage and interferon gamma (IFN γ) by T cells^[6], and it also has some antifibrotic properties^[7]. The risk for HCC increases with the severity of hepatic inflammation and chronic inflammation developing through the action of various inflammatory mediators is known as a cofactor of carcinogenesis^[8,9]. It also has been suggested that chronically HCV-infected patients who received either short- or long-term therapy with recombinant IL-10 showed a decrease in hepatic inflammation and fibrosis^[10,11]. The production of cytokines (including IL-10) is under genetic control and varies among individuals as a function of polymorphisms within the regulatory regions of the various genes that determine the transcriptional activation^[12-15]. The promoter of the *IL-10* gene contains three biallelic polymorphisms at positions -1082 (base G to A, dbSNP No. rs1800896), -819 (base C to T, dbSNP No. rs1800871), and -592 (base C to A, dbSNP No. rs1800872) from the transcription start site, and these influence the capacity to produce IL-10^[16]. *IL-10* gene polymorphisms have been reported to be associated with breast cancer^[17], cervical cancer^[18], multiple myeloma^[19], cutaneous malignant melanoma^[20], oral squamous cell carcinoma^[21] and gastric carcinoma^[22].

Over the last decade, a number of studies have assessed the association between the *IL-10* gene polymorphism and HCC risk in different populations; however, the results are inconsistent and inconclusive^[23-29]. Different methodologies have been used, but, in particular, most of the studies used a small sample size and it is therefore not surprising that there has been a lack of replication in the various studies. By using all the available published data to increase the statistical power, it was hypothesized that a meta-analysis might allow plausible candidate genes to be excluded and causative genes to be

identified with reliability. We have therefore taken a meta-analysis in which all the published case-control studies are processed to confirm whether the polymorphisms of *IL-10* gene promoter increased the risk of HCC.

MATERIALS AND METHODS

Literature search strategy

We searched the PubMed, Embase, China National Knowledge Infrastructure and Chinese Biomedicine databases for all articles on the association between *IL-10* polymorphisms and HCC risk (last search update 30th November 2010). The following key words were used: "Interleukin-10" or "IL-10", "liver cancer" or "hepatocellular carcinoma", and "polymorphism" or "variant". The search was without restriction on language, conducted on human subjects. The reference lists of reviews and retrieved articles were hand searched at the same time. We did not consider abstracts or unpublished reports. If more than one article was published by the same author using the same case series, we selected the study where the most individuals were investigated.

Inclusion and exclusion criteria

We reviewed abstracts of all citations and retrieved studies. The following criteria were used to include published studies: (1) evaluating the association between *IL-10* gene polymorphism and HCC; (2) case-control design; and (3) sufficient genotypes data were presented to calculate the odds ratio (OR) and confidence interval (CI). Participants could be of any age. Studies were excluded if one of the following existed: (1) the design was based on family or sibling pairs; (2) the genotype frequency was not reported; or (3) there was insufficient information for extraction of data. The HCC definition used in the individual studies was accepted and we documented this in our analysis.

Data extraction

All data were extracted independently by two reviewers (Wei Y and Liu F) according to the inclusion criteria listed above. Disagreements were resolved by discussion between the two reviewers. The following characteristics were collected from each study: first author, year of publication, country of the first or corresponding author, ethnicity, number of cases and controls, genotyping methods, matching variables, and evidence of Hardy-Weinberg equilibrium (HWE) (Table 1). Different ethnicities descents were categorized as Asian and non-Asian.

Statistical analysis

The statistical analysis was conducted using STATA 11.0 (Stata Corp LP, College Station, TX, United States); $P < 0.05$ was considered statistically significant. HWE in the controls was tested by the chi-square test for goodness of fit, and a $P < 0.05$ was considered as significant disequilibrium. For *IL-10*-1082 polymorphism and *IL-10*-819 polymorphism, analyses were performed for AA vs AG + GG

genotype and TT *vs* TC + CC genotype, respectively. Because lack of separated AG and GG genotype frequency for two studies, A allele *vs* G allele could not be performed for IL-10-1082 polymorphism. Similarly, T allele *vs* C allele could not be performed for IL-10-819 polymorphism. For IL-10-592 polymorphism, we examined the contrast of the allelic effect of C (minor allele) *vs* A (common allele), and also examined the contrast of CC *vs* AC + AA genotypes. These contrasts correspond to the recessive and dominant effects of the C allele, respectively.

The OR and 95% CI were estimated for each study in a random-effects model or in a fixed-effects model. Heterogeneity among studies was examined with the χ^2 -based Q testing and I^2 statistics^[30]. $P < 0.1$ was considered significant for the χ^2 -based Q testing and I^2 was interpreted as the proportion of total variation contributed by between-study variation. If there was a significant heterogeneity ($P < 0.1$), we selected a random-effects model (the DerSimonian and Laird method) to pool the data. If not, we selected a fixed-effects model (the Mantel-Haenszel method) to pool the data. Publication bias was examined with funnel plots and with the Begg's and Egger's tests^[31-33]. If there is evidence of publication bias, the funnel plot is noticeably asymmetric. For the Begg's and Egger's tests the significance level was set at 0.05.

RESULTS

Studies included in the meta-analysis

There were 72 papers relevant to the search words. *Via* the steps of screening the title and reading the abstract, eight studies were identified^[23-29,34]. Of these, one study was excluded because of no allele or genotype frequency; thus, seven eligible studies^[23-29] which included 1012 HCC cases and 2308 controls were found to match our inclusion criteria. Four of seven publications indicated HWE in their subjects^[23-25,27]; we calculated HWE for the remained three publications and found that only Migita's study relating to IL-10-1082 polymorphism was inconsistent with Hardy-Weinberg disequilibrium ($P = 0.004$). The flow chart of selection of studies and reasons for exclusion is presented in Figure 1. Studies had been carried out in China, Japan, Korea, Tunisia and the United States. Characteristics of studies included in the meta-analysis are presented in Tables 1 and 2.

Evaluation of IL-10 gene polymorphisms and association with HCC

There were six case-control studies^[23,24,26-29] which had been performed to study the IL-10-1082 A/G polymorphism and HCC risk. Of these, four studies were performed in Asians, one study in Americans and one in Africans. Because adequate sample populations were unavailable for the American and African groups, we performed ethnicity-specific meta-analysis in the Asian and non-Asian populations. The combined results based on all studies showed that there was no association between IL-10-1082 A/G polymorphism and HCC risk (AA *vs* AG + GG, OR = 1.11, 95% CI = 0.90-1.37). When

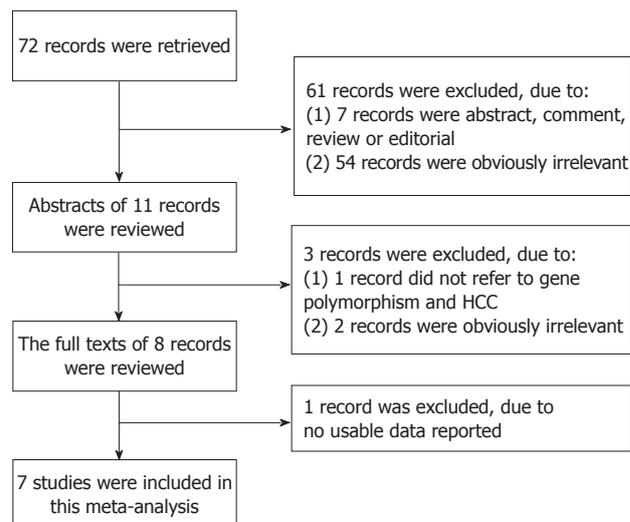


Figure 1 Flow chart of selection of studies and specific reasons for exclusion from the meta-analysis.

stratifying for ethnicity, the results were similar (Asian, OR = 1.12, 95% CI = 0.87-1.44; non-Asian, OR = 1.10, 95% CI = 0.75-1.60) (Figure 2).

There were four case-control studies^[25,26,28,29] which had been performed to study the IL-10-592 C/A polymorphism and HCC risk. Of these, all studies were performed in Asians. The combined results based on all studies showed that C allele carriers had an increased risk of HCC *vs* A allele carriers (OR = 1.29, 95% CI = 1.12-1.49) (Figure 3). Meanwhile, CC genotype carriers had an increased risk of HCC *vs* AC and AA genotype carriers (OR = 1.68, 95% CI = 1.25-2.26).

Only three studies were performed to study the IL-10-819 T/C polymorphism and HCC risk. The combined results based on all studies showed that there was no association between IL-10-819 T/C polymorphism and HCC risk (TT *vs* TC + CC, OR = 1.02, 95% CI = 0.79-1.32).

Sensitivity analysis

The influence of a single study on the overall meta-analysis estimate was investigated by omitting one study at a time, and the omission of any study made no significant difference, indicating that our results were statistically reliable.

Evaluation of heterogeneity and publication bias

Statistically significant heterogeneity was not observed between trials for all analysis with the χ^2 -based Q testing and I^2 statistics. Review of funnel plots could not rule out the potential publication bias for all analyses. Publication bias was not evident when the Begg's rank correlation method and the Egger's weighted regression method were used except for analyzing IL-10-1082 polymorphism and HCC risk (Table 3).

DISCUSSION

It has been recognized that the most important risk fac-

Table 1 Characteristics of studies included in the meta-analysis

Authors	Year	Design	Country	Ethnicity	No. of case	No. of control	Genotyping methods	Matching criteria
Bouzzargrou <i>et al.</i> ^[23]	2009	HCC	Tunisia	African	58	145	AS-PCR	Age, sex, geographical area
Ognjanovic <i>et al.</i> ^[24]	2009	PCC	United States	Caucasian	120	230	Taqman	Age, sex, race
Tseng <i>et al.</i> ^[25]	2006	HCC	Taiwan, China	Asian	208	528	PCR-RFLP	Race
Migita <i>et al.</i> ^[26]	2005	HCC	Japan	Asian	48	188	PCR-SSP	-
Nieters <i>et al.</i> ^[27]	2005	HCC	China	Asian	250	250	AS-PCR	Age, sex, race, district of residence
Heneghan <i>et al.</i> ^[28]	2003	HCC	China	Asian	98	175	-	Age, sex
Shin <i>et al.</i> ^[29]	2003	HCC	Korea	Asian	230	792	MAPA	-

HCC: Hospital-based case-control; PCC: Population-based case-control; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; PCR-SSP: Polymerase chain reaction and sequence-specific primer typing; AS-PCR: Allele-specific polymerase chain reaction; MAPA: Multiplex automated primer extension analysis.

Table 2 *Interleukin-10* gene polymorphism and hepatocellular carcinoma susceptibility

Gene	Polymorphism	HCC group genotype ¹			Control group genotype ²			HWE	Ref.
<i>IL-10</i>	-1082 G/A	AA	AG	GG	AA	AG	GG	Yes	[24]
<i>IL-10</i>	-1082 G/A	39	79	-	67	147	-	Yes	[23]
<i>IL-10</i>	-1082 G/A	24	24	10	56	68	21	No	[26]
<i>IL-10</i>	-1082 G/A	42	5	1	176	10	2	Yes	[27]
<i>IL-10</i>	-1082 G/A	130	119	-	115	135	-	Yes	[28]
<i>IL-10</i>	-1082 G/A	86	12	0	160	15	0	Yes	[28]
<i>IL-10</i>	-1082 G/A	201	28	1	675	112	5	Yes	[29]
<i>IL-10</i>	-592 C/A	AA	AC	CC	AA	AC	CC	Yes	[25]
<i>IL-10</i>	-592 C/A	93	84	31	259	223	46	Yes	[26]
<i>IL-10</i>	-592 C/A	17	23	8	85	78	25	Yes	[29]
<i>IL-10</i>	-592 C/A	89	101	26	384	299	65	Yes	[28]
<i>IL-10</i>	-592 C/A	49	38	11	95	60	19	Yes	[28]
<i>IL-10</i>	-819 T/C	TT	TC	CC	TT	TC	CC	Yes	[26]
<i>IL-10</i>	-819 T/C	17	23	8	85	78	25	Yes	[27]
<i>IL-10</i>	-819 T/C	130	119	-	115	135	-	Yes	[27]
<i>IL-10</i>	-819 T/C	49	38	11	95	60	19	Yes	[28]

¹Absolute number of patients; ²Absolute number of controls; OR: Odds ratio; HCC: Hepatocellular carcinoma; HWE: Hardy-Weinberg equilibrium.

Table 3 Pooled odds ratio for *Interleukin-10* gene polymorphism and hepatocellular carcinoma susceptibility in meta-analyses

Comparison	No. of study	OR (95% CI)	P-Publication bias			Heterogeneity test ²	
			P ¹ value	Egger ³	Begg ⁴	P value	I ²
IL-10-1082							
AA vs AG + GG	6	1.11 (0.90, 1.37)	0.32	0.004	0.02	0.44	0
IL-10-592							
CC vs AA + AC	4	1.68 (1.25, 2.26)	0.001	0.43	1	0.11	50.80%
C allele vs A allele	4	1.29 (1.12, 1.49)	0.001	0.51	0.73	0.86	0
IL-10-819							
TT vs TC + CC	3	1.02 (0.79, 1.32)	0.88	0.13	0.3	0.14	48.90%

¹Fixed effects models were used, weighted by the inverse variance. All statistical tests are two sided; ²P < 0.1 is considered statistically significant for Q statistics; I² is interpreted as the proportion of total variation contributed by between-study variation; ³Egger's test to evaluate publication bias, P < 0.05 is considered statistically significant; ⁴Begg's test to evaluate publication bias, P < 0.05 is considered statistically significant. OR: Odds ratio.

tor for the development of HCC is cirrhosis^[35]. Chronic infections with HBV and HCV are the most frequent causes of cirrhosis worldwide. A large number of cohort and case-control studies have shown that alcohol consumption causes liver cirrhosis and is an independent risk factor for HCC^[36,37]. Epidemiological studies reported elevated HCC risks associated with exposure to aflatoxins after adjustment for HBV exposure^[38]. Cigarette smoking has been causally associated with the risk of HCC^[37]. Although so many environmental factors are

found to correlate with the tumorigenesis of HCC, there still are a portion of patients without known risk factors who eventually developed HCC^[39]. Previous study had shown an interaction of environmental factors and genetic predisposition in the development of HCC^[40]. Therefore, genetic predisposition may contribute to the process of tumorigenesis.

A genetic predisposition to HCC has been suggested by many studies^[41,42]. Recent studies suggest that single nucleotide polymorphism may be related to the tu-

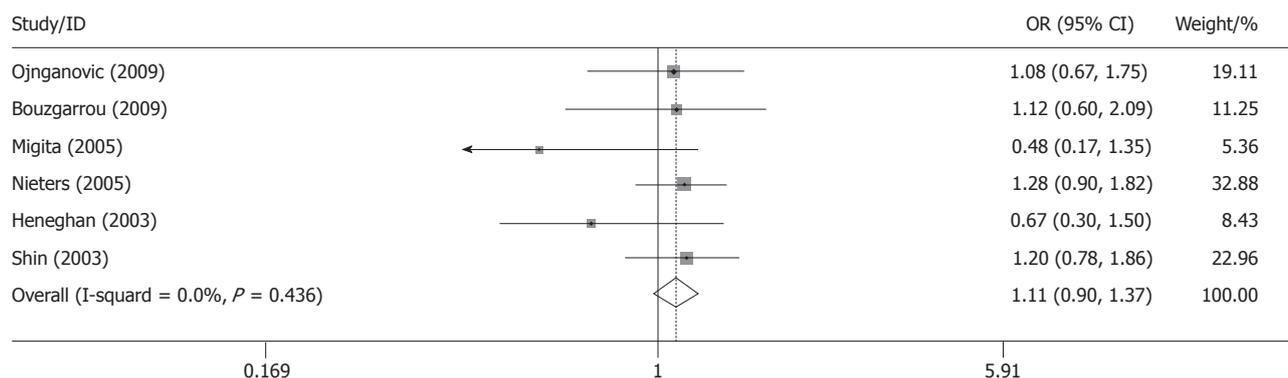


Figure 2 Odds ratios and 95% CI of individual studies and pooled data for the association of the Interleukin-10-1082 A/G polymorphism and hepatocellular carcinoma risk comparing AA genotype with AG + GG genotype.

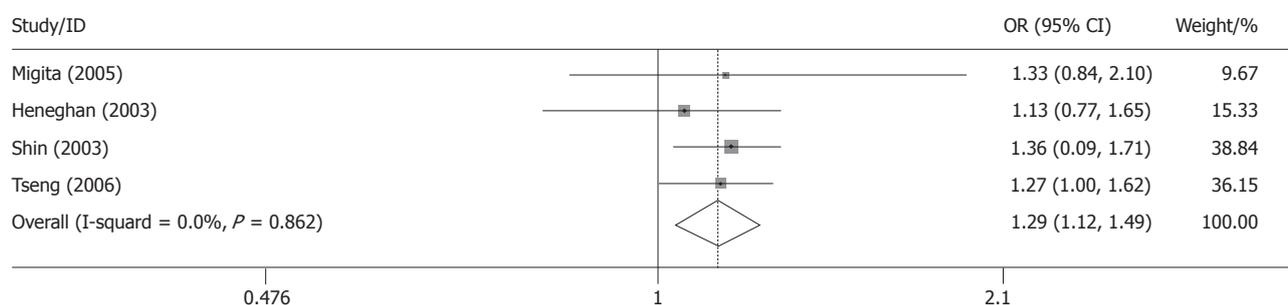


Figure 3 Odds ratios and 95% CI of individual studies and pooled data for the association of the Interleukin-10-592 C/A polymorphism and hepatocellular carcinoma risk comparing any C allele with A allele.

morigenesis of liver cancer^[43,44]. The *IL-10* gene and its encoded protein is an immunoregulatory cytokine that plays an anti-inflammatory action, which has three SNPs at positions -1082, -592 and -819 promoter region. Until recently, a number of studies had been performed to analyse *IL-10* polymorphisms and HCC risk. However, most of these studies were based on small sample sizes. Moreover, there were still some conflicting results. As a powerful statistical method, meta-analysis can provide a quantitative approach for pooling the results of different research on the same topic, and for estimating and explaining their diversity^[45,46].

The data from this meta-analysis clearly suggest that the *IL-10*-592 C allele is a genetic contributor to overall HCC susceptibility. We also observed that the C/C homozygote had a stronger association with HCC susceptibility than the C/A heterozygote and AA homozygote. Given the important roles of *IL-10* in inflammation and tumor development, it was biologically plausible that *IL-10*-592 C/A polymorphism was associated with the risk of HCC by modulating *IL-10* expression. Such evidence on the functionality of this polymorphism might lead to a better understanding of this association. The risk for HCC increases with the severity of hepatic inflammation and chronic inflammation developing through the action of various inflammatory mediators is known as a cofactor of carcinogenesis^[8,9]. As mentioned above, the function of *IL-10* is to inhibit immune response and inflammation. Meanwhile, previous studies

suggested that *IL-10*/-592*C allele may be a marker for lower *IL-10* production^[29,47]. The genetic polymorphism may affect the severity of hepatic inflammation and cause higher HCC risk due to lower *IL-10* production. In addition, this meta-analysis also suggests that *IL-10*-1082 A/G polymorphism was not associated with the risk of HCC. When stratifying for ethnicity, the results were similar. Similarly, no association was found between HCC susceptibility and *IL-10*-819 T/C polymorphism.

As previously described, ethnicity can strongly influence the distribution of cytokine gene polymorphisms^[48]. This suggests that there are racial differences in genetic risk; the different genetic backgrounds and different environments the different ethnicities lived in may contribute to the ethnic discrepancy. In the meta-analysis, studies comprising *IL-10* polymorphism (at position -592 and -819) and HCC risk were all performed in Asians. For *IL-10*-1082 polymorphism and HCC susceptibility, we stratified the result by race (Asian and non-Asian population), but the results were similar. This is probably because the number of studies from non-Asian populations were too small to detect the ethnic discrepancy, thus, caution should be adopted when explaining our results.

One of the major concerns in a sound meta-analysis is the degree of heterogeneity that exists between the component studies because non-homogeneous data are liable to result in misleading results. In the present study, the Q testing and I^2 statistics were carried out to test the

significance of heterogeneity. Fortunately, statistically significant heterogeneity was not observed between trials for all analysis with the χ^2 -based Q testing and I^2 statistics. Moreover, we performed a sensitivity analysis by removing one study each time and rerunning the model to determine the effect on each overall estimate. The estimates changed little, which implied that our results were statistically reliable.

However, there are still some limitations in this meta-analysis. (1) We did not test for gene-to-environment interactions because of the issue of multiple testing and the lack of sufficient studies. It is possible for specific environmental and lifestyle factors to alter those associations between gene polymorphisms and HCC risk; (2) as in most meta-analyses, these results should be interpreted with caution because the populations from 5 countries and controls were not uniform; (3) the number of studies and the number of subjects in the studies included in the meta-analysis by specific subgroups were small, thus, caution should be adopted when explaining our results; and (4) meta-analysis is retrospective research that is subject to methodological limitations. In order to minimize the bias, we developed a detailed protocol before initiating the study, and performed a meticulous search for published studies by using explicit methods for study selection, data extraction and data analysis. Nevertheless, our results should be interpreted with caution.

This meta-analysis suggests that the IL-10-592 C/A polymorphism may be associated with HCC among Asians. The pooled ORs in this study - both with respect to the IL-10-592*C allele and the IL-10-592/CC homozygote - suggest a modest but definite genetic effect. It is critical that larger and well-designed multicentre studies based on different ethnic groups are needed to confirm our results.

COMMENTS

Background

Interleukin-10 (IL-10) is an immunoregulatory cytokine produced by Th2 cells, monocytes/macrophages, and regulatory T cells. The promoter of the *IL-10* gene contains three biallelic polymorphisms at positions -1082 (base G to A, dbSNP No. rs1800896), -819 (base C to T, dbSNP No. rs1800871), and -592 (base C to A, dbSNP No. rs1800872) from the transcription start site. These changes have been implicated as risk factors for hepatocellular carcinoma (HCC), but individual studies have been inconclusive or controversial. The aim of this meta-analysis was to clarify the effect of IL-10 polymorphisms on the risk of HCC.

Research frontiers

To date, a number of studies have assessed the association between the *IL-10* gene polymorphism and HCC risk in different populations; however, the results are inconsistent and inconclusive. No quantitative summary of the evidence has ever been performed.

Innovations and breakthroughs

IL-10-592 A/C polymorphism may be associated with HCC among Asians, while IL-10-1082 G/A and IL-10-819 T/C polymorphisms could not alter susceptibility to HCC.

Applications

It can be seen from this paper that IL-10-592 A/C polymorphism could alter susceptibility to HCC. It suggests that a common variant in the functional region of a definitively meaningful gene had an effect on human disease, such as cancer.

Peer review

The meta-analysis aimed at assessing the association between *IL-10* gene polymorphisms and HCC. This is an appealing issue, leading to interesting results.

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S- Editor Sun H L- Editor O'Neill M E- Editor Li JY

Severe chronic diarrhea and maculopapular rash: A case report

Alessandra Elvevi, Federica Grifoni, Federica Branchi, Umberto Gianelli, Dario Conte

Alessandra Elvevi, Federica Branchi, **Dario Conte**, Gastrointestinal Unit 2, Fondazione IRCCS Cà Granda, Ospedale Maggiore Policlinico and University of Milan, 20121 Milan, Italy
Federica Grifoni, Hematology Unit, **Fondazione IRCCS Cà Granda**, Ospedale Maggiore Policlinico and University of Milan, 20121 Milan, Italy

Umberto Gianelli, Department of Pathology, Fondazione IRCCS Cà Granda, Ospedale Maggiore Policlinico and University of Milan, 20121 Milan, Italy

Author contributions: Elvevi A, Grifoni F, Branchi F, Gianelli U and Conte D contributed equally to this work; Elvevi A and Conte D wrote the paper.

Correspondence to: **Dario Conte, MD**, Gastrointestinal Unit 2, Fondazione IRCCS Cà Granda, Ospedale Maggiore Policlinico and University of Milan, 20121 Milan, Italy. dario.conte@unimi.it
Telephone: +39-02-55033418 Fax: +39-02-55033644

Received: September 23, 2010 Revised: February 26, 2011

Accepted: March 5, 2011

Published online: September 14, 2011

no therapeutic indications except for treatment of symptoms. The patient was strictly followed up because of the risk of aggressive evolution.

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Key words: Mast cells; Systemic mastocytosis; Bone marrow; Tryptase

Peer reviewer: Jean Paul Galmiche, MD, Professor, Department of Gastroenterology and Hepatology, Hôpital Hôtel Dieu, 44093 Nantes Cedex, France

Elvevi A, Grifoni F, Branchi F, Gianelli U, Conte D. Severe chronic diarrhea and maculopapular rash: A case report. *World J Gastroenterol* 2011; 17(34): 3948-3952 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i34/3948.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i34.3948>

Abstract

Systemic mastocytosis (SM) is a heterogeneous disease of the bone marrow characterized by abnormal growth, accumulation and activation of clonal mast cells (MCs). We report a case of SM with multi-organ involvement. A 30-year-old man presented with diarrhea, flushing, maculopapular rash with itching and weight loss. The upper and lower gastrointestinal endoscopies showed macroscopic involvement of stomach and duodenum; mucosal samples from stomach, duodenum, colon and distal ileum showed mucosal infiltration by large, spindle-shaped MCs with abnormal surface molecule expression (CD2 and CD25), a picture fully consistent with SM, according to the World Health Organization diagnostic criteria. A computed tomography scan showed diffuse lymphadenopathy, hepatosplenomegaly and diffuse small bowel involvement. Bone marrow aspirate and biopsy were diagnostic for SM; serum tryptase levels were increased (209 ng/mL, normal values < 20 ng/mL). The conclusive diagnosis was smouldering SM. There were

INTRODUCTION

Systemic mastocytosis (SM) is a heterogeneous disease of the bone marrow characterized by abnormal growth, accumulation and activation of clonal mast cells (MCs)^[1-3]. In most patients, SM is caused by mutations in the KIT oncogene (D816V, present in more than 80% of patients), which encodes for a tyrosine kinase protein involved in differentiation and proliferation of MCs. This mutation determines an abnormal differentiation, proliferation and clustering of neoplastic progenitors of MCs^[1-8]. Clinical features are related to histamine release (e.g., flushing, urticaria, itching, diarrhea, etc) or to uncontrolled growth and infiltration of clonal MCs in different organs (such as liver, spleen and bone marrow). The latter clinical findings must be divided into B- (Borderline Benign-Be watchful) and C-symptoms (Consider Cytoreductive therapy) (Table 1)^[2-5].

Bone marrow aspirate and biopsy represent the main diagnostic step when SM is suspected^[1-5,7,8]. According

to the World Health Organization (WHO) diagnostic criteria (Table 2), SM is diagnosed when the major and at least one minor criterion or three minor criteria are satisfied^[2,3].

At present there is no effective therapy for SM and the medical approach is aimed at symptomatic relief and improvement of quality of life. SM patients should avoid triggers for MC degranulation (e.g., exposure to heat, cold, acute emotional stress, very strenuous exercise, alcohol). Commonly used symptomatic drugs are H1 and H2 histamine receptor blockers, ketotifen, cromolyn sodium and anti-leukotriene drugs. Cytoreductive regimens (interferon alpha-2b, cladribine, tyrosine kinase inhibitors and hydroxyurea) are indicated in SM with C findings^[2,5,7-13].

CASE REPORT

A 30-year-old man presented with a ten-year history of maculopapular rash with itching and a six-month history of diarrhea (3-4 bowel movements per day with loose stools), flushing and weight loss.

The medical history was otherwise unremarkable, except for a non-steroidal anti-inflammatory drug-induced anaphylaxis.

Relevant findings at physical examination were represented by a diffuse maculopapular rash with itching (termed “urticaria pigmentosa”) (Figure 1), hepatomegaly (with the lower hepatic edge 3 cm below the costal margin), splenomegaly (with the lower splenic edge 2 cm below the costal margin) and a diffuse, superficial, painless lymphadenopathy, ranging from 2 to 4 cm in diameter.

Complete blood count, renal function tests, plasma electrolytes, liver function tests, clotting and thyroid function tests were normal as was plasma protein gel electrophoresis.

Serological and stool tests for bacterial and parasitic infections were negative. Past or current hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infections were ruled out by determining HBsAg and anti-HBc, anti-HCV and anti-HIV antibodies. Celiac disease was excluded on the basis of negative anti-endomysial and anti-transglutaminase antibodies with normal total IgA concentration. The panel for gastrointestinal neuroendocrine tumors was negative. Serum calcitonin levels were normal. Conversely, serum tryptase level was 209 ng/mL (reference value ≤ 20 ng/mL).

Abdominal ultrasonography showed hepatomegaly, splenomegaly, diffuse lymphadenopathy and diffuse small bowel dilatation with wall edema (Figure 2A and 2B). At upper and lower gastrointestinal endoscopy, there was a diffuse hyperemia with superficial erosions. Gastric, duodenal, distal ileal and colonic histology (Figure 3A, 3B and 3C) revealed diffuse mucosal MC infiltration, with spindle-shape and with abnormal surface molecule expression (CD2 and CD25) fully consistent with SM according to WHO criteria^[2,3].

In line with the current guidelines^[1-3,5], the patient

Table 1 Systemic mastocytosis findings related to mast cell infiltration and proliferation (modified from Valent *et al.*^[3])

B symptoms (Borderline benign-be watchful)
Hepatomegaly
Splenomegaly
Lymphadenopathy
Hypercellular marrow
Mast cell infiltration in bone marrow > 30%
Serum tryptase levels > 200 ng/mL
C symptoms (Consider cytoreductive therapy)
Anemia (Hb < 10 g/dL)
Thrombocytopenia (< 100 000/mm ³)
Neutropenia
Hepatopathy with ascites or portal hypertension
Splenomegaly with hypersplenism
Malabsorption with weight loss
Osteolysis with pathological bone fractures

Table 2 World Health Organization diagnostic criteria for systemic mastocytosis (modified from Valent *et al.*^[3])

Major criterion
Multifocal dense infiltrates of MCs (> 15 MCs in aggregates) in bone marrow biopsy and/or in sections of other extracutaneous organ(s)
Minor criteria
(1) > 25% of all MCs are atypical cells on bone marrow smears or are spindle-shaped in MC infiltrates detected on sections of extracutaneous organ(s)
(2) c-kit point mutation at codon 816 in the bone marrow or in another extracutaneous organ
(3) MCs in the bone marrow or in another extracutaneous organ express CD2 and/or CD25
(4) Serum tryptase levels > 200 ng/mL (this criterion is valid only if AHNMD-SM has been excluded)

MCs: Mast cells; AHNMD-SM: Associated hematopoietic clonal non-MC lineage disease systemic mastocytosis.

underwent a bone marrow aspirate and biopsy with evidence of diffuse MC infiltration, fully consistent with SM (Figure 4A, 4B and 4C). D816V mutation detection in the KIT oncogene was negative.

Disease staging was performed by both total body computed tomography scan, which confirmed hepatosplenomegaly, diffuse abdominal lymphadenopathy and diffuse small bowel involvement, and total skeleton X-ray, negative for osteolytic lesions. Osteoporosis was diagnosed on the basis of reduced bone mineral density.

In accordance with the diffuse organ involvement, serum tryptase levels > 200 ng/mL and B-findings (hepatosplenomegaly, lymphadenopathy, diarrhea and osteoporosis), the final diagnosis was of smouldering SM^[2-4]. No indication was given for cytoreductive regimen; the patient was instructed to avoid MC degranulation triggers and was given H1-H2 histamine receptor blockers and cromolyn sodium. A strict follow-up was planned aimed at early recognition of an aggressive SM evolution^[2-4,7,8,14]. The patient was evaluated at quarterly intervals for a nine-month period and then, because of clinical stability, twice a year.



Figure 1 Maculopapular rash (“urticaria pigmentosa”) in a systemic mastocytosis patient.

DISCUSSION

Systemic mastocytosis, a rare disease whose prevalence is unknown, can affect people at any age, with a slightly higher frequency in young men^[1-3].

According to the WHO classification, four SM variants have been identified^[1-3,5]: (1) **indolent SM** represents the most common form and is characterized by cutaneous and bone marrow involvement, without B or C findings; its prognosis is usually good. A rare subvariant, possibly progressing to a more aggressive SM type, is smoldering SM, characterized by B findings, diffuse organ involvement and serum tryptase levels > 200 ng/mL; (2) **aggressive SM**: this form affects 5% of SM patients and is characterized by the lack of cutaneous involvement. C findings are present and the prognosis is usually poor; (3) **associated hematopoietic clonal non-MC lineage disease SM (AHNMD-SM)** represents the second most frequent subtype of SM. To be diagnosed, WHO criteria for both SM and AHNMD must be fulfilled. Underlying blood disease is represented by myeloid disease in 80%-90% of cases and by lymphatic malignancy in the remaining 10%-20%. The prognosis is influenced both by AHNMD and SM subtype; and (4) **mast cell leukemia**: a very rare SM subtype characterized by C findings, percentage of neoplastic MCs at bone marrow biopsy > 20% and circulating neoplastic MCs. Its prognosis is usually poor.

As indicated by WHO diagnostic criteria, SM is diagnosed when the major criterion and at least one minor criterion or at least three minor criteria are satisfied^[2,3]. Based on the low disease prevalence and its wide clinical spectrum, SM can be difficult to diagnose.

Bone marrow aspirate and biopsy represent the cornerstones for SM diagnosis, the hallmark being the presence of multifocal, dense MC aggregates^[2,3]. To further improve MC recognition in bone marrow samples, immunohistochemical markers have been introduced. Among them, tryptase reactivity is considered the most sensitive, allowing the detection of even small MC infiltrates^[15,16]. Considering that virtually all MCs, irrespective of their maturation stage, activation status or tissue of localization, express tryptase, staining for this marker

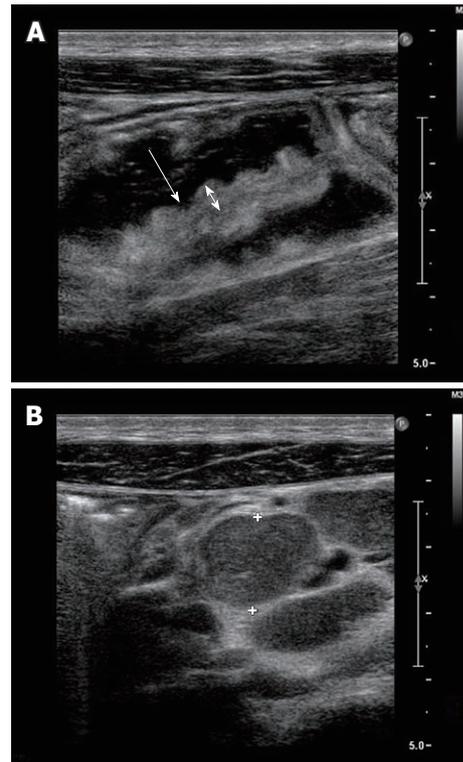


Figure 2 Abdominal ultrasound. A: Small bowel dilatation (white arrow) and wall edema (duble arrow) at ultrasonography (US). B: Abdominal lymphadenopathy at US (crosses refer to lymph node enlargement, 5 cm).

detects even those infiltrates that are primarily comprised of immature, nongranulated MCs^[17]. However, it must be emphasized that neither tryptase nor other immunohistochemical markers (e.g., CD 117) can distinguish between normal and neoplastic MCs^[18]. Conversely, immunohistochemical detection of aberrant CD2 or CD25 expression on bone marrow MCs appears to be a reliable diagnostic tool in SM, given its ability to detect abnormal MCs in all SM subtypes^[17]. The expression of even one of these two antigens represents a WHO minor diagnostic criterion.

Johnson *et al*^[19] enrolled 59 patients with clinically suspected SM; all of them underwent bone marrow examination, including immunophenotyping by immunocytochemistry and/or flow cytometry and molecular studies for KIT exon 17 mutations, and determination of serum tryptase level. Using the WHO criteria, in patients with suspected SM based on clinical and laboratory findings, the diagnosis of SM was possible in 90% of the cases. However, the major criterion was only observed in nearly 70% of patients. In an additional 30%, the diagnosis of SM could only be obtained by using ancillary testing, as specified by the WHO minor criteria. Noteworthy, the series from Johnson *et al*^[19] support the relevance of ancillary testing in obtaining the diagnosis of SM by bone marrow examination.

A further comment is warranted regarding the controversial role of serum tryptase level in the diagnostic SM algorithm. Tryptase is an enzyme stored in MC granules and released after MC degranulation. It is activated by acidic pH and presence of heparin. The bio-

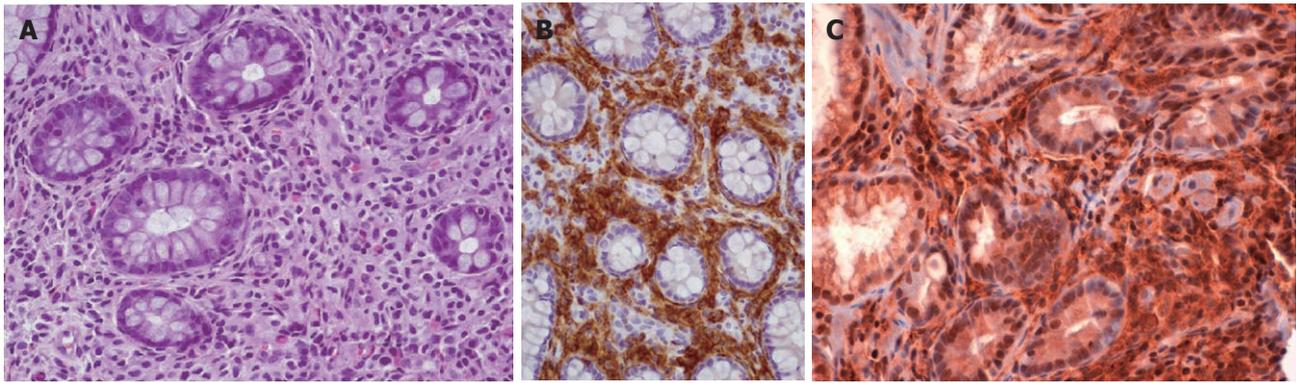


Figure 3 Colon biopsy in a systemic mastocytosis patient. A: Diffuse mast cell (MC) infiltrate (Hematoxylin-eosin, $\times 10$); B: The dense infiltrate is represented by MCs, whose detection is increased by positive immunohistochemical marker CD117; C: The dense infiltrate is represented by MCs, whose detection is increased by positive immunohistochemical marker CD25.

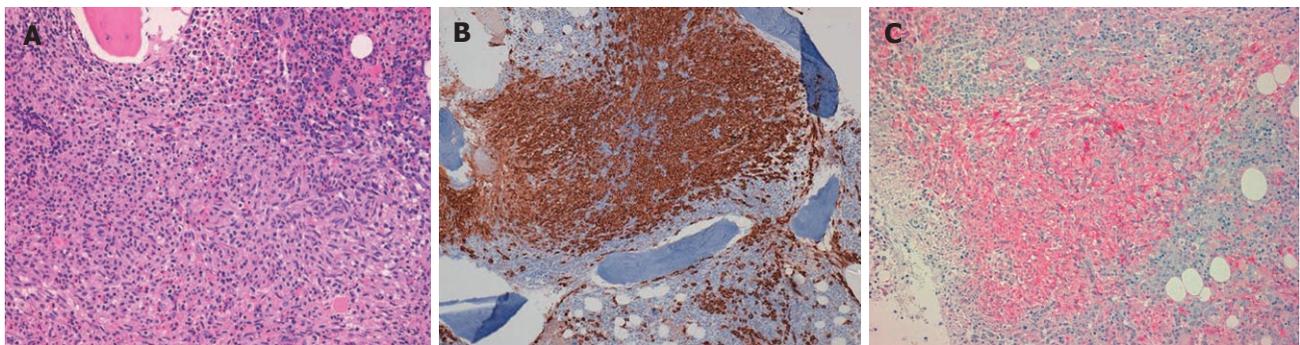


Figure 4 Bone marrow biopsy in a systemic mastocytosis patient. A: Diffuse mast cell (MC) infiltrate (Hematoxylin-eosin, $\times 10$); B: The dense infiltrate is represented by MCs, whose detection is increased by positive immunohistochemical marker CD117; C: The dense infiltrate is represented by MCs, whose detection is increased by positive immunohistochemical marker CD25.

logic activity of enzymatically active tryptase is still uncertain. Many potential substrates have been defined *in vitro*: anticoagulation, fibrosis and fibrolysis, kinin generation and destruction, enhancement of vascular permeability, airway smooth muscle hyperreactivity. However, it must be underlined that the *in vivo* relevance of these potential activities remains to be defined. Serum tryptase levels increase as a consequence of acute systemic anaphylaxis, SM and myeloproliferative diseases. In SM, serum tryptase levels represent a minor diagnostic criterion according to the WHO, but only if AHNMD-SM has been excluded^[20,21]. Valent *et al*^[3,5] have recently suggested that patients with clinical suspicion of SM having high serum tryptase levels should undergo a bone marrow examination in order to confirm the diagnosis. Furthermore, as tryptase levels are related to the burden of neoplastic MCs, their determination is of relevance in following up those SM patients given a cytoreductive regimen.

ACKNOWLEDGMENTS

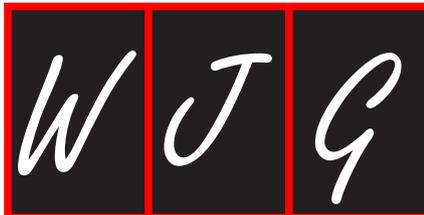
The authors would like to thank “Associazione Amici della Gastroenterologia del Granelli” for its continuous support.

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S- Editor Sun H L- Editor Logan S E- Editor Li JY



Recurrent abdominal complaints caused by a cecal neurofibroma: A case report

Willem Donk, Paul Poyck, Pieter Westenend, Wilco Lesterhuis, Fried Hesp

Willem Donk, Paul Poyck, Fried Hesp, Department of Surgery, Albert Schweitzer hospital, PO Box 444, 3300AK, Dordrecht, The Netherlands

Pieter Westenend, Laboratory for Pathology, Laan van Londen 1800, 3317 DA, Dordrecht, The Netherlands

Wilco Lesterhuis, Department of Gastroenterology, Albert Schweitzer hospital, PO Box 444, 3300AK, Dordrecht, The Netherlands

Author contributions: Donk W and Poyck P wrote the article, Westenend P revised the pathology section; Lesterhuis W did a thorough revision of the whole article; and Hesp F was the initiator of the publication and did a general revision of the article. **Correspondence to:** Willem Donk, MD, Department of Surgery, Albert Schweitzer Hospital, P.O. Box 444, 3300 AK, Dordrecht, The Netherlands. w.donk@asz.nl

Telephone: +31-78-6541111 Fax: +31-78-6179811

Received: January 22, 2011 Revised: March 25, 2011

Accepted: April 1, 2011

Published online: September 14, 2011

Abstract

Gastrointestinal involvement of neurofibromatosis type 1 (NF1, Von Recklinghausen's disease) is generally associated with the upper gastrointestinal tract. Abdominal manifestation of NF1 includes several tumors such as malignant peripheral nerve sheath tumors, gastrointestinal stromal tumors and ampulla of Vater tumors. However, colonic involvement in NF1 patients is rare. We report a case of a patient presenting with dysphagia, weight loss, intermittent abdominal pain and constipation caused by a single cecal neurofibroma obstructing the ileocecal valve. Also gastrointestinal involvement of the lower tract should be considered in patients with NF1 presenting with abdominal complaints.

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Key words: Neurofibromatosis type 1; Von Recklinghausen's disease; Colon; Neurofibroma; Treatment

Peer reviewer: Marco Scarpa, PhD, Dr., Department of

Surgical and Gastroenterological Sciences (Gastroenterology Section), University of Padova, via Giustiniani 2, Padova 35128, Italy

Donk W, Poyck P, Westenend P, Lesterhuis W, Hesp F. Recurrent abdominal complaints caused by a cecal neurofibroma: A case report. *World J Gastroenterol* 2011; 17(34): 3953-3956 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i34/3953.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i34.3953>

INTRODUCTION

Neurofibromatosis type 1 (NF1) or Von Recklinghausen's disease is an autosomal dominant genetic disorder with an incidence of 1 in 2600-3000 individuals^[1,2]. Neurofibromas are benign tumors arising from Schwann cells, caused by a mutation of the tumor suppressor gene NF1^[3,4]. Diagnosis of NF1 is made on clinical criteria, originally described by the National Institutes of Health Consensus Development Conference in 1987^[5]. The most typical features are café-au-lait macules and neurofibromas of the skin. Other organ systems can also be affected, including the cardiovascular system, eyes, bones and the gastrointestinal system. In this report we present a case of a patient with NF1 and recurrent abdominal complaints, caused by a neurofibroma in the cecal wall.

CASE REPORT

A 69-year-old woman with a history of Von Recklinghausen's disease was presented to the gastroenterologist with weight loss (9 kg in 3 mo), dysphagia and anorexia for several weeks. She also suffered from intermittent central abdominal pain for more than 1 year. There was no history of nausea, vomiting or pyrosis. For many years she had constipation, without visible blood loss or melaena. She underwent an appendectomy and a laparoscopic cholecystectomy at the age of 20 and 60, respectively. Despite her NF1, which affected her skin and bones, no other medical history was known. She did

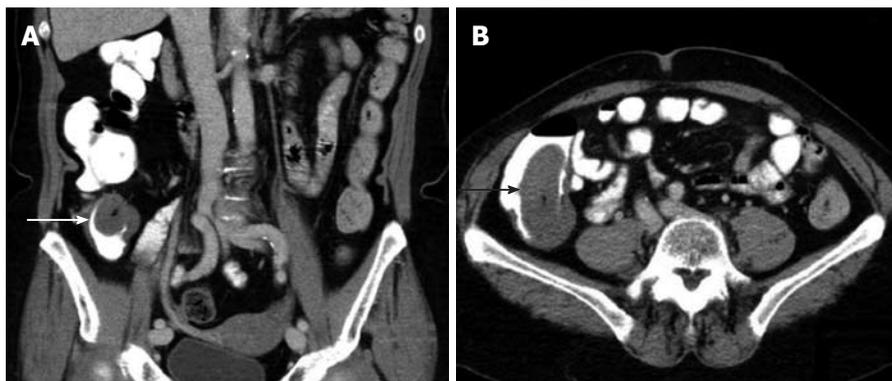


Figure 1 Coronal (A) and transversal coupe (B) of abdominal computed tomography in a patient with neurofibromatosis type 1 with a cecal mass (arrows).

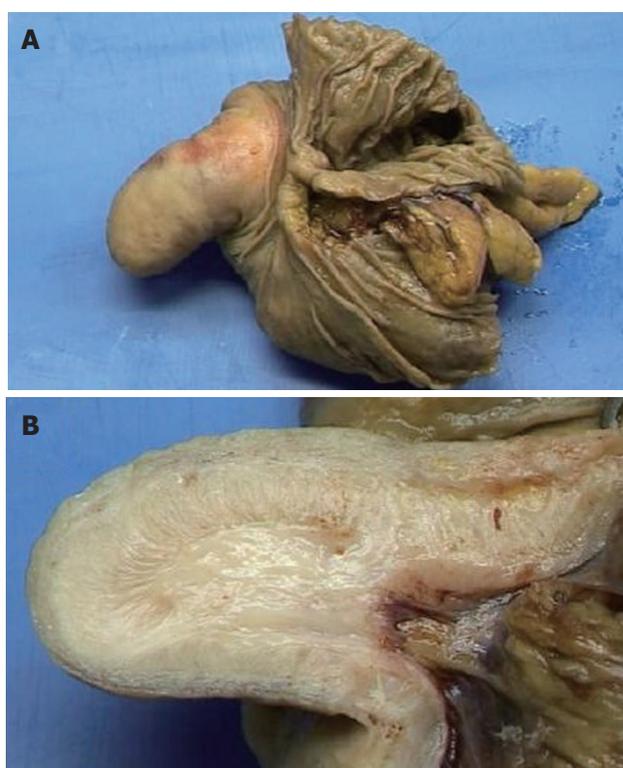


Figure 2 Opened resection specimen (A) and cut surface of the polyp (B).

not use any medication. Physical examination revealed no abnormalities of the abdomen, other than neurofibromas on her skin. Laboratory studies were normal.

Since the most prominent complaints were dysphagia and weight loss, a gastroduodenoscopy was performed, which did not show any abnormalities of the esophagus, stomach or duodenum. An abdominal ultrasound, however, demonstrated a 3.4 cm large tumorous process in the right lower quadrant. A computed tomography-scan of the abdomen showed the same process, localized in the cecal wall, invaginating into the cecal lumen (Figure 1). There were no signs of bowel obstruction or metastasis in mesenteric lymph nodes or liver.

Before a planned colonoscopy the clinical presentation deteriorated; our patient developed severe abdomi-

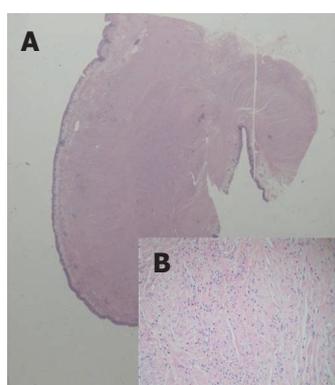


Figure 3 Overview of the polyp (A) and detail of the submucosal tumor (B), HE staining; original magnification, $\times 100$.

nal pain and a laparoscopic procedure was performed. Macroscopic inspection of the cecum revealed a palpable tumor inside the lumen. Further visual inspection of the abdominal cavity revealed no pathologic findings, such as mesenteric masses or liver metastasis. A laparoscopic right-sided hemicolectomy with an extracorporeal primary side-to-side anastomosis was performed. She recovered well and was discharged after five days. Soon her normal appetite returned, she regained her normal weight and had no more abdominal pain.

Pathologic study of the specimen revealed a polypoid cecal tumor of 3 by 5.5 cm (Figure 2), with a submucosal and intramucosal growing pattern. The lesion contained foci of spindle cells with a bundle-like growing pattern. Ganglion cells were absent. The lesion was positive for CD34, SMA and S100, but negative for P53. The morphological and immunohistochemical characteristics were consistent with the diagnosis of neurofibroma type 1 (Figures 3 and 4). The tumor was negative for CD117 and DOG-1 thus excluding a diagnosis of gastrointestinal stromal tumors (GIST).

DISCUSSION

NF1 can affect multiple organ systems, causing skin lesions, malignant peripheral nerve sheath tumors, intra-

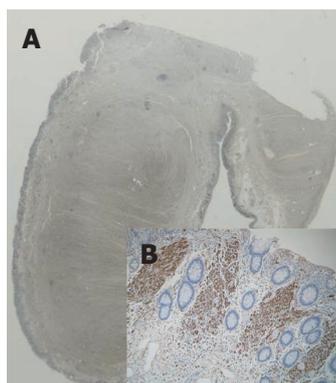


Figure 4 Overview of the polyp (A) and detail of the submucosal tumor (B), S100 staining; original magnification, $\times 100$.

cranial tumors and cranial vascular deformities, optic pathway gliomas, bone deformities, heart problems, hypertension and abdominal tumors^[1].

Five categories of abdominal neoplasms in association with NF1 have recently been described^[6]. The first category is benign or malignant neurogenic tumors, with neurofibromas as the most common and often asymptomatic (65%) neoplasm, originating from the mesenteric plexus. Several cases with abdominal neurofibroma have been described^[7-11]. Other neurogenic tumors are plexiform neurofibromas, malignant peripheral sheath tumors and ganglioneuromas^[6]. The second category is neuroendocrine tumors as carcinoids, pheochromocytomas and paragangliomas. In particular ampulla of Vater carcinoids are associated with NF1^[12,13]. The third category is GIST, which have been reported in up to one third of all patients with NF1^[14,15]. The pathogenesis of GIST is different from sporadic tumors^[3]. The fourth category is embryonal tumors, such as Wilms tumor, neuroblastoma and rhabdomyosarcoma. Finally, adenocarcinomas of the gastrointestinal tract have been detected in patients with NF1. Wood *et al.*^[6] (2005) reported a patient with NF1 and colon carcinoma and presented older reports suggesting an association between NF1 and colon carcinoma.

Gastrointestinal involvement of NF1 in patients is reported in 25% of all cases, almost always affecting the upper gastrointestinal tract. The jejunum and stomach are common sites of neurofibromas or associated tumors such as GISTs and ampulla of Vater carcinoids^[12,13]. Oesophagus and colon are rarely involved^[7]. We found 5 case reports and 1 patient in a series of 10 patients with a colonic neurofibroma^[7-11,17]. The most common presenting signs are abdominal pain, gastrointestinal bleeding, obstruction and palpable masses. The presence of one or more intestinal neurofibromas, however, does not imply that a patient has NF1. Only in 15% of cases where intestinal neurofibromas were found was the patient diagnosed with NF1^[7].

Pathologic study of a neurofibroma in the gastrointestinal tract reveals a tumor consisting of a mixture of spindle cells with wavy nuclei and strands of collagen as

well as Schwann cells, perineurial fibroblasts, endothelial cells and mast cells. Tumors positive for S100 are very suggestive for neurofibromas. These tumors must be distinguished from GISTs, schwannomas, perineuromas and leiomyomas^[18].

In this report we present a case of a patient with NF1 and recurrent abdominal complaints caused by a large cecal neurofibroma. Gastrointestinal involvement of NF1 can be found in patients with NF1 and abdominal pain, particularly in the upper gastrointestinal tract, but also colonic localisation has to be considered. Evaluation by endoscopic assessment or radiological imaging of the gastrointestinal tract is advised. Also a higher incidence of gastrointestinal malignancies in patients with NF1 has to be considered.

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S- Editor Tian L L- Editor O'Neill M E- Editor Li JY

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.

M H Ahmed, MD, PhD, Chemical Pathology Department, Southampton University Hospital NHS trust, Mail point 6, Level D, South Academic Block, Southampton SO16 6YD, United Kingdom

Basil Ammori, MD, Department of Surgery, Salford Royal Hospital, Stott Lane, Salford, Greater Manchester, M6 8HD, United Kingdom

Sung-Gil Chi, Professor, School of Life Sciences and Biotechnology, Korea University, #301, Nok-Ji Building, Seoul 136-701, South Korea

Olivier Detry, Assitant Professor, Department of Abdominal Surgery and Transplantation, University of Liège, CHU Sart Tilman B35, B-4000 Liège, Belgium

A M El-Tawil, MSc, MRCS, PhD, Department of Surgery, University Hospital of Birmingham, East Corridor, Ground Floor, Birmingham, B15 2TH, United Kingdom

Mitsuhiro Fujishiro, Dr, Department of Gastroenterology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

Fabio Grizzi, PhD, Laboratories of Quantitative Medicine, Istituto Clinico Humanitas IRCCS, Via Manzoni 56, 20089 Rozzano, Milan, Italy

Akio Inui, MD, PhD, Professor, Department of Behavioral Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

Toshiyuki Ishiwata, Associate Professor, Department of Pathology, Integrative Oncological Pathology, Nippon Medical

School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan

Bhupendra Kumar Jain, Dr., MS, Professor of Surgery and Head, Department of Surgery, GTB Hospital & University College of Medical Sciences, Delhi 110 095, India

Jae J Kim, MD, PhD, Associate Professor, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

Anastasios Koulaouzidis, MD, MRCP (UK), Day Case & Endoscopy Unit, Centre of Liver and Digestive Disorders, Royal Infirmary of Edinburgh, 51 Little France Crescent, Edinburgh, EH16 4SA, Scottish

Sang Yeoup Lee, MD, PhD, Associated Professor, Family Medicine Clinic, Pusan National University Yangsan Hospital, Medical Education Unit, Pusan National University School of Medicine, Beomeo-ri Mulgeum-eup, Yangsan, Gyeongsangnam-do 626-770, South Korea

María IT López, Professor, Experimental Biology, University of Jaen, araje de las Lagunillas s/n, Jaén 23071, Spain

Dr. Abdul-Wahed Meshikhes, MD, FRCS, Chairman & Consultant Surgeon, Department of Surgery, King Fahad Specialist Hospital, Amir Bin Thabit St, Dammam, 31444 Eastern Province, Saudi Arabia

Giuseppe Orlando, MD, PhD, Department of Health Sciences, Wake Forest Institute for Regenerative Medicine, 391 Technology Way, Winston Salem, NC 27101, United States

Christoph Reichel, Priv.-Doz., Dr., Head of the Gastroenterological Rehabilitation Center Bad Brückenau, Clinic Hartwald, German Pension Insurance Federal Office, Schlüchterner Str. 4, 97769 Bad Brückenau, Germany

Vittorio Ricci, MD, PhD, Department of Physiology, Human Physiology Section, University of Pavia Medical School, Via Forlanini 6, Pavia 27100, Italy

Philip Rosenthal, MD, Professor of Pediatrics & Surgery, UCSF, 500 Parnassus Avenue, Box 0136, MU 4-East, San Francisco, CA 94143-0136, United States

Events Calendar 2011

- January 14-15, 2011
 AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States
- January 20-22, 2011
 Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States
- January 27-28, 2011
 Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany
- January 28-29, 2011
 9. Gastro Forum München, Munich, Germany
- February 4-5, 2011
 13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany
- February 13-27, 2011
 Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia
- February 17-20, 2011
 APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver Bangkok, Thailand
- February 22, 2011-March 04, 2011
 Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada
- February 24-26, 2011
 Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland
- February 24-26, 2011
 2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil
- February 24-26, 2011
 International Colorectal Disease Symposium 2011, Hong Kong, China
- February 26-March 1, 2011
 Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada
- February 28-March 1, 2011
 Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates
- March 3-5, 2011
 42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States
- March 7-11, 2011
 Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States
- March 14-17, 2011
 British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom
- March 17-19, 2011
 41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany
- March 17-20, 2011
 Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States
- March 18, 2011
 UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States
- March 25-27, 2011
 MedicRes IC 2011 Good Medical Research, Istanbul, Turkey
- March 26-27, 2011
 26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States
- April 6-7, 2011
 IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States
- April 7-9, 2011
 International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy
- April 15-16, 2011
 Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany
- April 18-22, 2011
 Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States
- April 20-23, 2011
 9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea
- April 25-27, 2011
 The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia
- April 25-29, 2011
 Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States
- April 28-30, 2011
 4th Central European Congress of Surgery, Budapest, Hungary
- May 7-10, 2011
 Digestive Disease Week, Chicago, IL 60446, United States
- May 12-13, 2011
 2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom
- May 19-22, 2011
 1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain
- May 21-24, 2011
 22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venice, Italy
- May 25-28, 2011
 4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina
- June 11-12, 2011
 The International Digestive Disease Forum 2011, Hong Kong, China
- June 13-16, 2011
 Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy
- June 14-16, 2011
 International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia
- June 22-25, 2011
 ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain
- June 29-2, 2011
 XI Congreso Interamericano de Pediatría "Monterrey 2011", Monterrey, Mexico
- September 2-3, 2011
 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany
- September 10-11, 2011
 New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States
- September 10-14, 2011
 ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States
- September 30-October 1, 2011
 Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium
- October 19-29, 2011
 Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia
- October 22-26, 2011
 19th United European Gastroenterology Week, Stockholm, Sweden
- October 28-November 2, 2011
 ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States
- November 11-12, 2011
 Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan
- December 1-4, 2011
 2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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.

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Maximization of personal benefits

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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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- 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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- 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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Volume 17 Number 35
September 21, 2011



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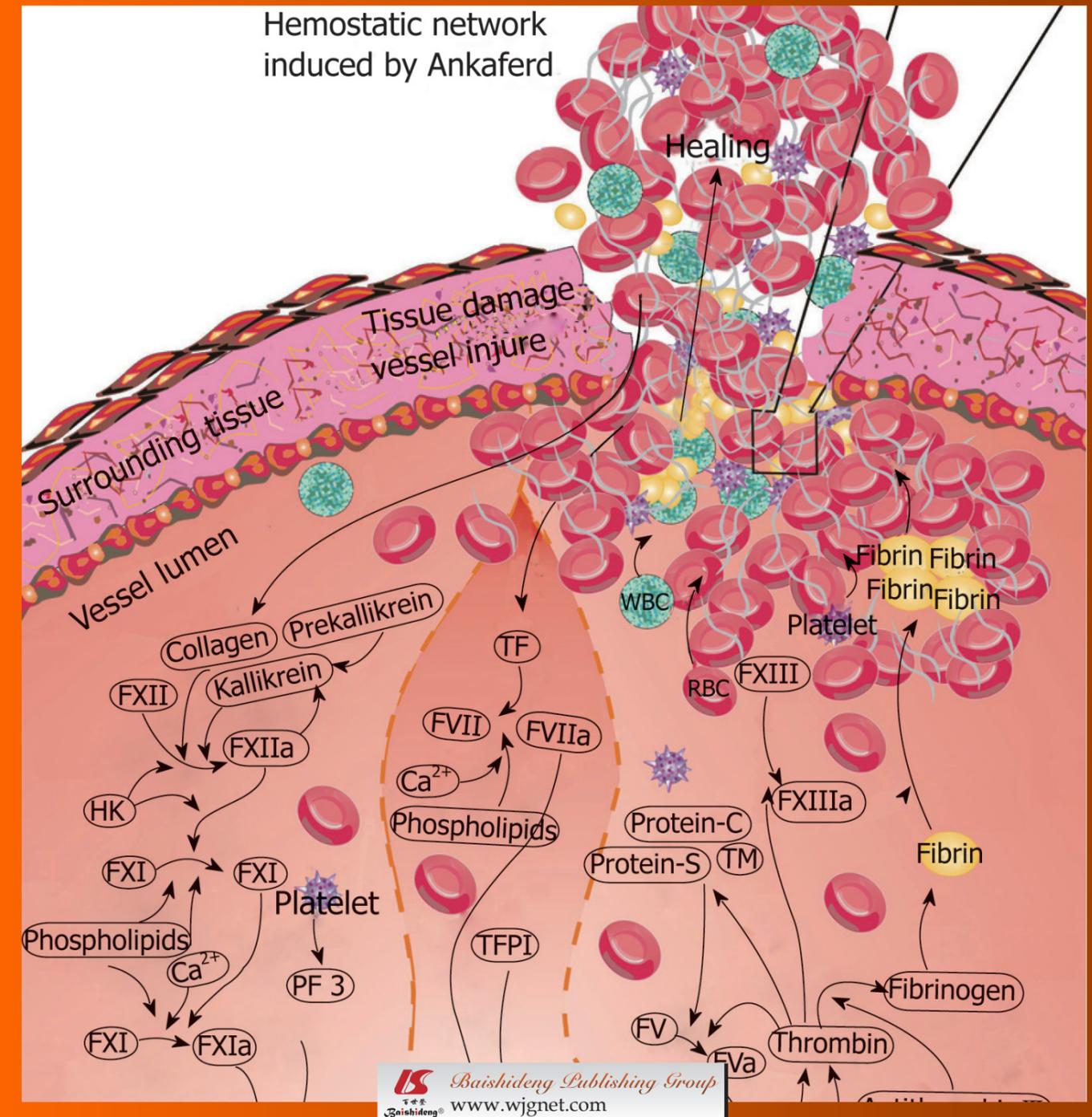
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ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

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ABOUT COVER Beyazit Y, Kekilli M, Haznedaroglu IC, Kayacetin E, Basaranoglu M. Ankaferd hemostat in the management of gastrointestinal hemorrhages.
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<http://www.wjgnet.com/1007-9327/full/v17/i35/3962.htm>

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

World Journal of Gastroenterology

LAUNCH DATE

October 1, 1995

RESPONSIBLE INSTITUTION

Department of Science and Technology of Shanxi Province

SPONSOR

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

EDITING

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

Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building, No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
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SUBSCRIPTION

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Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
<http://www.wjgnet.com>

PRINT SUBSCRIPTION

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

PUBLICATION DATE

September 21, 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.

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Update on endoscopic pancreatic function testing

Tyler Stevens, Mansour A Parsi

Tyler Stevens, Mansour A Parsi, Department of Gastroenterology and Hepatology, Digestive Disease Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195, United States

Author contributions: Stevens T wrote this paper; Parsi MA provided critical revision of the manuscript.

Correspondence to: Tyler Stevens, MD, Department of Gastroenterology and Hepatology, Digestive Disease Institute, The Cleveland Clinic Foundation, 9500 Euclid Ave, Desk Q3, Cleveland, OH 44195, United States. stevent@ccf.org

Telephone: +1-216-4451996 Fax: +1-216-4446284

Received: August 23, 2010 Revised: January 18, 2011

Accepted: January 25, 2011

Published online: September 21, 2011

Abstract

Hormone-stimulated pancreatic function tests (PFTs) are considered the gold standard for measuring pancreatic exocrine function. PFTs involve the administration of intravenous secretin or cholecystokinin, followed by collection and analysis of pancreatic secretions. Because exocrine function may decline in the earliest phase of pancreatic fibrosis, PFTs are considered accurate for diagnosing chronic pancreatitis. Unfortunately, these potentially valuable tests are infrequently performed except at specialized centers, because they are time consuming and complicated. To overcome these limitations, endoscopic PFT methods have been developed which include aspiration of pancreatic secretions through the suction channel of the endoscope. The secretin endoscopic pancreatic function test (ePFT) involves collection of duodenal aspirates at 15, 30, 45 and 60 min after secretin stimulation. A bicarbonate concentration greater than 80 mmol/L in any of the samples is considered a normal result. The secretin ePFT has demonstrated good sensitivity and specificity compared with various reference standards, including the "Dreiling tube" secretin PFT, endoscopic ultrasound, and surgical histology. Furthermore, a standard autoanalyzer can be used for bicarbonate analysis, which allows the secretin ePFT to be performed at any hospital. The secretin ePFT may complement imaging tests like endoscopic ultrasound (EUS) in the diagnosis of early chronic pancreatitis.

This paper will review the literature validating the use of ePFT in the diagnosis of exocrine insufficiency and chronic pancreatitis. Newer developments will also be discussed, including the feasibility of combined EUS/ePFT, the use of cholecystokinin alone or in combination with secretin, and the discovery of new protein and lipid pancreatic juice biomarkers which may complement traditional fluid analysis.

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Key words: Endoscopic pancreatic function test; Pancreatic function testing; Chronic pancreatitis; Pancreatic exocrine insufficiency

Peer reviewer: Rakesh Kumar, Associate Professor, Institute Of Liver And Biliary Sciences, New Delhi 110070, India

Stevens T, Parsi MA. Update on endoscopic pancreatic function testing. *World J Gastroenterol* 2011; 17(35): 3957-3961 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/3957.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.3957>

INTRODUCTION

Direct hormone-stimulated pancreatic function tests (PFTs) are the most sensitive and specific tests for assessing the pancreatic exocrine reserve^[1]. They involve administration of a gastrointestinal hormone, followed by collection and analysis of the resulting pancreatic secretions. Direct PFTs are categorized based on the hormonal stimulants used. The secretin PFT measures bicarbonate and volume, a reflection of duct-cell function. The cholecystokinin (CCK) PFT measures enzymes (e.g, lipase and trypsin), a reflection of acinar-cell function.

Direct PFTs have been performed for over 80 years using double-lumen gastroduodenal collection tubes (Dreiling tubes). The tubes are placed through the mouth, and positioned with the weighted tip passed the ligament of Treitz. The gastric lumen sits in the greater curvature of the stomach and collects gastric secretions to prevent acid contamination of the duodenum. The duodenal lu-

men collects the pancreatic secretions. In secretin PFT protocols, fluid is analyzed for bicarbonate concentration or output for an estimation of duct cell secretion. In CCK PFT protocols, fluid is analyzed for enzyme output for an estimation of acinar cell capacity. Dreiling tubes are long, floppy, and often difficult to place properly. Accurate placement requires prolonged manipulation under fluoroscopy, or endoscopic guide-wire placement^[2]. It is not uncommon for the test to take 2-3 h because of the long time required for tube placement and an additional 60-90 min required for fluid collection. Sophisticated laboratory techniques may be required for fluid analysis, which may not be universally available. Even among the few centers that perform PFTs, the test protocols are not standardized. Various combinations of hormones or analogs, dosing regimens, collection times, laboratory techniques, parameters for analyses, and diagnostic thresholds are used, making it difficult to compare results and assess the tests' performance. Although direct PFTs have been labeled the "gold standard" tests for assessing exocrine function, there is no "gold standard" direct PFT. Based on these limitations, direct PFTs are rarely performed, despite their potential usefulness for diagnosing mild and moderate exocrine insufficiency.

ENDOSCOPIC METHODS

Recently, endoscopes have been used to collect pancreatic fluid under direct visualization. Some investigators have collected pancreatic secretions using a catheter placed in the pancreatic duct at the time of endoscopic retrograde cholangiopancreatography (ERCP)^[3,4]. One potential advantage of this method is that pure pancreatic fluid is obtained, preventing contamination by other fluids found in the duodenum (e.g, bile, mucous, or food). In addition, a pancreatogram can be performed to detect structural abnormalities. A major limitation is that cannulation of the pancreatic duct is required, imparting a risk of acute pancreatitis. The time of fluid collection must also be relatively short (10-15 min). Bicarbonate secretion may not reach maximum until 25-40 min after secretin; thus, false positives are common^[5]. More recently, investigators have performed endoscopic methods in which fluid is aspirated through the suction channel of the endoscope. A common secretin ePFT protocol is as follows: Secretin is administered as an intravenous bolus dose of 0.2 mcg/kg. After sedation, the endoscope is passed through the mouth into the stomach. Gastric fluid is aspirated as completely as possible to prevent contamination of the duodenal contents. The scope is advanced into the duodenum and residual duodenal fluid is thoroughly suctioned. Timed aspirates of duodenal fluid (5-10 mL) are obtained through the suction channel into a fluid collection trap at 15, 30, 45, and 60 min. The fluid samples are placed on ice and taken to the hospital laboratory. The samples are analyzed using a chemistry autoanalyzer for bicarbonate concentration. The maximum bicarbonate concentration from all the samples is termed the peak bicarbonate. A peak bicarbonate concentration less than 80 millimolar is

considered abnormal. The following section will review the literature regarding the endoscopic secretin pancreatic function test (ePFT).

SECRETIN ePFT

Most of the recent validation studies of the ePFT have used secretin for hormonal stimulation. Ceryak *et al*^[6] were the first group to publish the results of a purely endoscopic secretin PFT, in a pilot study of 11 patients who had undergone ERCP for evaluation of abdominal pain. Duodenal aspirates were obtained every 10 min for one hour following intravenous secretin administration. In seven patients with a normal pancreatogram, the peak bicarbonate concentration was greater than 80 mmol/L. Conversely, three of the four patients with ductal changes of chronic pancreatitis (CP) did not achieve the 80 mmol/L threshold. Note that 80 mmol/L is a widely accepted bicarbonate threshold used in most Dreiling tube PFT protocols^[7]. In a similar study, secretin ePFT results were compared in patients with abdominal pain and low suspicion of CP, suspected early CP, and advanced CP^[8]. All patients in the low risk category had a bicarbonate concentration greater than 80 mmol/L. Most patients with calcific pancreatitis had bicarbonate concentrations less than 60 mmol/L. Most of the patients in the early CP category had values between 60 and 80 mmol/L. These studies suggested the feasibility of the secretin ePFT, and demonstrated that it distinguishes the presence or absence of CP when using the cut-off point of 80 mmol/L.

Validation of any new tests requires comparison with a gold standard method. Our group performed crossover studies comparing the secretin ePFT and Dreiling PFT in healthy subjects^[9] and patients evaluated for CP^[10]. The mean difference in peak bicarbonate was 0 mmol (95% CI-3, 9). There was a strong correlation between peak bicarbonate obtained by ePFT and Dreiling PFT ($r = 0.77$, $P < 0.001$). In addition, the time required to perform the ePFT was significantly less compared with the Dreiling PFT.

Structural and functional tests may be used synergistically in the diagnosis of pancreatitis. Past studies comparing PFT with ERCP have shown less than optimal concordance of structural and functional abnormalities. In a recent study, we compared endoscopic ultrasound (EUS) to secretin ePFT^[11]. We found significant inverse correlations of the EUS score with the secretin ePFT peak bicarbonate. However, the concordance of EUS with secretin ePFT in the group with mild EUS changes was only 72%.

The ePFT has been compared to the secretin magnetic resonance cholangiopancreatography (MRCP) in patients evaluated for CP^[12]. Among 24 patients with a normal ePFT, 15 had a normal MRCP pancreatogram, while nine patients had an abnormal MR pancreatogram. Among 12 patients with abnormal ePFT, seven had an abnormal MR pancreatogram, while five patients had a normal MR pancreatogram. Again, this suggests suboptimal correlation of structural and functional tests in the early phase of CP. Utilizing the MRCP functional assessment (duodenal fill-

ing after secretion), all 24 patients with normal ePFT had normal duodenal filling, and all 15 patients with abnormal ePFT had abnormal duodenal filling.

The secretin ePFT has been compared to histology in one retrospective study^[13]. Seventeen patients underwent a secretin ePFT within 12 mo before surgical resection or biopsy of the pancreas. There was a significant negative correlation between the ePFT peak bicarbonate concentration and the histological fibrosis score (Spearman $r = -0.57$). The ePFT was 86% sensitive and 67% specific for the diagnosis of fibrosis. The sensitivity and specificity were similar to those of EUS in the detection of histological fibrosis.

NEW DEVELOPMENTS

Shortened ePFT

A considerable limitation of the secretin ePFT is that it takes approximately 1 h to perform, with fluid collections at 0, 15, 30, 45, and 60 min after secretin injection. As such, we and others have studied shortened ePFT methods^[14,15]. In a retrospective analysis of 240 ePFT results, we found that measuring bicarbonate at 30 and 45 min provides 94% accuracy compared with the full hour long test. We currently administer secretin in the admitting area before transport to the endoscopy suite. By the time the patient is sedated, the scope inserted, and the stomach cleared of gastric fluid, we are able to efficiently collect duodenal aspirates at the 30 and 45 min time points. A careful luminal examination is performed between collections.

Combined EUS/PFT

A combination of structural and functional testing may be required to diagnose CP. We often perform a combined EUS and ePFT in the same endoscopic session. This involves performance of EUS following secretin stimulation, with collection of duodenal fluid at 15, 30, and 45 min. In 252 patients evaluated for suspected minimal change CP (no calcifications), 160 (63.5%) had concordant normal EUS and ePFT results, “ruling out” CP. Thirty-two patients (12.7%) had concordant abnormal EUS and ePFT results, “ruling in” the diagnosis^[16]. The remaining 60 patients had discordant results, which are more difficult to interpret. Patients with abnormal EUS and normal ePFT may have CP with preserved exocrine function. The significance of normal EUS with abnormal ePFT is uncertain, but may suggest a very early form of CP prior to the development of overt structural changes. Long-term studies are needed to better understand the significance of minimal or discordant functional and endosonographic changes.

Use of CCK for ePFT

Many pancreatic referral centers advocate that the CCK PFT is the most sensitive method for detecting early acinar cell loss from pancreatic fibrosis. Most CCK PFT protocols require continuous collection of pancreatic fluid using a gastroduodenal collection tube, with measurement of total enzyme output. Measurement of enzyme output

requires an accurate assessment of volume. Most CCK protocols use a second orogastric tube to perfuse an inert non-absorbable marker, such as polyethylene glycol (PEG). Measurement of recovered PEG from the duodenal juice produces a more accurate volume estimate.

Unlike more labor intensive methods that utilize perfusion markers, ePFT methods do not quantify volume, which would allow an accurate estimation of enzyme outputs. Instead, the ePFT collects timed samples of fluid and relies on concentration measurements. Studies of a CCK ePFT utilizing lipase concentrations have yielded mixed results. A pilot study found a threefold increase in lipase concentrations in healthy volunteers following continuous CCK stimulation (40 ng/kg per hour), with a mean peak lipase value of 1778847 IU^[17]. A subsequent study demonstrated that a peak lipase value of 780000 IU provided 83% sensitivity and 87% specificity for differentiating healthy subjects from patients with established CP^[18]. In a third study, CCK-stimulated endoscopic and Dreiling tube PFTs were compared with measurement of lipase concentrations^[19]. Both collection methods produced excellent discrimination between healthy volunteers and patients with moderate to advanced CP based on the ERCP Cambridge classification. A more recent study of the CCK ePFT yielded less satisfactory results^[11]. Although there was good separation between controls and those with advanced CP, there was substantial overlap in lipase results with the group with suspected early CP.

We have recently studied an ePFT using combined secretin and CCK to assess both duct-cell and acinar-cell function. The bicarbonate and enzyme results from the combined ePFT were compared using EUS as a reference standard^[20]. Of all the diagnostic parameters, peak bicarbonate and amylase appeared to optimize discrimination. Using logistic regression, a predictive score was developed including peak bicarbonate and peak amylase for prediction of CP. A predictive score threshold of 1213 yielded 82.8% sensitivity and 88.9% specificity. Further validation of this combined test is currently underway.

Use of autoanalyzers for bicarbonate measurement

In the secretin PFT, the standard technique for bicarbonate measurement has been back titration. Back titration involves gradual addition of defined quantities of hydrochloric acid to the pancreatic fluid sample until a pre-specified pH is obtained, allowing calculation of the original bicarbonate content of the fluid. Back titration is cumbersome and not available in most hospitals, whereas, chemistry autoanalyzers are widely available in all hospitals. We compared back titration versus an autoanalyzer for bicarbonate measurement in pancreatic fluid^[21]. There was high concordance between the methods (Lin's concordance coefficient = 0.96), suggesting that the autoanalyzer is a satisfactory method for bicarbonate measurement.

Measurement of proteins and lipids

Fluid analysis for PFTs has focused on the products of pancreatic exocrine secretion (bicarbonate and enzymes).

However, CP is disease of inflammation and fibrosis, not simply functional loss. Therefore, measurement of the byproducts of inflammation may be useful in diagnosing early CP, even before functional decline occurs. A recent study demonstrated the feasibility of measuring the entire complement of proteins from pancreatic fluid using gel electrophoresis followed by tandem mass spectrometry.^[22] The known functions of the discovered proteins were ascertained using gene ontology analysis. In this study, a total of 134 proteins were isolated from the pancreatic fluid, the majority of which were found in multiple samples. Further studies are underway to refine this proteomics approach, and to better understand the discriminative ability of these newly elucidated biomarkers for diagnosis.

Oxidative stress is known to have a role in pancreatic inflammation. Reactive oxidative molecules can cause damage to lipid membranes. Therefore, measurement of oxidized fatty acids may represent a useful biomarker for early CP. We have used a “lipidomics” approach to quantifying oxidized fatty acids in the serum and expressed pancreatic fluid during secretin ePFT, combined with tandem mass spectrometry. Oxidized fatty acids were differentially expressed in both the serum and fluid, suggesting a promising biomarker for early CP^[23,24]. Further work is needed to validate the use of protein or lipid measurement from pancreatic secretions.

ROLE OF THE ePFT

Endoscopic methods have simplified direct PFTs, and made them more accessible to clinicians and patients. However, there are acknowledged limitations. First, even when shortened protocols are used, the ePFT remains a time-consuming test, requiring 30-45 min of prolonged endoscopy. Second, the inability to accurately quantify fluid volume prevents calculation of enzyme output, arguably the optimal measure of acinar capacity. Finally, although intravenous sedation in low doses does not appear to substantially affect exocrine secretion, the effect of higher levels of sedation, as required for many patients with CP, has not been adequately studied.

The actual role of ePFT in the care of patients has yet to be defined. PFT has been considered a diagnostic test for early CP because mild changes in functional capacity may represent an early biomarker for pancreatic fibrosis. However, this is not universal in all patients. Past studies have shown that most patients with mild and severe CP have evidence of exocrine loss. However, some patients with advanced structural changes of CP have preserved exocrine function. We believe the ePFT serves as a complementary diagnostic modality with structural testing, as seen with the combined EUS/ePFT.

The ePFT may also be useful in investigating the cause of malabsorptive diarrhea. We frequently perform a fecal elastase test in the initial workup of patients with malabsorptive diarrhea. Fecal elastase levels are quite useful in diagnosing moderate and advanced exocrine insufficiency. However, if the fecal elastase result is equivocal or if mild exocrine insufficiency is considered, we often proceed

to secretin ePFT. We typically obtain secretin-stimulated duodenal aspirates before obtaining a small intestinal mucosal biopsy in patients evaluated for steatorrhea. In 12 patients who presented with painless steatorrhea, and who lacked structural features of CP on imaging tests, two patients (20%) were found to have concordant abnormal results, suggesting early CP with exocrine insufficiency^[16]. Conversely, 10 patients had a concordant normal EUS and ePFT, ruling out pancreatic insufficiency. Several of these patients were found to have other causes of steatorrhea, such as celiac disease or bacterial overgrowth.

Secretin ePFT may also be considered in patients with established CP to “stage” the disease and determine the need for exogenous enzymes. In our series of 38 patients with established severe CP who underwent EUS/ePFT, there were five patients (13.2%) who had a normal ePFT^[16]. These patients also lacked postprandial diarrhea. Based on the normal ePFT results, these patients were spared the cost and nuisance of taking pancreatic enzymes.

CONCLUSION

Endoscopic fluid collection has made hormone-stimulated pancreatic function tests much more accessible for routine clinical care. The incremental diagnostic utility of the secretin ePFT in the context of other sensitive structural tests, such as EUS and MRCP, remains to be proven. We have found the ePFT to be most useful in patients with suspicion of CP, but with minimal or equivocal radiographic abnormalities. In these patients, a combined secretin ePFT and endoscopic ultrasound is often performed as an efficient structural and functional assessment of the gland. We have also found the ePFT helpful in the workup of malabsorptive diarrhea, allowing a simultaneous small intestinal biopsy to screen for mucosal diseases that cause malabsorption. Further studies are underway to optimize ePFT protocols and to better define their role in the clinical care of patients.

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

Ankaferd hemostat in the management of gastrointestinal hemorrhages

Yavuz Beyazit, Murat Kekilli, Ibrahim C Haznedaroglu, Ertugrul Kayacetin, Metin Basaranoglu

Yavuz Beyazit, Division of Gastroenterology and Hepatology, Ankara Yüksek İhtisas Hospital Gastroenterology Clinic, Sıhhiye, Ankara 06100, Turkey

Murat Kekilli, Division of Gastroenterology and Hepatology, Ankara Yüksek İhtisas Hospital Gastroenterology Clinic, Sıhhiye, Ankara 06100, Turkey

Ibrahim C Haznedaroglu, Department of Hematology, Faculty of Medicine, Hacettepe University, Sıhhiye, Ankara 06100, Turkey

Ertugrul Kayacetin, Division of Gastroenterology and Hepatology, Yıldırım Beyazıt University, Altındag, Ankara 06100, Turkey

Metin Basaranoglu, Division of Gastroenterology and Hepatology, Teaching and Consulting, Ankara Yüksek İhtisas Hospital Gastroenterology Clinic, Sıhhiye, Ankara 06100, Turkey

Author contributions: Beyazit Y and Basaranoglu M provided literature search and figures besides designed and wrote the study; Kekilli M, Haznedaroglu IC and Kayacetin E commented on manuscript; Basaranoglu M is senior author.

Correspondence to: Dr. Metin Basaranoglu, Division of Gastroenterology and Hepatology, Ankara Yüksek İhtisas Hospital Gastroenterology Clinic, Sıhhiye, Ankara 06100, Turkey. metin_basaranoglu@yahoo.com

Telephone: +90-532-3448718 Fax: +90-212-6217580

Received: January 17, 2011 Revised: March 1, 2011

Accepted: March 8, 2011

Published online: September 21, 2011

Abstract

Gastrointestinal (GI) bleeding refers to any hemorrhage ascribed to the pathologies of the gastrointestinal tract, extending from the mouth to the anal canal. Despite the recent improvements in the endoscopic, hemostatic and adjuvant pharmacologic techniques, the reported mortality is still around 5%-10% for peptic ulcer bleeding and about 15%-20% for variceal hemorrhages. Although endoscopic management reduces the rates of re-bleeding, surgery, and mortality in active bleeding; early recurrence ratios still occur in around 20% of the cases even with effective initial hemostatic measures.

In this quest for an alternative pro-hemostatic agent for the management of GI bleedings, Ankaferd blood stopper (ABS) offers a successful candidate, specifically for "difficult-to-manage" situations as evidenced by data presented in several studies. ABS is a standardized mixture of the plants *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica*. It is effective in both bleeding individuals with normal hemostatic parameters and in patients with deficient primary and/or secondary hemostasis. ABS also modulates the cellular apoptotic responses to hemorrhagic stress, as well as hemostatic hemodynamic activity. Through its effects on the endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics, and wound healing, ABS is now becoming an effective alternative hemostatic medicine for gastrointestinal bleedings that are resistant to conventional anti-hemorrhagic measurements. The aim of this review is to outline current literature experience suggesting the place of ABS in the management of GI bleeding, and potential future controlled trials in this complicated field.

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Key words: Ankaferd blood stopper; Gastrointestinal bleeding; Hemostasis; Erythrocyte aggregation; Coagulation

Peer reviewers: Seng-Kee Chuah, MD, Division of Hepatogastroenterology, Kaohsiung Chang Gang Memorial Hospital, 123, Ta-Pei Road, Niasung Hsiang, Kaohsiung 833, Taiwan, China; Cuong D Tran, PhD, Research Fellow, Affiliate Lecturer, University of Adelaide, Gastroenterology Unit, Children, Youth and Women's Health Service, 72 King William Rd, North Adelaide, SA 5006, Australia

Beyazit Y, Kekilli M, Haznedaroglu IC, Kayacetin E, Basaranoglu M. Ankaferd hemostat in the management of gastrointestinal hemorrhages. *World J Gastroenterol* 2011; 17(35): 3962-3970 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/3962.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.3962>

INTRODUCTION AND BACKGROUND OF ANKAFERD BLOOD STOPPER

Ankaferd is a traditional herbal medicine that has been used in Anatolia as a hemostatic agent for centuries^[1]. Ankaferd is a standardized mixture of the plants *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica*, each of which have some effects on the endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics, and/or cell mediators^[1-4]. Ankaferd blood stopper (ABS), a novel topical hemostatic agent, has been approved in Turkey for clinical hemorrhages, when the conventional control of bleeding by ligation and/or conventional hemostatic measures is ineffective^[5,6]. ABS is clinically effective in bleeding individuals with normal hemostatic parameters and in patients with deficient primary hemostasis and/or secondary hemostasis^[7-10]. ABS modulates the cellular apoptotic responses to hemorrhagic stress as well as its hemostatic hemodynamic activity^[11], and has many effects on proteins of the tissue and blood. Dose-dependent reversible PAR-1 down-regulation is mediated by ABS and also induces sustained PAR-1 down-regulation in the presence of lipopolysaccharides (LPS). These findings are compatible with other investigations focusing on the endothelial hemostatic molecules, endothelial cell protein C receptor (EPCR) and PAI-1. ABS may act as a topical biological response modifier as along with its anti-hemorrhagic effects^[10].

Gastrointestinal (GI) bleeding is a potentially life-threatening condition and a common cause of hospitalization. Despite effective endoscopic treatments, it is responsible for a significant societal burden due to the associated morbidity, mortality and financial implications^[12]. Although endoscopic management does diminish the rates of re-bleeding, surgery, and mortality in active hemorrhage, early recurrence still occurs in around 20% of cases despite the effective initial hemostasis. Hence, there is an ongoing intensive search for novel techniques or treatments that are effective, safe and “potentially life-saving” in the distinct settings of GI bleedings. During the search for a complementary pro-hemostatic agent for the management of GI hemorrhages, accumulated evidence suggested that ABS could have an efficient place for the “difficult-to-manage” subtypes of GI bleedings^[13-22]. ABS may serve as an adjuvant and/or primary agent for this complicated area.

The aim of this review is to outline the current literature suggesting the place of ABS in the management of GI bleeding, and potential future controlled trials in this complicated field. Currently established standard medical and endoscopic therapeutic options with hemostatic approaches do not represent the primary scope of this paper.

ABS AS A MODERN TOPICAL HEMOSTATIC AGENT

The basic mechanism of action for ABS appears to be the formation of an encapsulated protein network that provides focal points for erythrocyte aggregation. Rather than affecting an individual clotting factor, this protein mesh af-

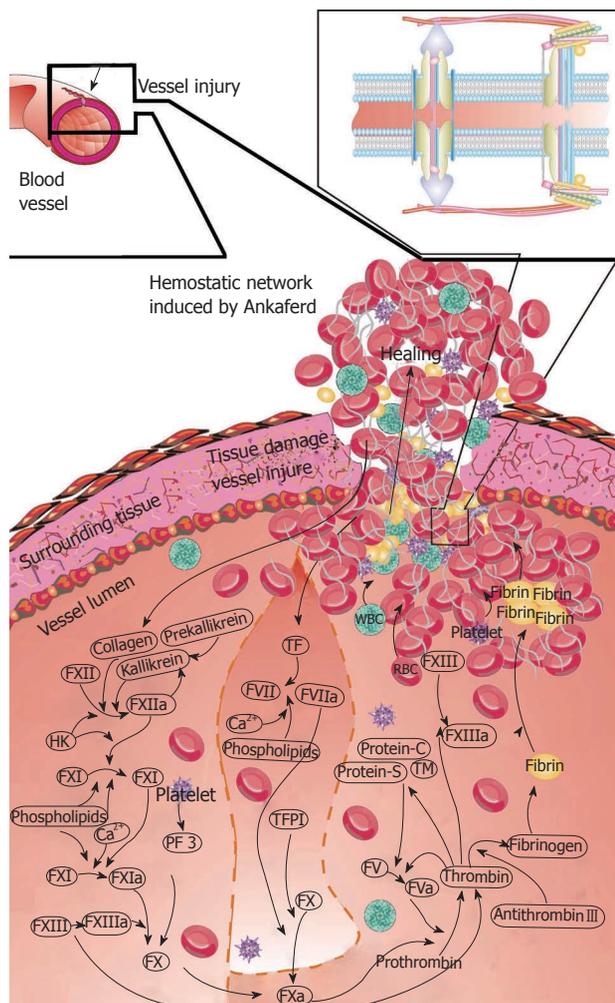


Figure 1 The basic mechanism of action for ankaferd blood stopper is the formation of an encapsulated protein network that provides focal points for erythrocyte aggregation. Ankaferd blood stopper (ABS)-induced formation of the unique protein network within the vital erythroid aggregation covers the entire physiological haemostatic process. Red blood cell (RBC) elements (such as spectrin and ankrin surface receptors, and internal ferrochelatase enzyme), related transcription factors (such as GATA-1) and RBC-related proteins (such as urotensin II) are the main targets of ABS. Those proteins and the required adenosine triphosphate bioenergy are included in the protein library of Ankaferd^[1].

fects the entire physiological hemostatic process that controls bleeding^[1,2]. Blood cells, particularly erythrocytes and activated leukocytes, were found to aggregate rapidly in the presence of ABS, thereby participating in the network formation (Figure 1). Macroscopic hemostatic actions of ABS may be explained by its rapid (< 1 s) induction of a protein network in human plasma and serum samples^[23]. ABS-induced formation of the protein network with vital erythroid aggregation covers the entire physiological hemostatic process^[1,2]. There are distinct important components of the ABS-induced hemostatic network. Vital erythroid aggregation takes place with the spectrin and ankyrin receptors on the surface of red blood cells. Those proteins, and the required ATP bioenergy, are included in the ABS protein library. Ankaferd also upregulates the GATA/FOG transcription system affecting erythroid functions. Urotensin-II is also an essential component of Ankaferd and represents the link between injured vascular endothe-

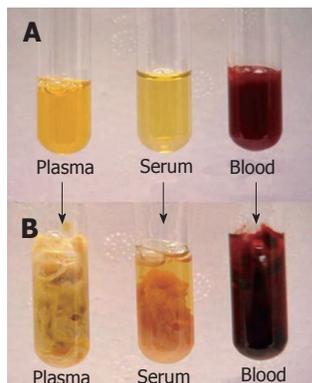


Figure 2 Ankaferd blood stopper-induced protein network formation with in less than 1 s. Plasma under the light microscopy before (A) and just after (B) Ankaferd application^[1].

lium, adhesive proteins, and active erythroid cells^[1,2]. These concepts have been developed *via* matrix-assisted laser desorption/ionization time of flight proteomic molecular analyses, cytometric arrays, transcription analysis, and Scanning electron microscopy ultrastructural examinations, as well as numerous investigations interacting with *in vivo* research settings^[23-26].

In vitro tests demonstrated that coagulation proteins were not individually affected by the addition of ABS to fresh normal plasma or serum, whereas plasma fibrinogen activity decreased from 302 to < 10 mg/dL, and fibrinogen antigen decreased from 299 mg/dL to < 30 mg/dL in parallel with thrombin time prolongation. Total protein, albumin and globulin levels decreased after the addition of ABS to fresh serum^[2,23]. These studies suggested that the ABS-induced network formation depends upon interactions between ABS and blood proteins, such as fibrinogen, and that ABS might affect fibrinogen and other proteins *via* agglutination of these molecules. Figure 2 depicts the macroscopic appearance of the protein network formation before and after adding ABS to human plasma, serum and whole blood.

Dose-dependent reversible PAR-1 down-regulation is mediated by ABS inside the human umbilical vein endothelial cells. ABS induces sustained PAR-1 down-regulation in the presence of LPS. These findings are compatible with our previous investigation focusing on the endothelial hemostatic molecules, EPCR and PAI-1. ABS has dual diverse dynamic reversible actions on EPCR and PAI-1 inside vascular endothelial cells also in the model of human umbilical vein endothelial cells. Sudden anti-hemorrhagic efficacy of ABS *via* immediate enhanced expression of pro-hemostatic PAI-1 and down-regulated anti-coagulant EPCR upon the exposure of ABS have been recognized as the unique hemostatic effects of ABS. The hemostatic function of PAR-1 is mainly prothrombotic. Significant PAR-1 down-regulation mediated by ABS indicated that ABS has balanced effects on global hemostasis^[2,26,27]. Coagulation proteins, namely factor II, V, VII, VIII, IX, X, XI and XIII, were not affected *in vitro* individually by ABS^[17]. Likewise, prothrombin time (PT) and activated partial thromboplastin time (aPTT) were normal

via the application of ABS. However, prolonged thrombin time (TT) was evident^[2]. Since PAR-1 is the most important thrombin receptor, depression of PAR-1 with ABS could explain the prolonged TT due to ABS^[10,27].

UPPER GASTROINTESTINAL BLEEDING

Peptic ulcer disease

Peptic ulcer is the most common cause of acute hemorrhage in the upper gastrointestinal tract, accounting for 28%-59% of all episodes of upper GI bleeding^[28,29]. Endoscopy with hemostatic therapy has clearly been shown to aid in proper diagnosis, prognosticate requirement for blood transfusions and, in the majority of instances, obviates the need for surgical intervention^[30,31]. Despite the improvements in achieving hemostasis, recurrent bleeding still occurs in about 15% to 20% of GI bleeding cases. Moreover, the reported mortality for patients with a bleeding peptic ulcer still amounts to 15%^[32]. Early effective hemostatic intervention is of great importance in the treatment of bleeding peptic ulcer disease, due to the high risk of morbidity and mortality. However, additional development is eagerly awaited for the therapeutic armamentarium of GI bleeding, which is safe, effective and easy applicable in difficult or intolerant patients^[33,34]. In this setting, ABS could be the candidate hemostatic agent in the controlling of peptic ulcer bleeding, based on the previous successful anecdotal reports in GI bleeding with various clinical outcomes^[13-22].

There is growing evidence in favor for the use of ABS in distinct states of GI bleeding, particularly in patients with bleeding due to peptic ulcer disease. In an observational study of "intention-to-treat" analysis by Ozaslan *et al.*^[35], five adult patients with bleeding peptic ulcer disease, in which ABS was used as a primary hemostatic agent due to difficulties or inappropriateness of the conventional measures, were reported to attained success in controlling of the bleeding within minutes. Similarly, Purnak *et al.*^[36] reported a successful hemostasis control in a patient with a bleeding peptic ulcer complicated with defective hemostasis. In this reported case, at the time of bleeding, the patient was under-treated with a cytotoxic chemotherapeutic agent leading to thrombocytopenia. Furthermore, platelet dysfunction and prolonged PT due to neutropenic sepsis had further complicated the hemostatic status. This "difficult-to-manage" situation was effectively controlled with topical ABS application and provided a critical time gain to the clinician until hemostasis could be returned to the normal level. Since ABS performs cellular hemostasis mainly through erythrocytes, it is reasonable to suggest that bleeding due to defective hemostasis (such as from a low platelet count, due to a warfarin overdose or because of chronic nonsteroidal anti-inflammatory drug use) could be controlled more efficiently with ABS as in this reported case^[36]. Likewise, the *in vivo* hemostatic effect of ABS with defective hemostasis, due to aspirin and low-molecular weight heparin administration, has been investigated in experimental models and ABS was found to be effective in shortening the bleeding duration and decreasing the amount of bleeding^[37]. Fur-

thermore, the first pediatric experience with ABS in an infant with bleeding peptic ulcer was recently demonstrated by Yarali *et al.*^[38]. Both of these reports seem to be encouraging for the justification of the use of ABS in peptic ulcer bleeding based on future controlled clinical trials.

Neoplastic upper GI bleedings

GI bleedings due to tumoral lesions (primary gastrointestinal tumors, direct local invasion by other malignancies, or metastatic disease to the gastrointestinal tract) are among the frequently encountered causes of GI bleeding, accounting for nearly 5% of severe upper GI bleeding cases^[39]. Severe bleeding is a bad prognostic sign for upper GI tumors, and endoscopic hemostasis in this setting is often a temporary measure prior to staging and surgical resection. Several methods have been used to control bleeding from gastroduodenal malignant lesions, including thermal contact probes (tumor probe, bipolar probes, or heater probe), epinephrine injection, laser coagulation and injection of sodium tetradecyl sulphate with a success rate of 66%-100%^[39-41]. Unfortunately, these intervention modalities were associated with high re-bleeding rates; up to 80% in a 1 mo period^[39,40].

In the setting of malignant GI bleeding, ABS was effectively used previously in several reports. Application of ABS successfully controlled GI bleeding within seconds in a patient with major GI bleeding from a recurrent lesion at the hepaticojejunostomy anastomosis following surgery for distal cholangiocellular carcinoma^[42]. In a case series by Kurt *et al.*^[43], topical application of ABS in seven patients with neoplastic upper GI hemorrhages, with appropriate bleeding control and post-procedural complications, were documented. In their summary, complete hemostasis was achieved in all of those patients within seconds of the endoscopic topical application of ABS, with no immediate complications. A recent report by Ozaslan *et al.*^[44] also supported the effectiveness of ABS in tumoral GI bleedings as a primary hemostatic agent. In their observational study, six patients suffering from malignant GI bleeding were reported to achieve hemostasis with topically applied ABS during endoscopy by a sclerotherapy needle or a heater probe catheter. The control of bleeding was obtained with ABS in five cases during the first endoscopic session, while the remaining one required a second application.

Apart from the mechanical hemostasis achieved by ABS, Turhan *et al.*^[45] disclosed that ABS decreases tumor vascularization in bleeding gastrointestinal carcinomas. In this report, topical ABS was applied in two patients with distinct tumoral GI bleedings due to gastric and rectal cancer. Tumor neo-vascularization/angiogenesis before and after the application of ABS were measured as tumor microvessel density (MVD). Topical ABS administration to the tumoral lesion resulted in complete control of the bleeding. Furthermore, ABS significantly decreased MVD measurements in both of the GI neoplastic tissues in comparison to the MVDs from the biopsy specimens before the ABS administration and the unexposed native neoplastic tissues of the stomach and rectum^[45]. Based on these preliminary findings, the authors suggested the

presence of a secondary, more sustained, mechanism of hemostasis induced by ABS beyond the initial protein network.

Although the management of tumoral GI bleeding in a pediatric population is a difficult to manage situation, ABS was also shown to be effective in a 10-year-old boy with esophageal tumor bleeding related to disseminated intravascular coagulation (DIC) during the post-chemotherapy period^[46]. Since the endoscopic procedure was contraindicated due to DIC and associated co-morbidities in this patient, nasogastric tubes were used for the topical application of ABS. The bleeding was stopped within a very short period of time following the 6 milliliters of topical ABS application, with no observation of re-bleeding or side effects.

Sphincterotomy bleeding

Endoscopic sphincterotomy (EST), which has become an essential procedure in therapeutic endoscopy for the management of pancreatic and biliary problems, raises concerns about procedure-related complications, such as hemorrhages, pancreatitis, cholangitis and perforation^[47]. Hemorrhage is one of the most frequently encountered, and sometimes fatal, complications of EST and the incidence is reported as 1%-10%^[47]. Though delayed hemorrhage may develop several days after EST, most of the bleedings occur just after EST. For this reason, effective control of intra-procedural hemorrhage is of great importance for the prevention of late post-EST bleedings. Several methods were suggested to control EST-related bleedings, with various grades of success^[48]. The classical therapeutic methods for the EST-induced hemorrhages are endoscopic, surgical and radiological interventions. The reported means of endoscopic management consist of: argon plasma coagulation, electrocoagulation, injection therapy with various agents, and hemoclipping^[49,50]. Since complete control of hemorrhages are not always possible *via* using those methods, novel hemostatic agents like ABS offer promising results in controlling post-sphincterotomy bleedings. We have recently reported the successful application of ABS in a 43-year-old woman that has underwent ERCP for cholangitis due to multiple bile duct stones^[13]. After mild sphincterotomy, early bleeding from the sphincterotomy site was observed. Despite management with electrocoagulation and injection therapy with epinephrine, the bleeding remained uncontrolled. Subsequently, we injected 3 mL of ABS *via* the working channel of the duodenoscope to the bleeding areas. After a rapid and effective hemostatic response was successfully achieved, the procedure was terminated. Figure 3 shows an early bleeding during the endoscopic sphincterotomy, which has been controlled *via* the topical application of ABS.

Mallory-Weiss syndrome

Mallory-Weiss syndrome (MWS) was determined to be the cause of upper GI bleeding in 3%-10% of cases^[51]. Bleeding in MWS usually stops spontaneously and patients can benefit from conservative medical treatment. Unfortunately, patients, especially those with stigmata of active

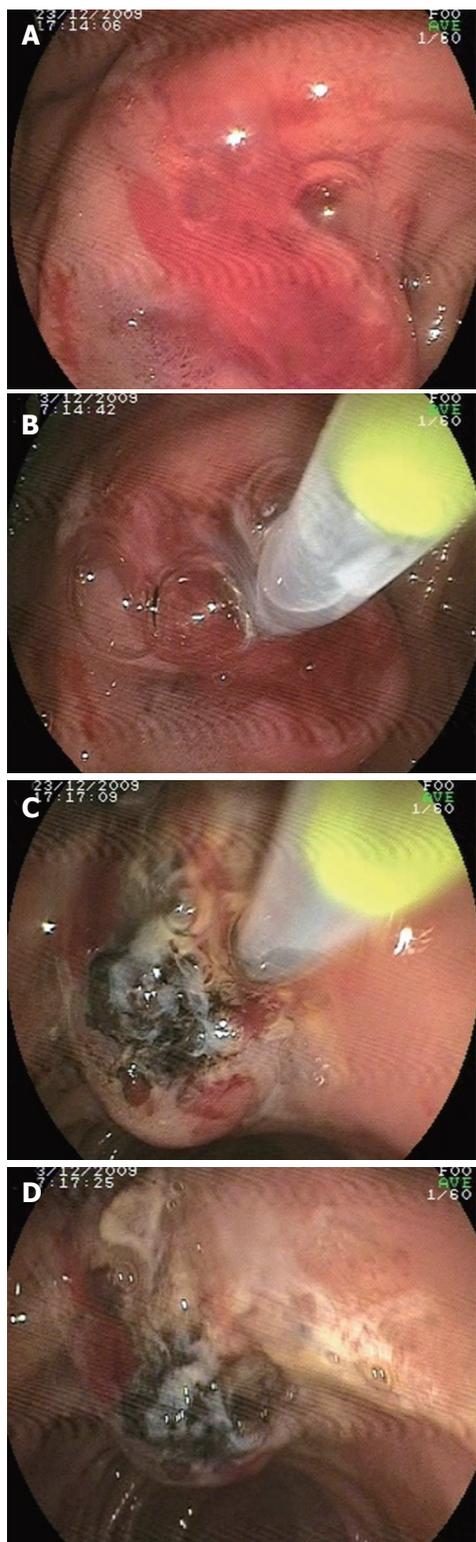


Figure 3 Ankaferd application during endoscopic sphincterotomy. A: Early bleeding during the endoscopic sphincterotomy; B: Ankaferd blood stopper (ABS) topically applied to the bleeding area; C: Hemorrhage was immediately controlled just after topical ABS administration; D: The bleeding site was covered by the hemostatic network related with the ABS application and hemorrhage was stopped.

bleeding and unstable vital signs at admission and/or associated co-morbid diseases, may require hemostatic intervention like hemoclip application, adrenaline injection and

band ligation^[52-54]. In a report by Ozaslan *et al.*^[35], a 62-year-old man with bleeding MWS was successfully treated with 13 mL of ABS. In another report, topical administration of ABS after unsuccessful combined endoscopic treatment in a warfarin-treated patient with bleeding MWS resulted in successful control after 7 mL of ABS application^[22]. This case demonstrates the effectiveness of ABS even in patients receiving anticoagulant therapy, which could possibly broaden the use of ABS in distinct states of gastrointestinal bleeding with hemorrhagic diathesis.

Dieulafoy's lesion

Dieulafoy's lesion (DL) is an uncommon, but important, cause of upper gastrointestinal bleeding consisting of a submucosal ectatic artery in the gastrointestinal tract and has a high mortality rate when diagnosis and treatment are delayed. It accounts for 0.3%-6.7% of all causes of upper GI bleeding^[55-57]. Endoscopic therapy is the current "standard-of-care" for patients with DL, because the lesions are commonly in an accessible localization with upper GI endoscopy^[56]. Unfortunately, endoscopic therapy sometimes fails to control active bleeding, resulting in hemorrhagic shock, circulatory collapse associated with increased morbidity, and even mortality. The first experience with ABS in a 63-year-old patient with DL was reported by Kurt *et al.*^[21]. In a recent paper, ABS was also shown to be effective in bleeding DL as an adjuvant modality in two patients^[22].

Variceal upper gastrointestinal bleeding

Variceal bleeding is one of the most serious and life-threatening complications of cirrhosis and portal hypertension, with mortality exceeding 50% in severe or advanced liver disease in acute variceal hemorrhage^[42]. Gastroesophageal varices are present in approximately 50%-60% of patients with cirrhosis. The prevalence of variceal hemorrhage is approximately 5%-15% yearly, and early variceal rebleeding has a rate of occurrence of 30%-40% within the first 6 wk^[29]. Despite urgent endoscopic and/or pharmacological therapy, variceal bleeding cannot be controlled, or recurs early, in about 10%-20% of patients with considerable morbidity and mortality rates^[58]. Although, endoscopic band ligation (EBL) and sclerotherapy are the choice of endoscopic treatment modalities for both active variceal bleeding and for secondary prophylaxis, application difficulties during active bleeding necessitated a search for new techniques and agents that are effective and safe. Furthermore, ease of administration, not requiring much experience and non-toxicity (even if the endoscopist could not locate the exact bleeding site), and injecting ABS to the close proximity to the suspected bleeding area may stop the variceal bleeding immediately. For that reason, ABS seems to offer a practical alternative in the setting of gastroesophageal variceal bleeding. Recently Tuncer *et al.*^[14] reported a patient with a fundal variceal hemorrhage that was effectively treated with 6 mL of ABS. Immediate hemostasis was achieved in 18 s without any further treatment. Control endoscopy was performed on day 5 that revealed clean surface

Table 1 Current data regarding the use of Ankaferd blood stopper in distinct states of gastrointestinal bleedings

Reference	Year	n	Diagnosis	Mean ABS volume (mL)
Ibis <i>et al</i> ^[20]	2008	1	Solitary rectal ulcer	10
Kurt <i>et al</i> ^[21]	2008	1	Dieulafoy lesion	12
Kurt <i>et al</i> ^[42]	2008	1	Distal cholangiocellular carcinoma	15
Tuncer <i>et al</i> ^[14]	2010	1	Fundal variceal bleeding	6
Ozaslan <i>et al</i> ^[17]	2009	1	Radiation colitis	20
Kurt <i>et al</i> ^[43]	2010	3	Rectum cancer	5, 14
		7	Gastric cancer	7, 9
Ozaslan <i>et al</i> ^[44]	2010	5	Gastric cancer	8
		1	Periampullary cancer	10
Beyazit <i>et al</i> ^[13]	2010	1	Sphincterotomy bleeding	3
Ozaslan <i>et al</i> ^[15]	2010	1	Variceal bleeding	10
Karaman <i>et al</i> ^[16]	2010	5	Colonic postpolypectomy bleeding	5-6
		2	Gastric postpolypectomy bleeding	5-6
		1	Oozing visible vessel at duodenum	5
Shorbagi <i>et al</i> ^[18]	2010	1	Radiation proctitis	20
Ozaslan <i>et al</i> ^[19]	2010	8	Radiation proctitis	20-30
Kurt <i>et al</i> ^[22]	2010	6	Gastric postpolypectomy bleeding	10
		2	Duodenal postpolypectomy bleeding	5, 9
		3	Colonic postpolypectomy bleeding	3, 17
		4	Gastric biopsy	10
		2	Dieulafoy lesion	47
		3	Radiation colitis	24
		2	GAVE	15
		1	Congestive gastropathy	10
Ozaslan <i>et al</i> ^[35]	2010	5	Peptic ulcer	2, 7
		2	Acute erosive gastropathy	5
		1	Esophageal ulcer	7
		1	Mallory-Weiss	13
Purnak <i>et al</i> ^[36]	2011	1	Peptic ulcer	20
Zulfikar <i>et al</i> ^[46]	2011	1	Esophageal cancer	6

ABS: Ankaferd blood stopper; GAVE: Gastric antral vascular ectasia.

fundal varices and a successful variceal obscuration by cyanoacrylate injection that was performed subsequently. Similarly, in a case report by Ozaslan *et al*^[15], a patient with alcoholic cirrhosis who developed severe bleeding during an elective EBL session due to immediate band slippage underwent endoscopic topical application of ABS, which was then associated with the cessation of the hemorrhage. Although both of these reports seem to be encouraging, further controlled randomized studies are required to validate the effectiveness of ABS in the therapy of gastroesophageal varices. Current data regarding the use of ABS in GI bleedings is summarized in Table 1.

LOWER GASTROINTESTINAL BLEEDING

Post-polypectomy bleeding

Bleeding following endoscopic polypectomy is the most common complication of colonic polypectomy^[59], occurring in 0.3%-6.1% of polypectomies in various reports^[60-62]. Bleeding can occur immediately following polypectomy or be delayed for hours or even up to 29 d^[63]. Acute bleeding is due to the involvement of an underlying artery or inadequate coagulation of the polyp stalk and is usually self limiting, although active arterial bleeding can occur acutely.

The effectiveness of ABS for post-polypectomy bleeding was shown by Karaman *et al*^[16] in 7 patients with post-polypectomy bleeding (5 cases of colonic, 2 cases of gastric

polypectomy). ABS application was reported to be performed as a first choice in 5 cases, and after failed attempts with endoscopic interventions in 2 patients. Bleeding following polypectomy was stopped with ABS application in all of the cases without any other complication or re-bleeding. In a recent case series by Kurt *et al*^[22], ABS application in a total of 11 patients (8 gastroduodenal, 3 colonic) with post-polypectomy bleeding resulted in the successful control of active bleeding.

Radiation colitis

Radiation proctitis (RP) is a relatively common late complication of pelvic radiation, commonly given for prostate, rectal, and gynecologic malignancies. The main symptoms of chronic RP are hematochezia (sometimes quite severe), urgency, constipation, tenesmus, diarrhea, and rectal pain. While mild cases may settle spontaneously over some months, severe hemorrhagic RP may require repeated blood transfusions and is difficult to treat with medical therapy such as sulfasalazine, corticosteroid enemas, and sucralfate (given orally or as an enema)^[64,65]. Currently, argon plasma coagulation (APC) and local application of formalin are being used as main successful measures for therapy of RP, while APC treatment offers a safe non-contact method of delivering hemostasis compared to formalin^[64-66]. Although complete healing of RP is not expected, even with APC or formalin, the measurement of efficacy with current treatments have been reported

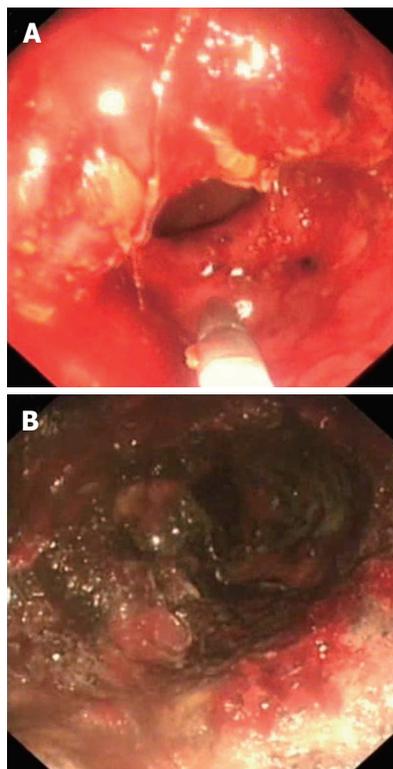


Figure 4 Endoscopic images of the distal rectum in a patient with radiation proctitis. A: Before Ankaferd blood stopper (ABS) application with fresh bleeding; B: After ABS application with grayish-yellow coagulum formation covering the diseased area.

as decreased rectal bleeding, reduced transfusion requirement, improvements in endoscopic appearance and quality of life for the patients. In this setting, ABS may offer an exciting alternative in the treatment of RP, due to its ease of application, non-toxicity, and speed of action. It has a short procedure time and very simple technique (only spraying targeted or even just close to the bleeding area), and does not require expensive equipment like APC. Moreover, it offers two unique advantages to other modalities that are used in APC therapy; it doesn't require precise localization of the site of bleeding when applied, and simple topical application over the whole lesion could suffice for the entire coating of the affected mucosa (Figure 4). The first case of successful ABS application in radiation colitis was reported by Ozaslan *et al*^[17] in a 71-year-old woman who had undergone pelvic radiotherapy due to cancer of the cervix. A total of 20 mL ABS was used with a sclerotherapy needle on the lesion and resulted in a greyish-yellow discoloration of the affected mucosa with cessation of bleeding. Three further sessions were carried out on a weekly basis to complete the healing with no signs of re-bleeding in the following days. At follow-up, the giant ulcerated lesion was reported to have almost disappeared, with only mild residual erosions and friability remaining. A difficult case of radiation proctitis that was managed by ABS was also reported by Shorbagi *et al*^[18] in a 70-year-old patient with failed management of both medical and endoscopic interventions with APC. In this report, approximately 20 mL of ABS solution was applied to the affected areas by using a disposable washing pipe,

which resulted in the immediate control of bleeding. The authors concluded that ABS would be a useful adjuvant to APC since, by controlling the active bleed, it may help to better localize and target telangiectasias. Kurt *et al*^[22] reported 3 patients with radiation colitis which was primarily managed with APC. Adjuvant application of ABS in these patients resulted in a more sustained control of bleeding. Aside from these reports, an observational study was also conducted in 8 patients with bleeding due to chronic RP in which ABS was applied as a primary therapy^[19]. In this study, ABS was instilled onto the bleeding areas by sclerotherapy needle or heater probe catheter, once a week, at a dose of 20-30 mL per session. ABS-induced hemostasis lasted for 1-8 d per session, and was achieved in seven of eight cases. In the eighth case, bleeding was only lessened. However, recurrence of bleeding was seen in all patients and ABS was found to be ineffective on telangiectasia at the last follow-up. As a result, ABS was only found to be effective in healing radiation-induced ulcers with no prolonged effect on bleeding telangiectasias due to RP.

Based on current observations, ABS may lead to the apparent healing of ulcers, but it might not be useful for the healing of telangiectasia or as a definitive therapy for bleeding in patients with chronic RP.

Solitary rectal ulcer

Solitary rectal ulcer (SRU) is a rare rectal disorder that can be present with bleeding, passage of mucus, straining during defecation, and a sense of incomplete evacuation^[67]. Although bleeding due to a rectal ulcer commonly stops spontaneously, re-bleeding is a major matter of concern, despite effective endoscopic interventions. Recently in a paper by Ibis *et al*^[20], topical application of 10 mL of ABS onto the ulcer through a disposable washing pipe resulted in successful control of the bleeding. Furthermore, complete healing of a bleeding SRU located adjacent to the anal canal prevented a potential risk for infection with fecal passage.

Neoplastic lower gastrointestinal bleeding

Colon cancer is the predominant cause of neoplastic bleeding. It accounts for up to 2%-9% of cases of hematochezia and is, by far, the most frequent cause of iron-deficiency anemia and the source of chronic lower GI bleeding^[68]. The bleeding is usually low-grade and recurrent, occurring as a result of erosions and ulceration on the surface of the tumor and often exacerbated by the use of NSAIDs. Although several endoscopic treatment modalities can be used to achieve hemostasis, when the bleeding tumoral lesion is identified in a colonoscopic examination, the majority of patients require surgical management due to increased re-bleeding rates, which can be as high as 80% up to 1 mo after the procedure. For that reason, alternative approaches are required, especially in inoperable cases or as a bridge to elective surgery. In this setting, ABS as a novel hemostatic agent could have a potential benefit in controlling bleeding from GI tumors. In a retrospective analysis, the effectiveness of ABS in lower GI bleeding due to rectal carcinoma was shown in three patients^[43]. Hemostasis was achieved in all three patients

within seconds following ABS application, with no adverse events.

CONCLUSION

ABS, which has long been used as a traditional folkloric medicinal plant extract, represents an effective alternative treatment modality as a modern topical hemostatic agent for GI bleeding either as an adjuvant or primary agent complementing conventional methods. Although ABS is still in the early developmental stages as a drug, observations from published series with encouraging results provide evidence for the preliminary safety and efficacy of ABS in distinct states of GI bleeding as a haemostatic agent^[13-22,35,42-46]. Future randomized controlled trials will elucidate whether ABS would be as much of a novel, safe and effective treatment option in the setting of GI bleeding.

ACKNOWLEDGMENTS

This article is dedicated to my clinical teachers Professor Dr. Abdullah Sonsuz, Professor Dr. Hakan Sentürk, Professor Dr. Hülya Cetinkaya, Professor Dr. Recep Öztürk, and Professor Dr. Sebati Özdemir in gratitude for having guided me into Clinical Hepatology.

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S- Editor Tian L L- Editor Rutherford A E- Editor Ma WH

A new look at anti-*Helicobacter pylori* therapy

Seng-Kee Chuah, Feng-Woei Tsay, Ping-I Hsu, Deng-Chyang Wu

Seng-Kee Chuah, Division of Gastroenterology, Kaohsiung Chang Gung Memorial Hospital, College of Medicine, Chang Gung University, 833 Kaohsiung, Taiwan

Feng-Woei Tsay, Ping-I Hsu, Division of Gastroenterology, Department of Internal Medicine, Kaohsiung Veterans General Hospital, National Yang-Ming University, 813 Kaohsiung, Taiwan

Ping-I Hsu, Department of General Medicine, School of Medicine, College of Medicine, Taipei Medical University, 110 Taipei, Taiwan

Deng-Chyang Wu, Department of Internal Medicine, Kaohsiung Medical University Hospital, 807 Kaohsiung, Taiwan

Author contributions: Chuah SK wrote the paper, Tsay FW and Wu DC revised the paper, Hsu PI drafted and approved the final version.

Correspondence to: Dr. Ping-I Hsu, Division of Gastroenterology, Department of Internal Medicine, Kaohsiung Veterans General Hospital, 386 Ta Chung 1st Road, 813 Kaohsiung, Taiwan. williamhsup@yahoo.com.tw

Telephone: +886-7-3462074 Fax: +886-7-3468237

Received: May 25, 2011 Revised: August 11, 2011

Accepted: August 15, 2011

Published online: September 21, 2011

Abstract

With the rising prevalence of antimicrobial resistance, the treatment success of standard triple therapy has recently declined to unacceptable levels (i.e., 80% or less) in most countries. Therefore, several treatment regimens have emerged to cure *Helicobacter pylori* (*H. pylori*) infection. Novel first-line anti-*H. pylori* therapies in 2011 include sequential therapy, concomitant quadruple therapy, hybrid (dual-concomitant) therapy and bismuth-containing quadruple therapy. After the failure of standard triple therapy, a bismuth-containing quadruple therapy comprising a proton pump inhibitor (PPI), bismuth, tetracycline and metronidazole can be employed as rescue treatment. Recently, triple therapy combining a PPI, levofloxacin and amoxicillin has been proposed as an alternative to the standard rescue therapy. This salvage regimen can achieve a higher eradication rate than bismuth-containing quadruple therapy in some regions and has less adverse effects. The best

second-line therapy for patients who fail to eradicate *H. pylori* with first-line therapies containing clarithromycin, amoxicillin and metronidazole is unclear. However, a levofloxacin-based triple therapy is an accepted rescue treatment. Most guidelines suggest that patients requiring third-line therapy should be referred to a medical center and treated according to the antibiotic susceptibility test. Nonetheless, an empirical therapy (such as levofloxacin-based or furazolidone-based therapies) can be employed to terminate *H. pylori* infection if antimicrobial sensitivity data are unavailable.

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Key words: Bismuth-containing quadruple therapy; Concomitant quadruple therapy; Hybrid (dual-concomitant) therapy; Rescue anti-*Helicobacter pylori* treatment; Sequential therapy

Peer reviewers: György M Buzás, MD, Department of Gastroenterology, Ferencváros Health Center, IX. District Polyclinic, Mester u 45, 1095 Budapest, Hungary; David J McGee, PhD, Associate Professor, Department of Microbiology and Immunology, Louisiana State University Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130, United States; Nawfal Hussein, PhD, Centre for Biomolecular Sciences, University of Nottingham, University Park, Nottingham, NG7 2RD, United Kingdom

Chuah SK, Tsay FW, Hsu PI, Wu DC. A new look at anti-*Helicobacter pylori* therapy. *World J Gastroenterol* 2011; 17(35): 3971-3975 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/3971.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.3971>

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is the main cause of gastritis, gastroduodenal ulcer disease, gastric adenocarcinoma and mucosa-associated tissue lymphoma. The Maastricht III Consensus Report has recommended that proton pump inhibitor (PPI)-clarithromycin-amoxicillin

or metronidazole treatment for 7 to 14 d is the first choice treatment for *H. pylori* infection^[1]. As a general rule for the treatment of other infectious diseases, clinicians should prescribe therapeutic regimens that have a per-protocol eradication rate $\geq 90\%$ for anti-*H. pylori* therapy^[2]. However, several large clinical trials and meta-analyses have shown that the eradication rate of the standard triple therapy has generally declined to unacceptable levels (i.e., 80% or less) recently^[3,4]. In some European countries, the success rates are disappointingly low with values of only 25%-60%^[5,6]. The reasons for this fall in efficacy with time are uncertain but may be related to the increasing incidence of clarithromycin-resistant strains of *H. pylori*^[3,4,7,8]. This article will review the most recent literature in an attempt to introduce novel first-line eradication regimens with a per-protocol eradication rate exceeding 90% and rescue regimens with an eradication rate exceeding 80%.

FIRST-LINE THERAPY

The main reasons for eradication failure of *H. pylori* infection include antibiotic resistance, poor compliance and rapid metabolism of PPI^[3]. Clarithromycin resistance is the major cause of eradication failure for standard triple therapy^[8]. Pooled data from 20 studies involving 1975 patients treated with standard triple therapy showed an eradication rate of 88% in clarithromycin-sensitive strains *vs* 18% in clarithromycin-resistant strains^[8]. Therefore, the background rate of clarithromycin resistance is critically important as its presence negatively impacts the efficacy of standard triple therapy. A systemic review showed that the rate of clarithromycin-resistant strains ranged from 49% (Spain) to 1% (Netherlands) worldwide^[9]. In areas with clarithromycin resistance of $< 10\%$ [i.e., Netherlands, Sweden, Ireland, Germany, Malaysia, Taiwan (South)], it is still possible to employ a standard triple therapy to achieve a per-protocol eradication rate $> 90\%$. However, standard triple therapies should be abandoned in areas with clarithromycin resistance $\geq 20\%$ [i.e., Spain, Turkey, Italy (Central), Alaska, China, Japan, Cameroon] because the per-protocol eradication rates of standard therapies are often less than 85% and the intention-to-treat eradication rates are usually less than 80%^[2-8,10].

Recently, several studies showed that a novel 10-d sequential therapy can achieve a promising success rate of 90%-94%^[11-13]. The regimen consists of a 5-d dual therapy with a PPI (standard dose, *b.i.d.*) and amoxicillin (1 g, *b.i.d.*) followed by a 5-d triple therapy with a PPI (standard dose, *b.i.d.*), clarithromycin (500 mg, *b.i.d.*) and metronidazole (500 mg, *b.i.d.*). Gatta *et al.*^[12] reported a rigorous systematic review that identified 13 trials evaluating 3271 patients. The data showed that sequential therapy achieved a 12% better absolute eradication rate than the standard triple therapy. A randomized, double-blind, placebo-controlled trial demonstrated that the per-protocol eradication rates of sequential therapy and standard triple therapy for clarithromycin-resistant strains were 89% and 29%, respec-

tively^[11]. However, it should be noted that most of the studies concerning sequential therapies were conducted in Italy. A recent trial in Korea showed that the per-protocol eradication rates of sequential therapy and standard triple therapy were 86% and 77%, respectively. The two therapies had comparable eradication rates^[14]. There is, therefore, a clear need for well-designed randomized trials from countries other than Italy to determine the real advantages of this novel therapy.

Concomitant therapy is another novel regimen which was proved successful in the presence of clarithromycin resistance^[15]. This is a 4-drug regimen containing a PPI (standard dose, *b.i.d.*), clarithromycin (500 mg, *b.i.d.*), amoxicillin (1 g, *b.i.d.*) and metronidazole (500 mg, *b.i.d.*) which are all given for the entire duration of therapy. This therapy is superior to standard triple therapy for *H. pylori* eradication^[15]. It is also less complex than sequential therapy as this regimen does not involve changing drugs halfway through. A head-to-head non-inferiority trial of 10-d sequential and 10-d concomitant therapy showed that they were equivalent (93.1% *vs* 93.0% by per-protocol analysis)^[16].

Recently, Hsu *et al.*^[17] reported a hybrid (dual-concomitant) therapy consisting of a dual therapy with a PPI (standard dose, *b.i.d.*) and amoxicillin (1 g, *b.i.d.*) for 7 d followed by a concomitant quadruple therapy with a PPI (standard dose, *b.i.d.*), amoxicillin (1 g, *b.i.d.*), clarithromycin (500 mg, *b.i.d.*) and metronidazole (500 mg, *b.i.d.*) for 7 d. The new therapy extends the duration of amoxicillin treatment to 14 d and concomitantly employs three antibiotics in the last 7 d of the treatment course. In 117 *H. pylori*-infected subjects, the novel therapy provided excellent eradication rates of 99% and 97% according to per-protocol and intention-to-treat analysis, respectively. It is important to note that the new therapy has a high efficacy in the treatment of *H. pylori* strains harboring dual resistance to clarithromycin and metronidazole. Several studies have shown that sequential therapy is ineffective in clearing *H. pylori* with dual resistance^[8]. The prolonged treatment duration of amoxicillin to 14 d in the hybrid therapy might account for the higher eradication rate in the face of *H. pylori* strains with dual resistance to clarithromycin and metronidazole. Further studies in populations with different levels of prevalence of clarithromycin and metronidazole resistance are needed to assess the efficacy of the new regimen.

Bismuth-containing quadruple therapy is an alternative first choice treatment for *H. pylori* infection recommended by the Maastricht III Consensus Report^[1] and the Second Asia-Pacific Consensus Guidelines for *H. pylori* Infection^[18]. Two studies each with more than 100 patients have shown eradication rates of $> 90\%$ with this combination given for 10 d^[19,20]. Recently, Malfertheiner *et al.*^[21] compared the efficacy of a 10-d bismuth-containing quadruple therapy (omeprazole, bismuth, metronidazole and tetracycline) and a 7-d triple therapy (omeprazole, clarithromycin and amoxicillin). The data indicated that the former had a higher eradication rate than the latter (93%

vs 70% by per-protocol analysis). Currently, the optimal treatment duration of bismuth-containing quadruple therapy remains unclear but a 10-14 d course is most commonly employed in clinical practice^[22].

Based on a large body of published clinical trials, a quinolone-based triple therapy is effective in the first-line therapy of *H. pylori* infection. The eradication rates of levofloxacin-based triple therapy ranged from 72% to 96%^[23]. The regimen might be considered in populations with clarithromycin resistance greater than 15%-20% and quinolone resistance less than 10%^[23]. However, a quinolone-based triple therapy is not generally recommended as first-line therapy at the moment due to concerns about the rising prevalence of quinolone-resistant strains in the first-line and second-line anti-*H. pylori* therapies. Furthermore, greater use of quinolone would likely result in the development of more quinolone-resistant pathogens causing respiratory and urogenital tract infections.

SECOND-LINE THERAPY

The Maastricht III Consensus Report recommended a bismuth-containing quadruple therapy regimen comprising a PPI, bismuth, metronidazole and tetracycline as second-line therapy^[1]. This rescue regimen fails in 5%-63% of patients with an average eradication rate of 76% on the basis of a pooled analysis^[24,26]. The prevalence of metronidazole-resistant strains, dose and duration of rescue therapy seem to be important variables for the efficacy of this treatment. In bismuth-containing quadruple regimens, PPI should be prescribed in the usual dose and twice a day, colloidal bismuth subcitrate 120 mg four times a day, tetracycline 500 mg four times a day and metronidazole 500 mg three times a day. A report from Korea showed that the two-week bismuth-containing quadruple therapy was more effective than the 1-wk treatment (83% *vs* 64% by intention-to-treat analysis)^[27].

Levofloxacin-based triple therapy consisting of levofloxacin (500 mg, *q.d.*), amoxicillin (1 g, *b.i.d.*) and a PPI (standard dose, *b.i.d.*) represents an encouraging strategy for second-line therapy. A meta-analysis by Saad *et al.*^[28] showed that a 10-d regimen of levofloxacin-based triple therapy was superior to a 7-d bismuth-based quadruple therapy. Another meta-analysis by Gisbert *et al.*^[29] demonstrated a borderline significance of higher *H. pylori* cure rates with levofloxacin-based triple regimens compared with quadruple therapies (81% *vs* 70%). Additionally, the study revealed fewer adverse effects with levofloxacin than with quadruple regimens (19% *vs* 44%). However, it is noteworthy that levofloxacin-based triple therapies seem less effective in Asia. Two randomized controlled trials from Taiwan and Hong Kong showed that levofloxacin-based triple therapy was comparable to quadruple therapy in the eradication efficacy of second-line therapy.^[30,31] The second-line therapy for patients who fail to eradicate *H. pylori* new first-line therapies (such as sequential therapy, concomitant therapy or hybrid therapy) remains unclear. However, a recent study showed that a le-

vofloxacin-based triple therapy with lansoprazole (30 mg, *b.i.d.*), levofloxacin (250 mg, *b.i.d.*) and amoxicillin (1 g, *b.i.d.*) achieved a high eradication rate in patients who failed to clear *H. pylori* with sequential therapy^[32].

THIRD-LINE THERAPY

Currently, a standard empirical third-line therapy is lacking. The Maastricht III Consensus Report recommended using bacterial culture with antimicrobial sensitivity tests to select antibiotics for third-line regimens^[1]. Cammarota *et al.*^[33] analyzed *H. pylori* isolates from 94 consecutive patients in whom *H. pylori* infection had persisted after two eradication attempts. Ninety-four subjects (100%) were resistant to metronidazole, 89 (95%) to clarithromycin, 29 (31%) to levofloxacin and five (5%) to tetracycline. No resistance to amoxicillin was found in any of the patients. Patients were then treated with a culture-guided, third-line regimen: 89 patients with a 1-wk quadruple regimen including omeprazole, bismuth, doxycycline and amoxicillin, and five patients with a 1-wk triple regimen containing omeprazole, amoxicillin and levofloxacin or clarithromycin. Overall, *H. pylori* eradication was obtained in 90% of subjects treated by the culture-guided therapy.

However, it has been reported that the sensitivity of culture is less than 60%^[34]. Additionally, *in vitro* antimicrobial sensitivity does not necessarily lead to eradication *in vivo* and *vice versa*. Recently, several empirical third-line therapies have been proposed to treat refractory *H. pylori* infection. A 10-d quadruple therapy comprising rabeprazole (20 mg *b.i.d.*), bismuth subcitrate (300 mg, *q.i.d.*), amoxicillin (500 mg, *q.i.d.*), and levofloxacin (500 mg, *q.d.*) achieved an eradication rate of 84% by both intention-to-treat analysis and per-protocol analysis in patients who failed to eradicate *H. pylori* with standard triple therapy and bismuth-based quadruple therapy^[35].

Rifabutin is an antituberculous agent, which can be administered with PPI and amoxicillin for 10-14 d to eradicate *H. pylori*. One study used rifabutin (150 mg, *b.i.d.*), amoxicillin (1 g, *b.i.d.*) and omeprazole (20 mg, *b.i.d.*) for 14 d as a third-line therapy^[36]. Per-protocol and intention-to-treat eradication was achieved in 11/14 patients (79%). It is noteworthy that serious myelotoxicity and ocular adverse events have been reported with rifabutin therapy^[37]. In addition, greater use of rifabutin would likely result in the development of more resistant strains to *Mycobacterium tuberculosis* and *Mycobacterium avium*.

Furazolidone-based therapy is another useful option to treat refractory *H. pylori* infection. A 7-d quadruple therapy consisting of lansoprazole (30 mg, *b.i.d.*), tripotassium dicitratobismuthate (240 mg, *b.i.d.*), furazolidone (200 mg, *b.i.d.*) and tetracycline (1 g, *b.i.d.*) has a high efficacy in third-line therapy with an eradication rate of 90% by both intention-to-treat and per-protocol analysis^[38]. The recommended regimens for *H. pylori* therapies are summarized in Table 1.

In conclusion, with the rising prevalence of antimicrobial resistance, the treatment success of standard

Table 1 Recommended regimens for *Helicobacter pylori* therapy

Treatment	Regimen
First-line therapy	
Standard triple therapy ¹	A PPI (standard dose, <i>b.i.d.</i>), clarithromycin (500 mg, <i>b.i.d.</i>) and amoxicillin (1 g, <i>b.i.d.</i>) for 7-14 d
Sequential therapy	A 5-d dual therapy with a PPI (standard dose, <i>b.i.d.</i>) and amoxicillin (1 g, <i>b.i.d.</i>) followed by a 5-d triple therapy with a PPI (standard dose, <i>b.i.d.</i>), clarithromycin (500 mg, <i>b.i.d.</i>) and metronidazole (500 mg, <i>b.i.d.</i>)
Concomitant therapy	A PPI (standard dose, <i>b.i.d.</i>), clarithromycin (500 mg, <i>b.i.d.</i>), amoxicillin (1 g, <i>b.i.d.</i>) and metronidazole (500 mg, <i>b.i.d.</i>) for 7-10 d
Hybrid therapy	A 7-d dual therapy with a PPI (standard dose, <i>b.i.d.</i>) and amoxicillin (1 g, <i>b.i.d.</i>) followed by a 7-d quadruple therapy with a PPI (standard dose, <i>b.i.d.</i>), amoxicillin (1 g, <i>b.i.d.</i>), Clarithromycin (500 mg, <i>b.i.d.</i>) and metronidazole (500 mg, <i>b.i.d.</i>)
Bismuth-containing quadruple therapy	A PPI (standard dose, <i>b.i.d.</i>), bismuth (standard dose, <i>q.i.d.</i>) tetracycline (500 mg, <i>q.i.d.</i>) and metronidazole (250 mg, <i>q.i.d.</i>) for 10-14 d
Second-line therapy	
Bismuth-containing quadruple therapy	A PPI (standard dose, <i>b.i.d.</i>), bismuth (standard dose, <i>q.i.d.</i>) tetracycline (500 mg, <i>q.i.d.</i>) and metronidazole (500 mg, <i>t.i.d.</i>) for 10-14 d
Levofloxacin-based triple therapy ²	A PPI (standard dose, <i>b.i.d.</i>), levofloxacin (500 mg, <i>q.d.</i>) and amoxicillin (1 g, <i>b.i.d.</i>) for 10 d
Third-line therapy	
Culture-guided therapy	A 10-d quadruple therapy comprising a PPI (standard dose, <i>b.i.d.</i>), bismuth (standard dose, <i>q.i.d.</i>) and two antibiotics selected by antimicrobial sensitivity tests
Levofloxacin-based quadruple therapy	A PPI (standard dose, <i>b.i.d.</i>), bismuth (standard dose, <i>q.i.d.</i>), levofloxacin (500 mg, <i>q.d.</i>) and amoxicillin (500 mg, <i>q.i.d.</i>) for 10 d
Rifabutin-based triple therapy	A PPI (standard dose, <i>b.i.d.</i>), rifabutin (150 mg <i>b.i.d.</i>) and amoxicillin (1 g <i>b.i.d.</i>) for 14 d
Furazolidone-based quadruple therapy	A PPI (standard dose, <i>b.i.d.</i>), tripotassium dicitratobismuthate (240 mg, <i>b.i.d.</i>), furazolidone (200 mg, <i>b.i.d.</i>) and tetracycline (1 g, <i>b.i.d.</i>)

¹Employed in areas with clarithromycin resistance < 10% and abandoned in areas with clarithromycin resistance ≥ 20%. ²Employed in patients who fail to eradicate *Helicobacter pylori* with standard triple therapy, sequential therapy, concomitant therapy or hybrid therapy. PPI: Proton pump inhibitor.

triple therapy has recently declined to unacceptable levels. Novel first-line anti-*H. pylori* therapies in 2011 include sequential therapy, concomitant quadruple therapy, hybrid (dual-concomitant) therapy and bismuth-containing quadruple therapy. After the failure of standard triple therapy, a bismuth-containing quadruple therapy or a levofloxacin-based triple therapy can be employed as rescue treatment. The best second-line therapy for patients who fail to eradicate *H. pylori* with first-line therapies containing clarithromycin, amoxicillin and metronidazole is unclear. However, a levofloxacin-based triple therapy is an accepted salvage treatment. With regard to third-line therapy of *H. pylori* infection, a culture-guided therapy has been recommended. If antimicrobial sensitivity data are unavailable, an empirical therapy (such as levofloxacin-based, rifabutin-based, or furazolidone-based therapies) can be employed to terminate *H. pylori* infection. In spite of many emerging therapies, none have been included into current major consensus so far. Hopefully, the upcoming Maastricht IV meeting will bring a wind of change.

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S- Editor Tian L L- Editor Webster JR E- Editor Xiong L

MicroRNAs as a potential prognostic factor in gastric cancer

Baruch Brenner, Moshe B Hoshen, Ofer Purim, Miriam Ben David, Karin Ashkenazi, Gideon Marshak, Yulia Kundel, Ronen Brenner, Sara Morgenstern, Marisa Halpern, Nitzan Rosenfeld, Ayelet Chajut, Yaron Niv, Michal Kushnir

Baruch Brenner, Ofer Purim, Institute of Oncology, Davidoff Center, Rabin Medical Center, Beilinson Hospital, Petach Tikva 49100, Israel

Baruch Brenner, Ofer Purim, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

Moshe B Hoshen, Miriam Ben David, Karin Ashkenazi, Ayelet Chajut, Michal Kushnir, Rosetta Genomics Ltd., Rehovot 76706, Israel

Moshe B Hoshen, Clalit Research Institute, Clalit Medical Services, Tel Aviv 69978, Israel

Nitzan Rosenfeld, Cancer Research UK, Cambridge Research Institute, Cambridge CB2 0RE, United Kingdom

Gideon Marshak, Institute of Oncology, Rabin Medical Center, Golda-Hasharon Hospital, Petach Tikva 49100, Israel

Yulia Kundel, Ronen Brenner, Institute of Oncology, Rabin Medical Center, Beilinson Hospital, Petach Tikva 49100, Israel

Gideon Marshak, Yulia Kundel, Ronen Brenner, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

Sara Morgenstern, Institute of Pathology, Rabin Medical Center, Beilinson Hospital, Petach Tikva 49100, Israel

Sara Morgenstern, Marisa Halpern, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

Marisa Halpern, Institute of Pathology, Rabin Medical Center, Golda-Hasharon Hospital, Petach Tikva 49100, Israel

Yaron Niv, Department of Gastroenterology, Rabin Medical Center, Beilinson Hospital, Petach Tikva 49100, Israel

Yaron Niv, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978 Israel

Author contributions: Brenner B, Hoshen MB and Purim O contributed equally to this work; Brenner B and Chajut A designed the research; Brenner B, Hoshen MB and Purim O analyzed the data; David MB, Ashkenazi K, Morgenstern S, Halpern M and Kushnir M performed the research; Brenner B, Purim O, Kundel Y, Mahrshak G and Brenner R collected the data; Brenner B, Hoshen MB, Purim O, Rosenfeld N, Niv Y and Kushnir M wrote the paper.

Correspondence to: Baruch Brenner, MD, Institute of Oncology, Davidoff Center, Rabin Medical Center, Beilinson Campus, Petach Tikva 49100, Israel. brennerb@clalit.org.il

Telephone: +972-3-9378005 Fax: +972-3-9378045

Received: February 17, 2011 Revised: April 15, 2011

Accepted: April 22, 2011

Published online: September 21, 2011

Abstract

AIM: To compare the microRNA (miR) profiles in the primary tumor of patients with recurrent and non-recurrent gastric cancer.

METHODS: The study group included 45 patients who underwent curative gastrectomies from 1995 to 2005 without adjuvant or neoadjuvant therapy and for whom adequate tumor content was available. Total RNA was extracted from formalin-fixed paraffin-embedded tumor samples, preserving the small RNA fraction. Initial profiling using miR microarrays was performed to identify potential biomarkers of recurrence after resection. The expression of the differential miRs was later verified by quantitative real-time polymerase chain reaction (qRT-PCR). Findings were compared between patients who had a recurrence within 36 mo of surgery (bad-prognosis group, $n = 14$, 31%) and those who did not (good-prognosis group, $n = 31$, 69%).

RESULTS: Three miRs, miR-451, miR-199a-3p and miR-195 were found to be differentially expressed in tumors from patients with good prognosis vs patients with bad prognosis ($P < 0.0002$, 0.0027 and 0.0046 respectively). High expression of each miR was associated with poorer prognosis for both recurrence and survival. Using miR-451, the positive predictive value for non-recurrence was 100% (13/13). The expression of the differential miRs was verified by qRT-PCR, showing high correlation to the microarray data and similar separation into prognosis groups.

CONCLUSION: This study identified three miRs, miR-451, miR-199a-3p and miR-195 to be predictive of recurrence of gastric cancer. Of these, miR-451 had the strongest prognostic impact.

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Key words: MicroRNA; Prognosis; Recurrence; Gastric cancer

Peer reviewer: Ondrej Slaby, PhD, Assistant Professor, Masaryk Memorial Cancer Institute, Department of Comprehensive Cancer Care, Zlutý kopec 7, 65653 Brno, Czech Republic

Brenner B, Hoshen MB, Purim O, David MB, Ashkenazi K, Marshak G, Kundel Y, Brenner R, Morgenstern S, Halpern M, Rosenfeld N, Chajut A, Niv Y, Kushnir M. MicroRNAs as a potential prognostic factor in gastric cancer. *World J Gastroenterol* 2011; 17(35): 3976-3985 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/3976.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.3976>

INTRODUCTION

Gastric cancer is a highly aggressive and lethal malignancy. It accounts for 8.6% of all new cancer cases worldwide, and is the second leading cause of cancer deaths. Of the estimated 930 000 people newly diagnosed with gastric cancer each year, some 700 000 will die of the disease^[1]. Although surgery is the standard treatment of localized gastric cancer, the results are often disappointing, with recurrence rates as high as 70% after successful complete (R0) resection. Attempts to improve outcome with adjuvant therapy have yielded only modest success. Trials of postoperative chemoradiation or perioperative chemotherapy report only a 10%-15% absolute reduction in the risk of recurrence^[2,3]. Moreover, the adjuvant therapy regimens used in these studies were themselves associated with significant morbidity and even mortality.

These findings emphasize the need for better selection of patients for the various treatment strategies. For example, patients with a good prognosis may be spared adjuvant therapy whereas those with a poor prognosis may receive such treatment or even be offered investigational programs. However, at present, the prognosis of the individual patient is determined solely by the extent of local tumor spread [Tumor, Node Status, Metastasis (TNM) staging]^[4]. Other factors, such as the patient's age and sex, tumor grade and presence of vascular invasion and perineural spread, add little to the ability of clinicians to distinguish between patients with a good and bad prognosis^[5-7]. A nomogram incorporating multiple clinical and pathological parameters that was created to predict survival after R0 resection, but it has not been adopted by the medical community^[8]. Clearly, novel effective prognostic markers are still lacking in gastric cancer.

MicroRNAs (miRs) are short non-coding RNAs, 17-22 nucleotides in length, which regulate gene expression and thereby play significant roles in human development and various pathological conditions^[9-11]. The expression of miRs is dynamic and corresponds to the physiological situation, raising the possibility that miR profiles derived from tumoral specimens may be able to serve as diagnostic or prognostic biomarkers^[12-15]. Indeed, recent studies have shown an association of miR expression and different malignancies^[16-20]. Their prognostic role in gastric cancer is unknown, but preliminary findings are encouraging^[21-26].

The aim of the present study was to compare the miR profiles in surgically resected primary gastric cancer tumors between patients with and without recurrence to evaluate their prognostic impact.

MATERIALS AND METHODS

Patients

The study population consisted of patients with histologically confirmed adenocarcinoma of the stomach who were operated on and followed-up at the two hospitals of the Rabin Medical Center. Other inclusion criteria were as follows: treatment between 1995 and 2005, to ensure the quality of the surgical specimens on the one hand and adequate follow-up on the other; absence of distant spread; and minimum of 36 mo follow-up in those without recurrence, to reliably estimate disease-free survival. Patients with cardiac tumors extending into the gastroesophageal junction were eligible, but not patients with predominantly esophageal or gastroesophageal junction tumors (Siewert classification I - II)^[27]. All patients underwent potentially curative gastrectomies with clear margins (R0 resection). To isolate a prognostic from a predictive effect, patients who received any adjuvant or neoadjuvant therapy were excluded. Eligible patients were identified from the database of the two Institutes of Oncology at the Rabin Medical Center. The study was approved by the local Institutional Review Board.

Follow-up

The study was retrospective; therefore, the follow-up schedule for the individual patients was determined by the treating physician. At our center, patients with gastric cancer are routinely followed once every 3 to 6 mo in the first three years, regardless of the stage of their disease. Time to recurrence or death is defined from the date of surgery. At each visit, patients undergo a medical history, physical examination, and measurement of serum carcinoembryonic antigen level. Imaging tests and endoscopies are performed when clinically indicated.

For the present study, eligible patients were divided into two groups: those in whom the disease recurred during the first 36 mo of follow-up (bad-prognosis group) and those who did not have a recurrence within this period (good-prognosis group).

Pathology

Formalin-fixed paraffin-embedded blocks of the surgical specimens from the initial gastrectomies of the eligible patients were retrieved from the archives of the two Institutes of Pathology at the Rabin Medical Center. After initial patient identification, all original histological slides were reviewed, and an appropriate block containing > 50% tumor was retrieved. In the cohort used for this study the median tumor content was 78% with a range of 50%-95%. From each block, 10 slices of 10 μ m each were collected in one 1.5mL tube for RNA extraction and miR analysis. Histological type and grade, as well as

other significant tumor features (e.g., perineural invasion), were determined by a pathologist on hematoxylin-eosin-stained slides prepared from the first and/or last sections of the sample.

RNA extraction

Total RNA was extracted as described previously^[28]. Briefly, the sample was incubated several times in xylene at 57°C to remove excess paraffin and then washed several times with ethanol. Protein degradation was performed by incubation of the sample in a proteinase K solution at 45°C for a few hours. The RNA was extracted using acid phenol/chloroform and then precipitated with ethanol; DNase was introduced to digest DNA. Total RNA quantity and quality were measured using a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE).

Array platform

Custom miR microarrays have been described previously^[15]. Briefly, ~900 DNA oligonucleotide probes representing miRs (Sanger database version 10 and additional miRs predicted and validated by Rosetta Genomics) were spotted in triplicate on coated microarray slides (Nexterion[®] Slide E, Schott, Mainz, Germany), using the BioRobotics MicroGrid II microarrayer (Genomic Solutions, Ann Arbor, MI) according to the manufacturer's directions. 3.5 µg of total RNA were labeled by ligation of an RNA-linker, p-rCrU-Cy/dye (Eurogentec Inc., San-Diego, CA; Cy3 or Cy5) to the 3' end. Slides were incubated with the labeled RNA for 12-16 h at 55°C and then washed twice. Arrays were scanned using Agilent DNA Microarray Scanner Bundle (Agilent Technologies, Santa Clara, CA) at a resolution of 10 µm with 100% and 10% laser power. Array images were analyzed using SpotReader software (Niles Scientific, Portola Valley, CA). Microarray spots were combined and signals normalized as described previously^[28]. Two types of positive controls were included in the experimental design: (1) synthetic small RNAs were spiked into each RNA sample before labeling to verify labeling efficiency; and (2) probes for abundant small RNAs were spotted to validate RNA quality.

Signal calculation and normalization

The RNA fluorescence data from the slide corresponding to each patient were loaded into a single database. Microarray spots were combined and signals were normalized as described previously^[15]. Data were log-transformed and analyzed in log-space. Therefore, the expression level or signal of an individual miR referred to the normalized value. The miR profile of each patient was visually compared with the median value for all patients. Eleven samples for which the readings were clearly incomparable (i.e., overall pattern too noisy) were excluded. These samples did not differ in their survival patterns from the 45 samples that were kept for statistical analysis ($P = 0.28$ by log-rank test). Only samples that passed this analysis were included in further analyses.

Polymerase chain reaction validation

For the purposes of signal verification, 15 miRs were selected for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Nine were selected as differential miR probes and six as non-differential probes, for signal normalization. The six miRs (hsa-let-7c, hsa-miR-222, hsa-miR-22, hsa-miR-15b, hsa-miR-425 and hsa-miR-34a) that were selected for normalization had low variability across all samples in the microarray experiment and were used as endogenous controls. Linear normalization was applied as follows: the mean cycle threshold (C_T) for the miRs used for normalization was calculated for each sample and the difference between the mean C_T for each sample and the mean of the mean C_T s was subtracted from all C_T s measured for that sample. Twenty samples, 10 from the good-prognosis group and 10 from the poor-prognosis group, were analyzed. MiR amounts were quantified using a recently described qRT-PCR method^[29]. RNA was incubated in the presence of poly (A) polymerase (PAP; Takara-2180A), $MnCl_2$ and ATP for 1 h at 37°C. Then, using an oligodT primer harboring a consensus sequence, reverse transcription was performed on total RNA using SuperScript II RT (Invitrogen, Carlsbad, CA). This was followed by cDNA amplification by RT-PCR; the reaction contained a miR-specific forward primer, a TaqMan probe complementary to the 3' of the specific miR sequence as well as to part of the polyA adaptor sequence, and a universal reverse primer complementary to the consensus 3' sequence of the oligodT tail. The C_T , i.e., the PCR cycle at which the probe signal reached the threshold, was determined for each well. To allow comparison with results from the microarray, each value received was subtracted from 50. The $50-C_T$ (50_{CT}) expression for each miR for each patient was compared with the log signal obtained by the microarray method. The microarray and PCR readings for each miR were correlated over all patients. Differential expression analysis for good *vs* bad prognosis, and Kaplan-Meier survival analysis were also performed for the PCR data.

Data analysis and statistics

The clinical and pathological data of the eligible patients whose surgical specimens were deemed suitable for the tissue analysis were entered into an electronic database created for this purpose and anonymized. The miR measurements were performed by trained personnel who were blinded to the patients' clinical data.

Data were split by the prognostic grouping of the patients with or without a recurrence within 36 mo of surgery. A total of 112 miRs had a median signal that passed the minimal threshold of 300 units in at least one group. For each of these, the distributions of readings in the two groups were compared using the Wilcoxon-Mann-Whitney two-sample rank-sum test. The threshold for P -value significance was selected by setting a Benjamin-Hochberg false discovery rate (FDR) of 0.1, yielding a value of 0.0235. The fold-change between the two groups (i.e., the

Table 1 Epidemiological and clinicopathological characteristics *n* (%)

	<i>P</i> value ¹	Bad prognosis (<i>n</i> = 14)	Good prognosis (<i>n</i> = 31)	All patients (<i>n</i> = 45)
Age (yr)	0.36	74	75.5	75
Median (range)		57-86	47-88	47-88
Sex	0.31			
Male		11 (79)	18 (58)	29 (64)
Female		3 (21)	13 (42)	16 (36)
Ethnicity	0.46			
Ashkenazi		12 (86)	22 (71)	34 (76)
Sephardic		2 (14)	9 (29)	11 (24)
Surgery type	0.02 ^a			
Partial gastrectomy		3 (21)	18 (58)	21 (47)
Subtotal gastrectomy		2 (14)	4 (13)	6 (13)
Total gastrectomy		4 (29)	4 (13)	8 (18)
Esophagogastrectomy		5 (36)	5 (16)	10 (22)
Tumor location	0.14			
Proximal		7 (50)	12 (39)	19 (42)
Distal		3 (21)	17 (55)	20 (44)
Diffuse		4 (29)	2 (6)	6 (13)
T stage	0.001 ^b			
T1		0 (0)	6 (23)	6 (13)
T2		1 (7)	12 (37)	13 (29)
T3		12 (86)	13 (40)	25 (56)
T4		1 (7)	0 (0)	1 (2)
N Stage	0.014 ^c			
N0		5 (36)	23 (74)	28 (62)
N1		6 (43)	7 (23)	13 (29)
N2		3 (21)	1 (3)	4 (9)
TNM Stage	0.036			
I		1 (8)	14 (45)	15 (34)
II		4 (31)	11 (35)	15 (34)
III		8 (62)	6 (19)	14 (32)
Grade	0.6			
I		0 (0)	4 (13)	4 (9)
II		6 (43)	15 (48)	21 (47)
III		8 (57)	12 (39)	20 (44)
Examined lymph nodes	0.89			
≤ 10		6 (43)	13 (42)	19 (42)
> 10		7 (57)	18 (58)	26 (58)
Mucin secretion	1			
Yes		2 (14)	4 (13)	6 (13)
No		12 (86)	27 (87)	39 (87)
Signet	0.900			
Yes		2 (14)	4 (13)	6 (13)
No		12 (86)	27 (87)	39 (87)
Vascular invasion	0.085			
Yes		5 (36)	3 (10)	8 (18)
No		9 (64)	28 (90)	37 (82)
Perineural invasion	0.64			
Yes		2 (14)	3 (10)	5 (11)
No		12 (86)	28 (90)	40 (89)
Site of recurrence ²	1.4 × 10 ⁻⁵			
Locoregional		3 (21)	0 (0)	3 (6)
Distant		7 (50)	1 (3)	8 (18)
Combined		4 (29)	3 (10)	7 (16)

¹Comparison between patients with recurrence of gastric cancer within three years from surgery (bad prognosis) and patients without a recurrence (good prognosis), χ^2 test. ²Four patients had a recurrence more than three years from surgery and were therefore included in the good-prognosis group. ^a $P < 0.05$, partial gastrectomy *vs* others; ^b $P < 0.05$, stages T1 + T2 *vs* T3 + T4; ^c $P < 0.05$, nodes N1 or N2 *vs* N0. TNM: Tumor, Node Status, Metastasis; N0: No node.

ratio of the median expression levels) was calculated for

each miR; miRs were deemed differentially expressed if the *P* value was below the significance threshold and the fold-change was at least 2.0.

The cohort was divided into two groups according to the expression signal (above or below the median) of each of the most significant miRs. Kaplan Meier survival curves were then used to compare the two groups, and *P* values were obtained by a log-rank test. Additionally, to adjust for multiple-hypothesis testing, the miR profiles were randomly shuffled between patients. Specifically, miR profiles were randomly associated with clinical data at $N_{\text{repeat}} = 200$ times; in each repeat and for each miR, patients were divided into two groups (i.e., miR signal above/below the median), and log-rank *P* values were recalculated using the (randomly associated) clinical follow-up data. The lowest *P* value for each random set was recorded. The *P* values obtained were ranked, and the placement of the true *P* value within this list ($\text{rank}_{\text{true}}$) was determined, generating an adjusted *P* value, $P_{\text{adjusted}} = \text{rank}_{\text{true}}/N_{\text{repeat}}$. The re-sampling method was used to evaluate conclusions of complex analyses, such as combinations of miRs.

Stepwise Cox regression was used to analyze combined survival patterns (combinations of miRs and combinations of clinical and demographic features) on multivariate analysis. The inclusion criterion was $P < 0.05$, and the exclusion criterion was $P > 0.1$. The coefficients of the Cox fit were used to create a composite risk score for each patient. A score threshold that produced optimal separation between good and bad prognosis was used for Kaplan-Meier analysis.

The overall goal of this research was to reliably predict non-recurrence after surgery, i.e. to achieve a high positive predictive value (PPV, number of patients correctly predicted to have no recurrence/all patients predicted to have no recurrence). After choosing the most relevant miR, its predictive value was optimized by finding the threshold that maximized the PPV with high sensitivity for detection of non-recurrence. The Kaplan-Meier analysis was then repeated on the basis of this separation, and the log-rank test was repeated to measure separation.

RESULTS

Clinical predictors of outcome

A total of 69 patients who fulfilled all the eligibility criteria, and from whom paraffin blocks were available, were identified retrospectively from the database of the Institutes of Oncology of the Rabin Medical Center. Fifty-six of the samples had a tumor content of at least 50% and were analyzed for miR expression using microarrays (see section 2.5). In 45 of them (80%), reliable miR expression data were obtained; these samples were included in the statistical analysis. The samples were derived from 14 patients (31%) who had a recurrence of the disease within 36 mo of surgery (bad-prognosis group), and 31 (69%) who did not (good-prognosis group). Four patients had a recurrence more than 36 mo after surgery and were included in the good-prognosis group. The median duration of follow-up for the patients without recurrence was

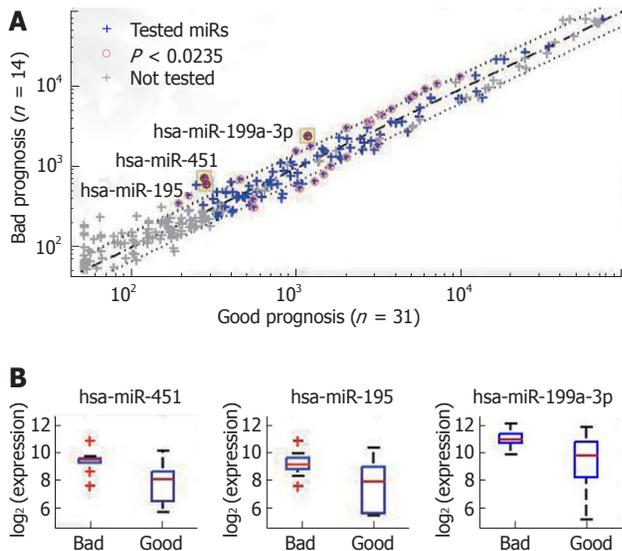


Figure 1 Differential expression of microRNAs between gastric cancer patients with good prognosis (no recurrence within 36 mo from surgery) or bad prognosis (recurrence within 36 mo). A: Median expression data (in normalized fluorescence units) are shown for all microarray probes (crosses). MiRs with low expression (below 300 units) in both groups and control probes were not tested for expression differences (grey crosses); 112 miRs (blue crosses) were tested using a rank-sum (Wilcoxon-Mann-Whitney) test. Twenty-six miRs had a $P < 0.0235$ (pink circles), corresponding to a false discovery rate = 0.1. Of these, three miRs (highlighted) also had a fold-change of > 2 : miR-451, miR-195 and miR-199a-3p. B: Box-plots of the expression levels (in \log_2 normalized fluorescence units) of these three miRs in the good/bad prognosis groups. Plots show the median (horizontal line), 25th to 75th percentile (box), extent of data (“whiskers”, extending up to 1.5 times the inter-quartile range), and outliers (red crosses, values outside the range of the whiskers). miR: MicroRNA.

86 mo (range: 40-194 mo).

The patients’ clinicopathological characteristics are summarized in Table 1. Analysis of the clinical variables with the pathological tumor features of the two groups revealed that TNM stage, T and N stages, and surgery type correlated significantly with bad prognosis. No correlation was noted for patient age, sex, or ethnicity, tumor grade, location, or histological type, or preoperative carcinogenic embryonic antigen level.

Molecular predictors

Three miRs had a significant difference in expression in the tumor samples of the patients with a bad prognosis and in the samples of the patients with a good prognosis: miR-451, miR-199a-3p, and miR-195 (Figure 1 and Table 2). The largest fold-change and the most significant difference were obtained for miR-451, with a P value of 0.0012 (rank-sum test), which is much lower than the P -value significance threshold (0.0235) after correction for multiple hypothesis testing (with FDR = 0.1). Dividing the samples according to the median expression level of miR-451 generated two groups with significantly different rates of disease-free survival ($P = 0.001$, log-rank test). To correct for multiple hypothesis testing, we randomly reassigned the patient follow-up data to the miR expression profiles and then tested all miRs for significance using the log-rank test. Out of the 200 random re-assignments of miR expression patterns, none generated

miR	P value	Fold change	Median value	
			Good prognosis	Bad prognosis
miR-451	0.0002 (0.0046)	2.66 (3.14)	260 (18.9)	690 (20.6)
miR-195	0.0046 (0.017)	2.17 (3.29)	270 (17.6)	580 (19.4)
miR-199a-3p	0.0027 (0.045)	2.15 (1.97)	1100 (20.1)	2300 (21.1)

Comparison between patients with recurrence of gastric cancer within three years from surgery (bad prognosis) and patients without a recurrence (good prognosis). P values were calculated by a Wilcoxon-Mann-Whitney rank-sum test. Only miRs that passed the false discovery rate = 0.1 threshold ($P < 0.0235$) and had a fold-change greater than 2 (in the microarray data) are listed. Values in parenthesis show the same statistics for the quantitative real-time polymerase chain reaction verification set. Cycle threshold (C_t) values are the inverses of the log signals; therefore, median values are given in $50-C_t$ to maintain the same sense as the array data.

a P value as low as that obtained for miR-451 with the real data (hence, an adjusted $P < 0.005$).

To obtain a better predictive value, we optimized the cutoff threshold for miR-451 expression. Using a threshold of 181 normalized fluorescence units, we were able to identify a group of patients ($n = 13$) without a single case of recurrence within 36 mo ($P = 0.0009$, log-rank test; Figure 2A). All 13 were included among the 31 patients in the good prognosis group. The sensitivity for identifying non-recurrence was 42% [13/31, 95% Confidence Interval (CI): 28%-56%] and the specificity was 100% (14/14, 95% CI: 78%-100%). The PPV was 100% (13/13, 95% CI: 75%-100%), and the negative predictive value (NPV) was 44% (14/32, 95% CI: 28%-60%). This group included 3 of the 6 patients (50%) in the good-prognosis group with stage III disease, and 5 of the 11 patients in that group (45%) with stage II disease.

A fair correlation was noted between the differentially expressed miRs ($r \sim 0.6$), except between miR-199a-3p and miR-195 ($r = 0.86$). This finding suggested that these miRs are independent predictors and that their linear combination could increase the predictive value. Indeed, using logistic regression, the combination of miR-451 and miR-199a-3p produced an excellent separation ($P = 0.00003$). In no case, out of 200 random re-assignments, was a combination of any two miRs found to be as good a predictor of prognosis as this combination with the real data (adjusted $P < 0.005$).

Combining clinical and molecular markers

Stage is the most typical and often the strongest clinical predictor of prognosis in gastric cancer. A possible confounding factor could be a correlation of miR expression with stage. Therefore, to remove the effect of stage, we subdivided the patient population by stage. We found that miR-451 was an excellent predictor of poor prognosis even within the subset of patients with stage III cancer (log-rank $P = 0.026$, Figure 2B). For stages I - II alone, the result was not significant owing to lack of statistical power (only one case of recurrence of stage I disease and four cases of stage II).

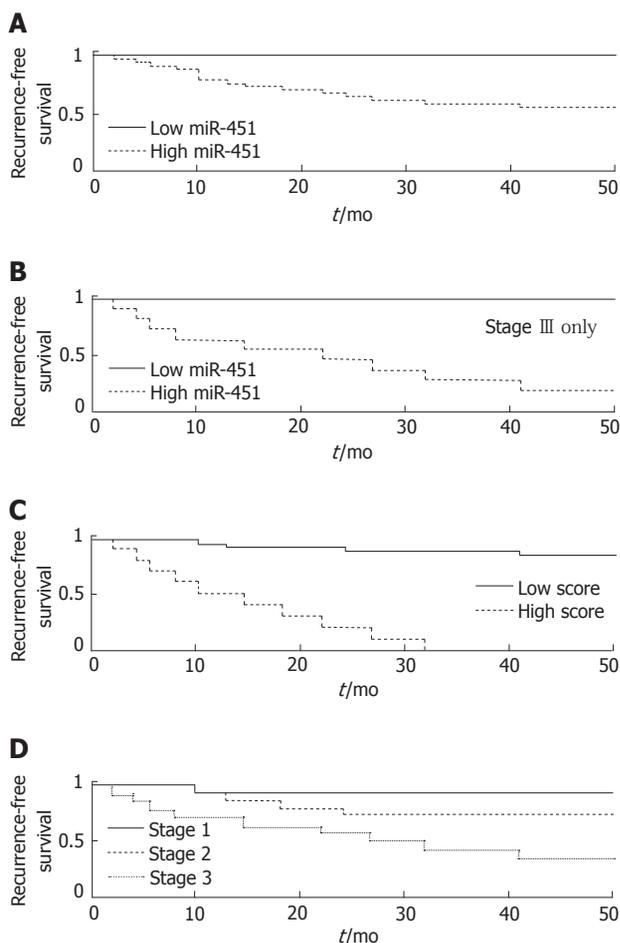


Figure 2 Kaplan-Meier model of disease recurrence for gastric cancer patients, showing the fraction without disease recurrence as function of time from surgery. A: The population was divided into groups with high expression ($n = 32$) or low expression ($n = 13$) of miR-451, based on best separation (log-rank $P = 0.0009$). B: Recurrence for patients with stage III gastric cancer only, grouped by low expression ($n = 3$) or high expression ($n = 11$) expression of miR-451, based on best separation (log-rank $P = 0.026$). C: Population grouped by composite score, calculated as $0.827 \cdot \log_2(\text{miR-451 expression}) + 1.57 \cdot \text{stage}$, using Cox regression coefficients. The threshold used (11.86) maximizes the separation between high score ($n = 10$, poor prognosis) and low score ($n = 35$, good prognosis). Although the positive predictive value with this threshold was lower than obtained using miR-451 alone (panel A), and recurrence-free survival of the good-prognosis group was not 100% as obtained for miR-451 (4 of the 35 patients in the low-score group had a recurrence before 36 mo), the negative predictive value increased to 100% (all 10 cases in the high-score group had recurrence by 36 mo) and the separation was much more significant ($P = 2 \cdot 10^{-10}$). D: Population split by stage. $P = 0.00013$ between stages I and III (log-rank test). miR: MicroRNA.

Using the Cox proportional hazards model, we created a composite score, with improved separation. The most significant separation was obtained for a combination of miR-451 expression with stage; the score was defined by the Cox coefficients as $0.827 \cdot \log_2(\text{miR-451 normalized signal}) + 1.57 \cdot \text{stage}$. Lower scores, corresponding to lower values of miR-451 expression and lower stage, indicated a better prognosis. The separation into prognostic groups based on score values was excellent (log-rank $P = 2 \cdot 10^{-10}$, Figure 2C), and much better than that for either miR alone ($P = 0.0002$, see above) or stage alone ($P = 0.00013$,

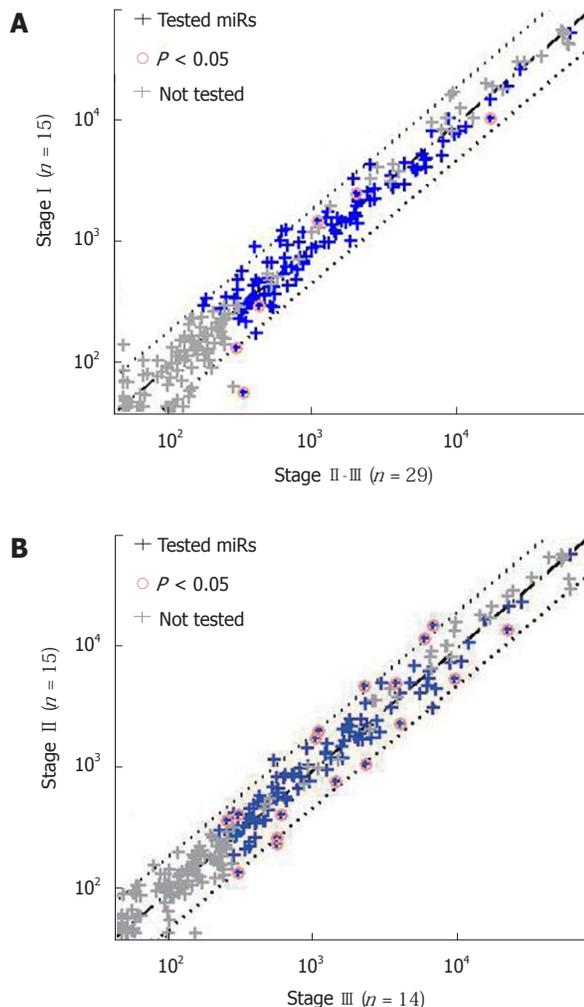


Figure 3 Differential expression of miRs in gastric cancer tumors by clinical disease stage (at surgery). Median expression data (in normalized fluorescence units) are shown for all microarray probes (crosses). MiRs with low expression (below 300 units) in both groups and control probes were not tested for expression differences (grey crosses). A: 112 miRs (blue crosses) were tested by rank-sum test for expression between stage I tumors and stages II-III tumors. None of the miRs passed the false discovery rate (FDR) threshold of 0.2; 6 miRs had a $P < 0.05$ (pink circles). B: 111 miRs (blue crosses) were tested for expression between stage II tumors and stage III tumors. None of the miRs passed the FDR threshold of 0.2; 17 miRs had a $P < 0.05$ (pink circles). Diagonal lines show the equal median expression (dashed line) and the twofold change in median expression (dotted lines). miR: MicroRNA.

Figure 2D). On fine tuning the score threshold, we found that a score of < 9.5 identified a good-prognosis group with a PPV of 100% (17/17, 95% CI: 80%-100%). None of the 17 patients had had a recurrence in 36 mo (sensitivity = 55%, 95% CI: 33%-69%). Among the patients with a score of > 9.5 were all those with a recurrence (14/14, specificity = 100%, 95% CI: 78%-100%), for a NPV of 50% (14/28, 95% CI: 32%-67%). The combination of miR expression with stage was further justified by the finding that miR-451, as well as miR-199a-3p and miR-195, were not differentially expressed between stage I and stages II-III tumors, between stage II and stage III tumors (Figure 3), or between stages I-II and stage III tumors (not shown), with miR-199a-

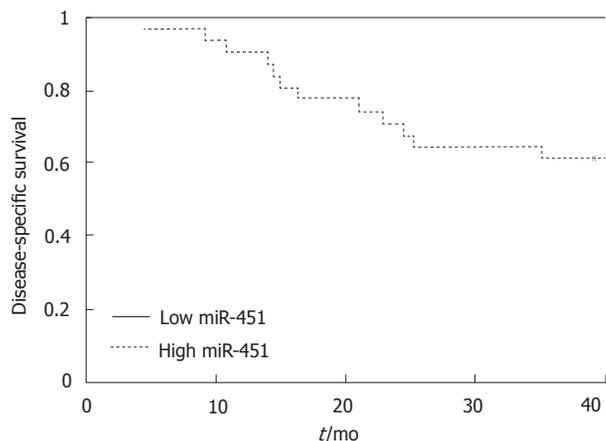


Figure 4 Kaplan-Meier model of disease-specific survival for patients with gastric cancer, grouped by high expression ($n = 32$) or low expression ($n = 13$) of miR-451, using the threshold of miR-451 expression that optimized the positive predictive value (181 normalized fluorescence units, $P = 0.005$). Among the patients whose tumors expressed low levels of miR-451, no disease-specific deaths occurred within 36 mo of surgery.

3p showing a nonsignificant upregulation in stage III.

Survival

Splitting the population by miR451 expression, using the same threshold value of 181 normalized fluorescence units, also led to a clear separation of the patients by survival. All 13 patients with low miR-451 expression survived for 36 mo (PPV = 100%, 95% CI: 75%-100%), whereas 12 of the 32 patients with a high miR-451 expression died within 36 mo (NPV = 37%, CI: 23%-55%; $P = 0.005$, Figure 4). These values suggest a specificity for survival of 100% for miR-451 expression (12/12, 95% CI: 76%-100%) and a sensitivity of 39% (13/33, 95% CI: 25%-56%).

Validation by qRT-PCR

The expression of a subset of miRs in a subset of samples was verified by qRT-PCR. Overall, the same miRs that showed significant differential expression in the microarray analysis were also differentially expressed by qRT-PCR (Table 2, Figure 5A). We focused on the miR with the strongest differential expression in the two prognosis groups, miR-451. The expression levels of miR-451 measured by the two platforms were highly correlated (Figure 5B; Pearson correlation coefficient, 0.83). Patients in the good-prognosis group had a lower expression of miR-451 by both microarray and qRT-PCR analysis. Using a simple threshold on the qRT-PCR signals (at $50_{Ct} = 19$), we found that signals below this threshold were characteristic only of patients with a good prognosis (PPV for non-recurrence of 100%, 95% CI: 53%-100%) and identified half these patients (sensitivity of 50%, 95% CI: 24%-76%), with a specificity of 100% (95% CI: 72%-100%) and NPV of 50% (95% CI: 41%-84%). There was a clear difference in prognosis between patients with signals above or below this threshold (Figure 5C, log-rank test; $P = 0.015$).

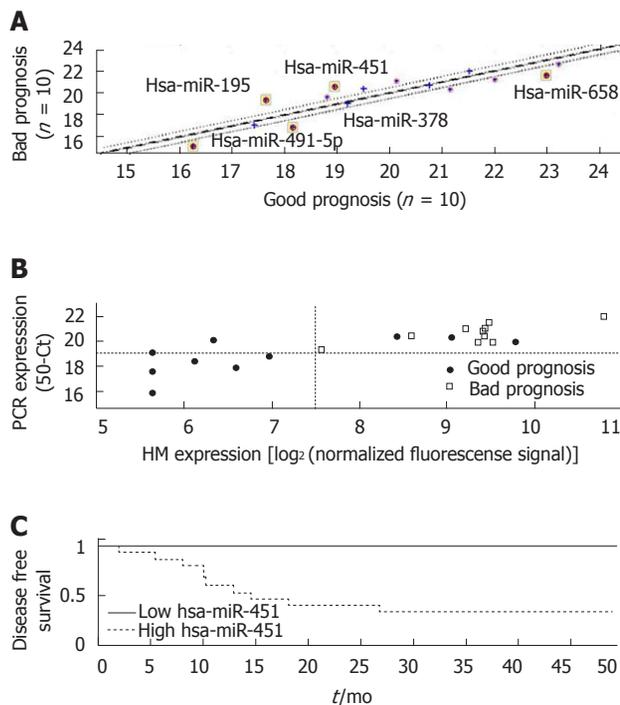


Figure 5 Validation by quantitative real-time polymerase chain reaction: A: Differential expression of microRNAs (miRs) between a subset of the gastric cancer samples from patients with good prognosis ($n = 10$) or bad prognosis ($n = 10$). Median expression data are shown for all probes tested. Cycle threshold (C_T) values are the inverses of the log signals; therefore, median values are given in $50-C_T$ (50_{Ct}) to maintain the same sense as the array data; B: Correlation of expression signals of miR-451 between microarrays (\log_2 normalized fluorescence units) and quantitative real-time polymerase chain reaction (qRT-PCR) (50_{Ct}). Good-prognosis cases are represented by filled circles, bad-prognosis cases by empty squares. Dotted lines indicate the thresholds of the miR-451 signal at normalized fluorescence of 181 units [$\log_2(181) = 7.5$] and at $50_{Ct} = 19$. The correlation coefficient (Pearson) between the signals is 0.83; C: Recurrence in 20 cases, measured by qRT-PCR, grouped by $50_{Ct} < 19$ (solid line, $n = 5$) or $50_{Ct} > 19$ (dotted line, $n = 15$). $P = 0.015$ by log-rank test. MiR: MicroRNA; PCR: Polymerase chain reaction; HM: Human custom microRNA-Microarray.

DISCUSSION

The results of the present study indicate that the expression levels of three miRs, miR-451, miR-199a-3p, and miR-195, may help to differentiate patients with gastric cancer with a good or bad prognosis. Specifically, tumors from patients who remained free of recurrence for at least 36 mo from surgery had significantly lower levels of these miRs than tumors from patients who had a recurrence. The miR with the most significant difference was miR-451, and the combination of the miR-451 with miR-199a-3p values provided even better predictive information. The prognostic role of miR-451 was both independent of, and additive to, the currently most important prognostic factor in gastric cancer, tumor stage. Finally, higher levels of miR-451 were found to be associated not only with recurrence but also with worse survival.

Two recent studies have highlighted the importance of miR-451 in gastric cancer. Takagi *et al.*^[30] evaluated tumor samples from 43 patients and found that miR-451 levels were lower in the gastric cancer cells than in adjacent non-malignant cells. Bandres *et al.*^[22], in a study of

21 patients with stage III disease receiving postoperative chemoradiation, also found lower levels of miR-451 in the gastric cancer cells. The lower levels were correlated with a higher risk of recurrence and death after resection of the primary tumor. These results were confirmed in a cohort of 24 patients with stage I-IV disease. Our study also highlights the role of miR-451 in gastric cancer, but as opposed to the findings of Bandres *et al.*^[22], lower, not higher levels of miR-451 were associated with better outcome. This discrepancy may be explained by the different study populations: in our cohort, only 30% of the patients had stage III disease and none had received postoperative treatment. Therefore, the patients in the earlier study^[22] were at a much higher risk of recurrence. Moreover, given that Bandres *et al.*^[22] were evaluating a treated population, the miR-451 expression in their study may well have had a predictive impact. Indeed, they found that overexpression of miR-451 was associated with increased radiosensitivity. The different results between the studies might also be attributable to differences in the methods of selecting and handling the tissues from which RNA was extracted, and the actual percentage of tumor in the specimens. Bandres *et al.*^[22] did not provide these details, but in the present study, more than 30% of the samples were found to be inadequate for investigation. The correlation of our qRT-PCR results with the microarray platform results suggests not only internal consistency, but also a stable process for miR measurement. Lastly, and probably most importantly, the small sample sizes and the essentially preliminary nature of the results in both studies, and in that of Takagi *et al.*^[30], may explain the inconsistencies among them. For example, other recent studies of the miR expression profile of gastric cancer did not find a differential expression of miR-451 in the malignant cells^[31,32].

While the actual impact of miR-451 on patient outcome is unclear, there are preliminary clues pointing to the possible mechanisms whereby it may influence cell function. In the study by Takagi *et al.*^[30], *in vitro* analysis suggested that miR-451 inhibits tumor growth and induces tumor sensitivity to 5-fluorouracil by interacting with messenger RNAs (mRNAs) of the insulin receptor substrate-1 (IRS-1) and beta-actin. In the study by Bandres *et al.*^[22], overexpression of miR-451 reduced cell proliferation and increased sensitivity to radiotherapy, apparently via downregulation of mRNA and protein levels of the macrophage migration inhibitory factor oncogene. Two other studies have shown that miR-451 is involved in the regulation of the multi-drug resistance 1 (MDR-1) gene and, thereby, in tumor resistance to various chemotherapeutic agents, most notably doxorubicin^[33,34]. However, the studies reported opposite effects: in one study, MDR-1 expression increased in the presence of miR-451^[29], and in the other, it decreased^[34]. Tsuchiya *et al.*^[35] found that miR-451 is essential for epithelial cell polarity by affecting the translocation of the beta-1 integrin protein. Clearly, as a single miR may target multiple mRNAs simultaneously, and several miRs may target a single mRNA

simultaneously, the interactions of miRs with their target mRNAs are expected to be very complex. Hence, it is not surprising that a number of unrelated mechanisms have already been postulated for a single miR such as miR-451, and it is likely that it may indeed be involved in multiple cell processes, like the ones described.

There are several strengths and weaknesses of the present study that need to be addressed. First, the sample size, though small, was nevertheless somewhat larger than in previous studies. Second, we used very strict criteria for selection of the study population: all patients were operated in a single medical center, none received adjuvant therapy, and all were closely followed for at least three years. Third, several statistical tests were performed to reduce the risk of randomly choosing a “statistically significant” biomarker from the hundreds tested, a risk that is typical of studies screening for novel biomarkers (multiple hypothesis testing). Fourth, qRT-PCR was used to verify the appropriate identification of significant signals and suggested a method for future adaptation of our findings in the hospital laboratory setting. Another strong point of this study is that it provides meaningful predictive values, which may have important clinical implications. Informed decision-making using a test with a high PPV can spare patients unnecessary and sometimes toxic treatment. We were able to identify a group of samples with low signals of miR-451 for which the PPV for non-recurrence was 100%. According to the current standard, a substantial proportion of patients with gastric cancer receive adjuvant therapy. Thus, our finding, if validated, suggests that those with low miR-451 expression do not require adjuvant chemotherapy because their risk of recurrence is low. Our sample size was insufficient for adequate independent validation, and further studies, in larger cohorts, are needed. In addition, although the estimated PPV was 100%, our confidence interval was still quite wide. We are currently in the process of formulating a follow-up validation study wherein the prognostic impact of miR-451 will be tested in an independent cohort. The critical importance of such validation is further emphasized by the large variability of the available data on the prognostic role of various miRs in gastric cancer. In fact, there is only a minimal overlap between the different miR signatures that have been reported to have a prognostic impact in gastric cancer^[21,26].

In summary, this study showed that three miRs, miR-451, miR-199a-3p and miR-195, might serve as biomarkers of the risk of recurrence of gastric cancer after resection. One of them, miR-451, seems to hold the most promise for further evaluation. Our results add to the accumulating evidence on the role of miR-451 in gastric cancer. Further research in this direction is warranted. Within this setting, we have recently embarked on a validation study for the results presented here.

ACKNOWLEDGMENTS

We would like to thank Dr. Tamara Druzd for her assis-

tance in analysis of the pathological slides.

COMMENTS

Background

Surgery is the standard treatment of localized gastric cancer but its results are often disappointing. Our current ability to determine the prognosis of such individual patients, and hence their need for adjuvant therapy is limited. MicroRNAs (miRs) are short non-coding RNAs that regulate gene expression and are therefore involved in various physiological and pathological conditions, including cancer.

Research frontiers

The expression of miRs is dynamic and therefore these molecules may serve diagnostic or prognostic biomarkers in various malignancies. Indeed, recent studies have suggested that various miR molecules may have a prognostic role in gastric cancer. In this study, three miRs, miR-199a-3p, miR-195, and especially miR-451, were found to associated with the risk of recurrence after the resection of gastric cancer.

Innovations and breakthroughs

Several attempts have been made to identify miRs that may predict patient outcome in gastric cancer. The current study showed that three miRs might indeed serve as biomarkers for the risk of recurrence of gastric cancer after resection. These results are based on a reasonably sized cohort of 45 patients with strict eligibility criteria, two independent methods to measure miR expression levels, and multiple statistical testing to reduce the risk of randomly choosing a "statistically significant" biomarker. All these have resulted in meaningful predictive values, and most importantly, the authors were able to identify a group of patients who had no risk of recurrence at all.

Applications

The results of this study add to the accumulating data on the prognostic role of miRs in gastric cancer. Once validated, these results may allow a better prognostication of patients after resection of gastric cancer and an improved selection of patients for adjuvant therapy.

Terminology

MiRs are short non-coding RNAs, 17-22 nucleotides in length, which regulate gene expression and thereby play significant roles in human development and various pathological conditions, including cancer.

Peer review

The proposed study is well designed, well written, and uses appropriate methods.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Xiong L

Dynamic changes and surveillance function of prion protein expression in gastric cancer drug resistance

Ji-Heng Wang, Jing-Ping Du, Ying-Hai Zhang, Xiao-Jun Zhao, Ru-Ying Fan, Zhi-Hong Wang, Zi-Tao Wu, Ying Han

Ji-Heng Wang, Jing-Ping Du, Xiao-Jun Zhao, Ru-Ying Fan, Zhi-Hong Wang, Zi-Tao Wu, Ying Han, Department of Gastroenterology, Beijing Army General Hospital, Beijing 100700, China

Ying-Hai Zhang, Department of General Surgery, Jiamusi University Medical College, Jiamusi 154004, Heilongjiang Province, China

Author contributions: Wang JH and Du JP performed the majority of experiments; Wang ZH, Wu ZT provided vital reagents and analytical tools; Zhang YH, Zhao XJ and Fan RY collected the chemotherapy for gastric cancer data and performed follow up work; Han Y designed the study and wrote the manuscript.

Supported by National Natural Science Foundation of China, No. 30672063; China Postdoctoral Science Foundation Funded Project, No. 20080431404; and China Postdoctoral Special Fund, No. 200801038

Correspondence to: Ying Han, MD, Beijing Army General Hospital, No. 5 Nanmengcang Road, Dongcheng district, Beijing 100700, China. ying1000@beihua.edu.cn

Telephone: +86-10-66721168 Fax: +86-10-66721168

Received: February 22, 2011 Revised: May 19, 2011

Accepted: May 26, 2011

Published online: September 21, 2011

Abstract

AIM: To explore the dynamic changes of prion protein (PrPc) in the process of gastric cancer drug resistance and the role of PrPc expression in the prognosis of gastric cancer patients receiving chemotherapy.

METHODS: A series of gastric cancer cell lines resistant to different concentrations of adriamycin was established, and the expression of PrPc, Bcl-2 and Bax was detected in these cells. Apoptosis was determined using Annexin V staining. Western blotting and immunohistochemistry were performed to detect the expression of PrPc in patients receiving chemotherapy and to explore the role of PrPc expression in predicting the chemosensitivity and the outcome of gastric cancer patients receiving chemotherapy. Follow-up was performed for 2 years.

RESULTS: PrPc expression was increased with the increase in drug resistance. Bcl-2, together with PrPc, increased the level of anti-apoptosis of cancer cells. Increased PrPc expression predicted the enhanced level of anti-apoptosis and resistance to anticancer drugs. PrPc expression could be used as a marker for predicting the efficacy of chemotherapy and the prognosis of gastric cancer. Increased PrPc expression predicted both poor chemosensitivity and a low 2-year survival rate. Contrarily, low PrPc expression predicted favorable chemosensitivity and a relatively high 2-year survival rate.

CONCLUSION: PrPc expression is associated with histological types and differentiation of gastric cancer cells; The PrPc expression level might be a valuable marker in predicting the efficacy of chemotherapy and the prognosis of gastric cancer patients receiving chemotherapy.

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Key words: Prion protein; Gastric cancer; Drug resistance; Chemotherapy; Apoptosis

Peer reviewer: Jean Paul Galmiche, MD, Professor, Department of Gastroenterology and Hepatology, Hôpital Hôtel Dieu, Nantes cedex 44093, France

Wang JH, Du JP, Zhang YH, Zhao XJ, Fan RY, Wang ZH, Wu ZT, Han Y. Dynamic changes and surveillance function of prion protein expression in gastric cancer drug resistance. *World J Gastroenterol* 2011; 17(35): 3986-3993 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/3986.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.3986>

INTRODUCTION

Prion protein (PrPc or PrP) is a pathogen that causes bovine spongiform encephalopathy (also known as “mad cow disease”). In 1982, Prusiner^[1] isolated the protease-

and heat-resistant protein from the brains of scrapie-infected hamsters. PrPc is encoded by the host prion gene but not by the prion gene. Thereafter, a new field of biomedicine, prion biology, was founded, and a new concept of pathology, protein conformation diseases, was also established. In 2003, our study showed that PrPc was associated with the multidrug resistance of gastric cancer^[2,3], and thereafter, a series of studies have been conducted to explore the role of PrPc in the differentiation, proliferation and metastasis of cancer cells and to deeply investigate the structural domains of PrPc. These studies have achieved great progress in prion biology and pathogenesis^[4-13]. The present study aimed to investigate PrPc expression in gastric cancer cell lines and its role in predicting the outcome of gastric cancer patients undergoing chemotherapy. Our study may elucidate the clinical significance of PrPc over-expression in cancers and provide a potential indicator for predicting the outcome of cancer patients.

MATERIALS AND METHODS

Materials

Gastric cancer cell lines (KATO III, BGC-823, GES, HGC-27, AGS, SGC7901 and MGC803) were purchased from the Academy of Military Medical Sciences and were maintained in our department. PrPc and β -actin monoclonal antibodies (Sigma, United States), enhanced chemiluminescence (ECL) kit (Amersham Pharmacia) and immunohistochemistry kit (Beijing biodev-tech Co., Ltd) were used in this study.

A total of 185 patients with gastric cancers at stages I to IV (6th edition of the UICC TNM classification and Lauren classification) were recruited. The characteristics of these patients are summarized in Table 1. There were 134 men and 51 women included in this study. The age of these patients ranged from 33 to 76 years, with a mean age of 54.2 years. The cancers of these patients were pathologically proven to be moderately or well-differentiated ($n = 43$), poorly differentiated ($n = 33$), undifferentiated ($n = 46$), mucinous ($n = 16$) and mixed cancers ($n = 47$). In addition, normal gastric tissues without cancer infiltration were used as controls.

Methods

Establishment of adriamycin-resistant gastric cancer cell lines^[10]: Adriamycin-resistant SGC7901 cells were selected by a stepwise increase in adriamycin concentration from 0.04 $\mu\text{g}/\text{mL}$ to 0.6 $\mu\text{g}/\text{mL}$. In brief, the SGC7901 cells were maintained in medium containing 0.04 $\mu\text{g}/\text{mL}$ adriamycin for 48 h. Then, the medium was refreshed with adriamycin-free medium. When normal growth was observed, the medium was replaced with that containing 0.04 $\mu\text{g}/\text{mL}$ adriamycin followed by incubation for 48 h. The above mentioned procedures were repeated 3-5 times, and then the adriamycin concentration was increased once every 6-10 d. Finally, SGC7901 cells that were resistant to 0.04, 0.18, 0.32, 0.46 and 0.6 $\mu\text{g}/\text{mL}$ adriamycin were obtained. Each adriamycin-resistant

SGC7901 cell line was collected and stored in liquid nitrogen for the synchronization of experiments. The sensitivity of cells to adriamycin was determined by methyl thiazolyl tetrazolium assay followed by calculation of the half-maximal inhibitory concentration (IC₅₀). The resistance index (RI) was calculated as follow: $\text{RI} = \text{IC}_{50\text{drug-resistant cells}} / \text{IC}_{50\text{parental cells}}$.

Detection of the expression of PrPc, Bcl-2 and Bax in each adriamycin-resistant SGC7901 cell line by Western blotting:

After recovery from storage in liquid nitrogen, cells were maintained in fresh medium. Total proteins were extracted from cells in the logarithmic growth phase of growth, and the protein concentration was determined. Then, 40 μg total proteins were separated in sodium dodecylsulphate (SDS) polyacrylamide gels and transferred onto nitrocellulose membranes. The primary antibodies used to detect the target proteins were anti-PrP, anti-Bcl-2 and anti-Bax monoclonal antibodies and anti- β -actin polyclonal antibody; horseradish peroxidase-conjugated goat anti-mouse IgG was used as the secondary antibody. The bands were visualized using the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, United States) according to the manufacturer's instructions, and protein concentration was quantified by densitometry with Total-Lab 2.0 software.

Flow cytometric analysis of apoptosis in each adriamycin-resistant SGC7901 cell line:

Cells in the logarithmic phase of growth were transferred to a 6-well plate. After adherence, cells were maintained in medium with 0.7 $\mu\text{g}/\text{mL}$ adriamycin at 37 °C for 48 h. The medium was then replaced with fresh medium containing 5 μL Annexin V-FITC and 1.5 mmol CaCl_2 , and the cells were incubated for 15 min at 37 °C. Subsequently, the cells were re-suspended in 490 μL binding buffer and 5 μL PI staining solution and incubated for 10 min at 4 °C. Apoptosis was then detected by flow cytometry, and the apoptosis index (AI) was calculated as follows: $\text{AI} = (\text{number of early and late apoptotic cells}) / \text{total number of cells} \times 100\%$.

Detection of PrPc expression in different gastric cancer cell lines by Western blotting:

Gastric cancer cells in the logarithmic growth phase of growth were harvested and the total protein was extracted, after which the proteins were separated on SDS polyacrylamide gels and transferred onto nitrocellulose membranes. The primary antibodies used to detect the target proteins were anti-PrP, anti-Bcl-2 and anti-Bax monoclonal antibodies and anti- β -actin polyclonal antibody; horseradish peroxidase-conjugated goat anti-mouse IgG was used as the secondary antibody. The bands were visualized using the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, United States) according to manufacturer's instructions.

Detection of PrPc expression in different gastric cancers by tissue microarray:

Anti-PrP monoclonal antibody (Sigma, Swampscott, MA, United States) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody

Table 1 Demographics of gastric cancer patients at baseline (mean \pm SD)

Stage	<i>n</i>	Surgery + chemotherapy	Comprehensive treatment	KPS before treatment	KPS (2 yr later)	ZPS before treatment	ZPS (2 yr later)	CR + PR (%)	Death
I	52	43	9	87.12 \pm 12.66	77.31 \pm 19.72	1.019 \pm 0.772	1.346 \pm 1.254	74.6	2
II	66	41	25	77.73 \pm 11.12	72.12 \pm 16.56	1.803 \pm 1.131	2.167 \pm 1.344	52.1	1
III	47	32	15	66.81 \pm 23.35	41.06 \pm 35.20	2.383 \pm 1.314	2.830 \pm 1.872	36.8	17
IV	20	12	8	57.00 \pm 16.46	33.00 \pm 31.64	2.650 \pm 1.014	3.650 \pm 1.459	15.0	9

Male: 134; Female: 51; Mean age: 54.2 \pm 21.5. KPS: Karnofsky Performance Scale; ZPS: Zubrod-ECOG-WHO Performance Status; CR: Complete remission; PR: Partial remission.

were used for immunohistochemical detection of PrPc with the StreptAvidin Biotin Complex staining kit according to the manufacturer's instructions. The bands were visualized using diaminobenzidine substrate. The primary antibody was replaced with phosphate-buffered saline as the negative control.

Analysis was performed with Image-Pro Plus 5.0 software. Five high-power fields were randomly selected, and 100 cells were counted. The proportion of PrP-positive cells per sample was calculated, and tissue grading was performed as follows: < 5% as negative (score 0); 5%-25% as "+" (score 1); 26%-50% as "++" (score 2); 51%-75% as "+++" (score 3), and > 75% as "++++" (score 4). Scores of greater than 3 were regarded as strongly positive.

Detection of PrPc expression in gastric cancer tissues by Western blotting: Gastric cancer tissues, including 52 adenocarcinomas, 26 undifferentiated cancers, 33 mixed cancers, 17 poorly differentiated cancers and 11 precancerous lesions (severe atrophic gastritis), were obtained from 139 patients with gastric cancers who underwent biopsy or gastrectomy. Total protein was extracted with Trizol, the protein concentration was determined, and the proteins were separated by SDS-PAGE. Anti-PrP monoclonal antibody (Sigma, Swampscott, MA, United States) and anti- β -actin polyclonal antibody were used as primary antibodies and horseradish peroxidase-conjugated goat anti-mouse IgG as the secondary antibody.

Relationship between PrPc expression and the efficacy of chemotherapy in gastric cancer patients

The treatment strategies included surgery + chemotherapy and surgery + chemotherapy + interventional therapy. The treatment efficacy was recorded as complete remission (CR), partial remission (PR), stable disease, and progressive disease according to the criteria developed by the World Health Organization (WHO). The overall efficacy was calculated as CR + PR. According to the Karnofsky Performance Scale (KPS), the patients were stratified into one of the following groups: the improvement group (the increase in KPS score was greater than 10 after treatment), the deterioration group (the decrease in KPS score was greater than 10 after treatment), and the stability group (the changes in KPS score were less than 10 after treatment). The performance status was evaluated according to Zubrod-ECOG-WHO, and survival was followed up for 2 years. The demographics of these patients

at baseline and after 2 years of follow-up were summarized in Table 1, respectively. PrPc expression in the patient samples was detected by immunohistochemistry and Western blotting, and the patients were divided into PrPc-positive and PrPc-negative groups. The whole protocol was approved by the Institutional Review Board of the Ethics Committee of our hospital. Informed consent was obtained before the study.

Statistical analysis

Statistical analysis was performed using SPSS software. Quantitative data were analyzed using the *t* test, and qualitative data were analyzed using the χ^2 test. A value of *P* < 0.05 was considered statistically significant.

RESULTS

PrPc, Bcl-2 and Bax expression in gastric cancer cells resistant to different concentrations of adriamycin

The RI of gastric cancer cells resistant to adriamycin were increased accompany with the deepened concentrations which the value of RI 0.92 \pm 0.072 *vs* the adriamycin concentration 0 μ g/mL, 1.478 \pm 0.098^a *vs* 0.04 μ g/mL, 3.096 \pm 0.201^a *vs* 0.18 μ g/mL, 4.5 \pm 0.334^a *vs* 0.32 μ g/mL, 5.086 \pm 0.302^a *vs* 0.46 μ g/mL, 6.65 \pm 0.354^a *vs* 0.6 μ g/mL (^a*P* \leq 0.01 *vs* 0 μ g/mL group).

PrPc, Bcl-2 and Bax expression in gastric cancer cells resistant to different concentrations of adriamycin are shown in Figure 1. Analysis indicated that the expression of PrPc and Bcl-2 were markedly increased (*P* < 0.01) in an adriamycin concentration-dependent manner, while that of Bax was significantly reduced (*P* < 0.01). The results indicated that the resistance index was 3.096 when the adriamycin concentration was 0.18 μ g/mL.

Apoptosis of gastric cancer cells resistant to different concentrations of adriamycin

The AI is displayed in Figure 2. A significant difference in the AI was not noted between the 0.04 and 0 μ g/mL adriamycin groups (*P* > 0.05). In SGC7901 cells resistant to 0.18 μ g/mL adriamycin, the anti-apoptotic capability was significantly increased and drug sensitivity markedly decreased with an RI of 3.096 (*P* < 0.001) when compared with SGC7901 cells without adriamycin treatment.

PrPc expression in different gastric cancer cell lines

PrPc expression in different gastric cancer cell lines is

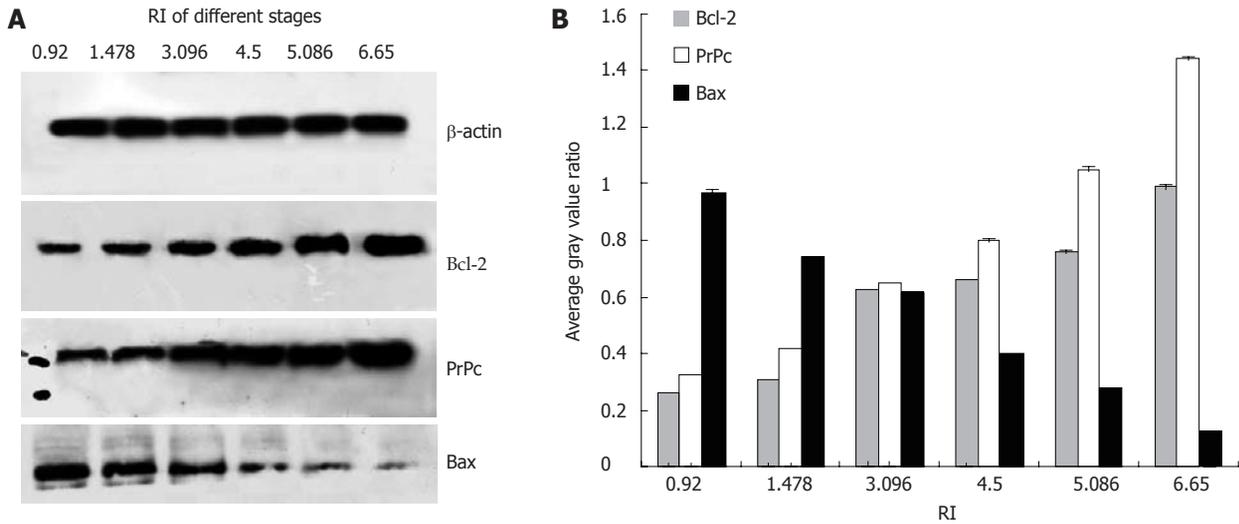


Figure 1 Expressions of PrPc, Bcl-2 and Bax in gastric cancer cells resistant to different concentrations of adriamycin. Analysis indicated that the expressions of PrPc and Bcl-2 were markedly increased ($P < 0.01$), but that of Bax was significantly reduced ($P < 0.01$), which were in an adriamycin concentration dependent manner. Resistance index was 3.096 when the adriamycin concentration was 0.18 $\mu\text{g/mL}$. PrPc: Prion protein; RI: Resistance index.

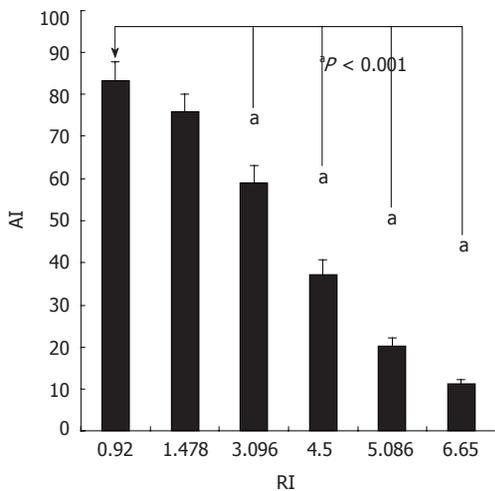


Figure 2 Apoptosis index of gastric cancer cells resistant to adriamycin of different concentrations. In the SGC7901 cells resistant to 0.18 $\mu\text{g/mL}$ adriamycin, the anti-apoptotic capability was significantly increased with the resistance index of 3.096 ($P < 0.001$) when compared with SGC7901 cells without adriamycin treatment. AI: Apoptosis index; RI: Resistance index.

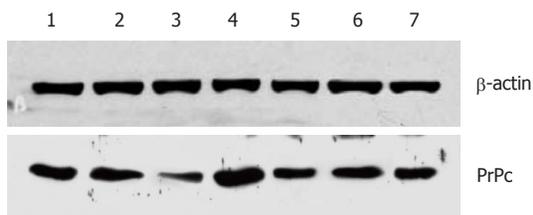


Figure 3 Prion protein expression in different gastric cancer cell lines. 1: KATO III; 2: BGC-823; 3: GES; 4: HGC-27; 5: AGS; 6: SGC7901; 7: MGC80. Results showed that the highest prion protein (PrPc) expression was found in undifferentiated gastric cancer cells (HGC-27), followed by poorly differentiated gastric cancer cells (KATO III), and the lowest PrPc expression in human gastric epithelial immortalized GES-1 cells. PrPc: Prion protein.

presented in Figure 3. Results showed that the highest PrPc expression was found in undifferentiated gastric

cancer cells (HGC-27) followed by poorly differentiated gastric cancer cells (KATO III), and the lowest PrPc expression was in GES-1 immortalized human gastric epithelial cells. These results were consistent with tissue microarray and Western blotting analyses of the cancer tissues. Of note, low PrPc expression was noted in immortalized GES-1 cells, which was different from the results from the tissue microarray analysis.

PrPc expression using tissue microarray assay

PrPc expression in the gastric cancers, precancerous lesions and normal tissues are presented in Figures 3, 4 and 5, respectively. χ^2 tests revealed that the percentage of PrPc-positive cells in gastric cancers and precancerous lesions were dramatically increased when compared with normal tissues ($P < 0.01$). The percentage of PrPc-positive cells in adenocarcinoma, mucinous carcinoma, undifferentiated cancer and poorly differentiated cancer are displayed in Figure 4. Analysis showed that PrPc-positive cells were related to the types of gastric cancer ($P < 0.05$). The proportion of PrPc-positive cells in undifferentiated cancer was significantly higher than that in adenocarcinoma ($P < 0.01$), but no significant differences in PrPc positive cells were found between mucinous carcinoma and poorly differentiated adenocarcinoma (Figure 5).

PrPc protein expression in different types of gastric cancers

PrPc expression in the cancer and normal tissues is shown in Figure 6, and the immunohistochemistry for PrPc in gastric cancer is shown in Figure 7. Results showed that PrPc expression in gastric adenocarcinoma and mixed carcinoma was relatively low, and no significant difference in PrPc expression was observed between the remaining types of gastric cancer. Furthermore, PrPc expression in normal gastric tissues was much lower than that in gastric cancers.

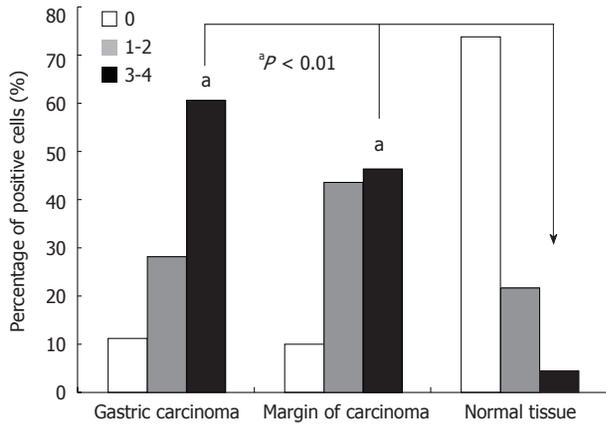


Figure 4 Prion protein expression in gastric cancers, precancerous lesions, and normal tissues. χ^2 test revealed that the number of prion protein positive cells in gastric cancers and precancerous lesions were dramatically increased when compared with normal tissues ($^aP < 0.01$). PrPc: Prion protein.

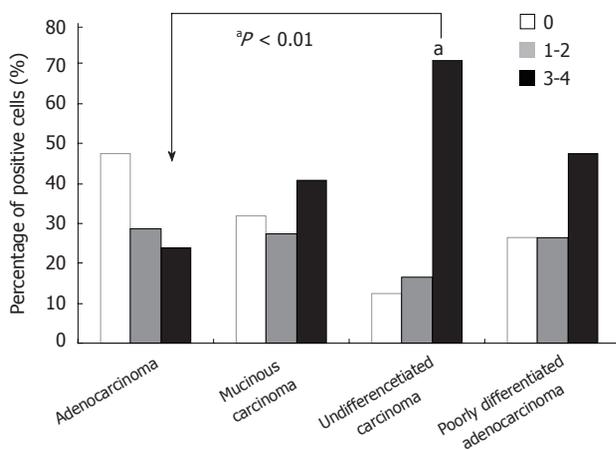


Figure 5 Prion protein expression in different type of gastric cancer. The proportion of prion protein positive cells in undifferentiated cancer was significantly higher than that in adenocarcinoma ($^aP < 0.01$), but no significant difference in PrPc positive cells was found between mucinous carcinoma and poorly differentiated adenocarcinoma. PrPc: Prion protein.

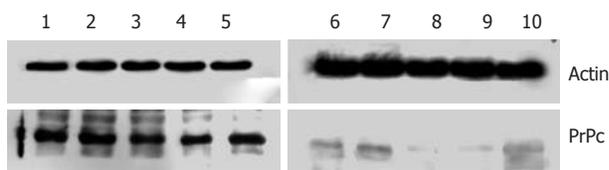


Figure 6 Prion protein expression in gastric cancers and normal tissues. The prion protein (PrPc) expression in gastric adenocarcinoma and mixed carcinoma was relatively low, and no significant difference in PrPc expression was noted between the remaining types of gastric cancer. Furthermore, PrPc expression in normal gastric tissues was much lower than that in gastric cancers. PrPc: Prion protein. 1: Tissue of atrophic gastritis; 2: Tissue of undifferentiated gastric cancer; 3: Tissue of gastric adenocarcinoma; 4: Tissue of mixed type carcinoma; 5: Tissue of poorly differentiated gastric cancer; 6-10: Corresponding normal gastric tissue.

Association of PrPc expression and chemotherapeutic efficacy in gastric cancer patients

The demographics of 185 patients at baseline are presented in Table 1, and those after 2 years of follow-up

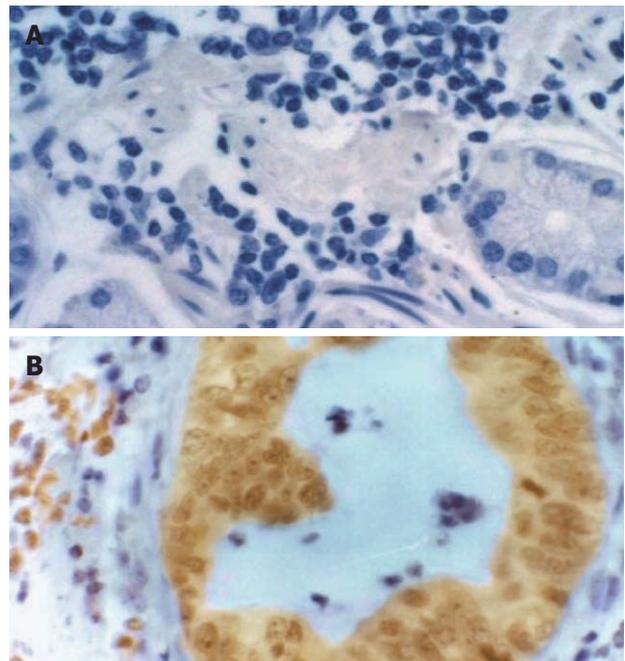


Figure 7 Prion protein expression in human gastric cancer ($\times 200$). A: Negative control; B: Positive results.

PrPc expression	Case	CR	PR	NC	PD	CR + PR (%)
0	59	7	25	18	9	32 (54.24)
1	30	2	8	13	7	10 (33.33)
2	39	1	9	19	10	10 (25.64)
≥ 3	57	0	11	21	25	11 (19.29)

PrPc: Prion protein; CR: Complete remission; PR: Partial remission; NC: Stable disease; PD: Progressive disease.

are presented in Table 2. Analysis showed that PrPc expression was significantly correlated with the pathological type of gastric cancer ($P < 0.05$). PrPc expression in undifferentiated cancers was significantly higher than that in moderately or well-differentiated cancer and mixed cancers ($P < 0.01$). However, no significant difference in PrPc expression was observed between poorly differentiated cancers and mucinous cancer ($P > 0.05$). The relationship between PrPc expression and the efficacy of chemotherapy are shown in Tables 2 and 3. The curative effects, KPS scores, and performance statuses were also evaluated. As shown in Table 2, PrPc expression was associated with the efficacy of chemotherapy. Patients with higher PrPc expression had a poorer response to chemotherapy ($P < 0.05$). As shown in Table 3, PrPc expression was associated with survival ($P < 0.05$). High PrPc expression predicted poorer prognosis, where a lower 2-year survival rate and higher mortality rate were observed when compared with low PrPc expression group. Table 4 showed KPS scores at diagnosis and after 2 years of follow-up in patients with different levels of PrPc expression. Results showed the overall survival status was

Table 3 Survival status of patients with different prion protein expression after chemotherapy

PrPc expression	n	6 mo	1 yr	2 yr	Death mortality n (%)
0	59	0	11	45	3(5.10)
1	30	0	8	20	2(6.67)
2	39	3	7	17	7(17.95)
≥ 3	57	5	12	25	17(29.82)

PrPc: Prion protein.

deteriorated (KPS score was decreased more than 10), and the decrease in KPS score was profound in patients with strongly positive PrPc expression ($P < 0.01$) when compared with other patients.

DISCUSSION

PrPc expression in gastric cancer cells and tissues

The expression of prion protein tends to occur in rapidly regenerating or poorly differentiated tissues^[14-18]. Pammer *et al*^[19] detected PrPc expression in gastrointestinal mucosa by immunohistochemistry, and results showed that PrPc expression was significantly increased in *H. pylori*-induced gastritis, suggesting that prion infection and replication could occur in gastrointestinal epithelial cells. In the present study, PrPc expression was detected through tissue microarray assays in normal gastric tissues, gastric cancers and precancerous lesions. Results revealed weak or no PrPc expression in normal gastric tissues, but hemocytes showed high PrPc expression; this observation could be attributed to normal tissues with some inflammation being used for detection, and positive expression was mainly found in infiltrated lymphocytes. PrPc expression was detected in gastric cancer tissues, adjacent normal tissues and normal tissues using tissue microarray assay based on tissue classification, e.g., negative group, positive group (+ to ++) and strongly positive group (+++ to ++++). χ^2 tests revealed that PrPc expression in gastric cancers and precancerous lesions was dramatically increased when compared with normal tissues ($P < 0.01$). These findings imply that PrPc expression is closely associated with cell status. When cellular functions are active, including malignant transformation and excessive proliferation, PrPc expression is increased, which has been confirmed by Liang *et al*^[9]. Moreover, PrPc expression was related to pathological types of gastric cancer. The proportion of PrPc-positive cells in undifferentiated cancer was significantly higher than that in gastric adenocarcinoma, suggesting that cancer differentiation is also correlated with PrPc expression, and the poorer the differentiation, the higher the level of PrPc expression. These results also indicate the relationship between cellular functions and PrPc expression: more active cellular function results in greater active proliferation and differentiation, and higher grades of malignancy mean higher levels of PrPc expression.

PrPc expression was detected by immunohistochemistry in 185 patients with gastric cancers, and PrPc ex-

Table 4 Karnofsky performance scale score of patients with different prion protein expression levels

PrPc expression	n	Before treatment	2 yr later	Difference
0	59	85.25 ± 11.40	73.90 ± 22.40	11.35
1	30	81.67 ± 17.34	69.33 ± 25.68	12.34
2	39	74.10 ± 18.77	60.77 ± 31.81	13.33
≥ 3	57	63.90 ± 18.87	45.93 ± 32.00	17.97

PrPc: Prion protein.

pression in gastric cancers was significantly higher than that in normal gastric tissues. Strongly positive PrPc expression was found in 26 undifferentiated gastric cancers, while the well-differentiated adenocarcinoma had a low positive rate of PrPc expression (42.45%). However, negative or weak PrPc expression was also observed in gastric cancers, while positive, but not strongly positive, PrPc expression was detected in normal gastric tissues. These results were consistent with the tissue microarray assays.

PrPc expression was detected in different gastric cancer cell lines. The highest PrPc expression was found in an undifferentiated cancer cell line (HGC-27 cells) followed by a poorly differentiated cell line (KATO III cells). The lowest PrPc expression was detected in the immortalized normal human gastric epithelial cell line (GES cells).

In our study, PrPc expression was detected in different types of gastric cancer by immunohistochemistry and western blot and in different gastric cancer tissues from patients and different gastric cancer cell lines using tissue microarray assay. In this study, PrPc expression was associated with cell status and differentiation, which is consistent with previous reports^[9,20].

Dynamic changes in PrPc in the process of anti-cancer drug resistance

PrPc is closely related to tumor development. PrPc expression has been shown to be related not only to cancer cell proliferation but also to drug resistance and cancer cell metastasis^[21]. Meslin *et al*^[22] showed that PrPc gene silencing in ADM-resistant MCF-7 breast cancer cells increased the sensitivity of those cells to TRAIL-induced apoptosis. In addition, Bcl-2 expression was inhibited and pro-apoptotic Bax expression was increased following PrPc gene silencing, resulting in enhanced apoptosis. Our *in vitro* experiments further validated this result. After pulse induction of SGC7901 cells with different concentrations of adriamycin, PrPc, Bcl-2 and Bax expression and apoptosis were determined in adriamycin-resistant cells. Results showed that PrPc expression increased and apoptosis decreased with the increase in resistance to adriamycin. Our experiments not only show the dynamic changes in PrPc, Bcl-2 and Bax expression in gastric cancer cells resistant to various concentrations of adriamycin, but they also reveal that PrPc over-expression decreases apoptosis in cancer cells and reduces their sensitivity to drugs. It has been confirmed that PrPc is an anti-apoptotic gene that can cause synergistic effects with

Bcl-2. PrPc has been shown to be able to dimerize with the C-terminus of Bcl-2^[23,24]. When PrPc expression is up-regulated, the Bcl-2/Bax ratio is increased and is accompanied by the suppression of p53 and Bax, resulting in anti-apoptosis^[25-29]. Bcl-2 is expressed in almost all cell types, while PrPc expression is rarely observed in normal cells. This observation also determines the significance of PrPc expression in clinical practice. Our results confirmed the above findings^[2,10].

In another study, Meslin *et al.*^[22] detected PrPc expression in breast cancer patients that were non-responsive to an estrogen receptor inhibitor. Results of the study of the efficacy of chemotherapy indicated that patients with suppressed PrPc expression show favorable sensitivity to chemotherapy. This study was very important in monitoring the sensitivity of cancers to chemotherapy and drug resistance. In addition, detection of PrPc expression may become an indicator in diagnosing cancers and in monitoring the therapeutic efficacy. Our results confirmed that PrPc over-expression predicts gastric cancer cell drug resistance.

In addition, PrPc expression is closely related to the apoptosis pathway. Up-regulation of PrPc expression reduces apoptosis by increasing Bcl-2 expression^[2,3]. A study showed that the N-terminus of PrPc in metastatic gastric cancer with high PrPc expression could promote invasion and metastasis, which was partially associated with extracellular signal-regulated kinase/mitogen extracellular kinase pathway activation and subsequent transcriptional activation of MMP1^[5]. Furthermore, PrPc over-expression promoted the proliferation of gastric cancer cells and was strongly related to the incidence and development of gastric cancer^[6,7]. These *in vitro* experiments confirmed that the up-regulation of PrPc expression predicted enhanced proliferation and reduced apoptosis in gastric cancer cells, which resulted in drug resistance.

The role of PrPc expression in monitoring the therapeutic efficacy of gastric cancer

In the present study, positive PrPc expression was associated with the pathological type of gastric cancer. Mixed gastric cancer and undifferentiated gastric cancer had relatively high PrPc expression, which was consistent with tissue microarray assay results and the detection of PrPc expression in different gastric cancer cell lines. Results from the follow-up revealed that the patients with strongly positive PrPc expression showed poor sensitivity to chemotherapy and that the 2-year survival rates and KPS scores were significantly decreased. However, those with negative PrPc expression showed high sensitivity to chemotherapy, and the 2-year survival rates and KPS scores were remarkably higher than those with positive PrPc expression. Thus, PrPc could be a marker for the prognosis and response to chemotherapy in gastric cancer patients. In the National Center for Biotechnology Information gene bank (<http://www.ncbi.nlm.nih.gov/geo/>), results show PrPc expression in gastric cancer is higher than that in normal tissues (high-density oligonucleotide microarray, $P < 0.05$), and researchers speculate

PrPc may be a useful resource for future development of therapeutic targets and diagnostic markers for gastric cancer (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2685>)^[30]. This result also confirms our previous findings that PrPc expression in gastric cancer can promote multidrug resistance and metastasis^[2,3] and that PrPc over-expression in gastric cancer patients predicts tolerance to chemotherapy and a low survival rate. Long-term follow-up should be conducted to investigate the chemotherapeutic efficacy.

In summary, we conclude that: (1) PrPc expression is associated with gastric cancer pathological type and cancer cell differentiation; (2) PrPc exerts synergistic effects with Bcl-2 to decrease apoptosis, resulting in drug resistance; (3) Gastric cancer patients with PrPc over-expression are resistant to chemotherapy and have a poor prognosis. We speculate that PrPc might be one of the markers used for monitoring the response to chemotherapy and the prognosis of gastric cancer.

COMMENTS

Background

Prion Protein (PrPc or PrP) is a pathogen causing bovine spongiform encephalopathy (BSE, also known as "mad cow disease"). PrPc is encoded by host prion gene but not by the prion gene. In 2003, the authors' study showed that PrPc was associated with multi-drug resistance of gastric cancer and thereafter a series of studies have been conducted to explore the role of PrPc in the differentiation, proliferation and metastasis of cancer cells and to investigate the structural domains of PrPc. The present study aimed to investigate the PrPc expression in gastric cancer cell lines and the role of PrPc expression in predicting outcome of gastric cancer patients undergoing chemotherapy.

Research frontiers

PrPc is closely related to tumor development. Study has demonstrated PrPc expression is related with not only cancer cell proliferation, but also the drug resistance and metastasis of cancer cells associated with multi-drug resistance of gastric cancer. When PrPc expression is up-regulated, Bcl-2/Bax ratio is increased accompanied by suppression of p53 and Bax resulting in anti-apoptosis. Up-regulation of PrPc expression predicted enhanced proliferation and anti-apoptosis of gastric cancer cells resulting in drug resistance. In the National Center for Biotechnology Information gene bank, results show PrP expression in gastric cancer is higher than that in normal tissues, and researchers speculate PrP may be a useful resource for future development of therapeutic targets and diagnostic markers for gastric cancer.

Innovations and breakthroughs

In the article "Over-expression of PrPc and its antiapoptosis function in gastric cancer" to be published on March 24, 2006, the authors found that PrPc might play a role as an effective antiapoptotic protein through Bcl-2-dependent apoptotic pathways in gastric cancer cells. According to results from this article, the authors conclude that PrPc and Bcl-2 have synergistic anti-apoptotic effects leading to increased resistance to chemotherapy. The PrPc expression level might be a valuable marker in predicting the efficacy of chemotherapy and prognosis of gastric cancer patients receiving chemotherapy. The findings were based on a series of studies which have been conducted to explore the role of PrPc in the differentiation, proliferation and metastasis of cancer cells and to investigate the structural domains of PrPc.

Applications

PrPc expression could be used as a marker for predicting the efficacy of chemotherapy and prognosis of gastric cancer. PrPc over-expression in gastric cancer patients predicts poor chemosensitivity and poor prognosis. Therefore, the PrPc expression level might be a valuable marker in predicting the efficacy of chemotherapy and prognosis of gastric cancer patients receiving chemotherapy.

Terminology

PrPc is a pathogen causing BSE (also known as "mad cow disease"). The prion (from proteinaceous infectious only) is devoid of informational nucleic acids and

consists of an 'infectious' protein that is capable of converting a normal host protein termed PrPc, or simply PrP, into a likeness of itself. PrPc is encoded by host prion gene but not by prion gene.

Peer review

The authors explore the dynamic changes of PrPc in the process of gastric cancer drug-resistance and the role of PrPc expression in the prognosis of gastric cancer patients receiving chemotherapy. The study revealed that: (1) PrPc expression is associated with gastric cancer pathological type of and cancer cell differentiation; (2) PrPc exerts synergistic effects with Bcl-2 to decrease apoptosis, resulting in drug resistance; and (3) Gastric cancer patients with PrPc over-expression are resistant to chemotherapy and have a poor prognosis. These findings suggest that PrPc might be one of the markers used for monitoring the response to chemotherapy and the prognosis of gastric cancer.

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S- Editor Sun H L- Editor O'Neill M E- Editor Xiong L

Association of overexpression of TIF1 γ with colorectal carcinogenesis and advanced colorectal adenocarcinoma

Shilpa Jain, Shashideep Singhal, Franto Francis, Cristina Hajdu, Jin-Hua Wang, Arief Suriawinata, Yin-Quan Wang, Miao Zhang, Elizabeth H Weinshel, Fritz Francois, Zhi-Heng Pei, Peng Lee, Ru-Liang Xu

Shilpa Jain, Franto Francis, Cristina Hajdu, Miao Zhang, Zhi-Heng Pei, Ru-Liang Xu, Department of Pathology, New York University School of Medicine, New York, NY 10016, United States

Shashideep Singhal, Department of Medicine, The Brooklyn Hospital Center, Brooklyn, NY 11201, United States

Arief Suriawinata, Dartmouth-Hitchcock Medical Center, Lebanon, NH 03756, United States

Jin-Hua Wang, NYU Cancer Institute, New York University School of Medicine, New York, NY 10016, United States

Yin-Quan Wang, Department of General Surgery, the First Hospital of Shanxi Medical University, 030001 Taiyuan, Shanxi Province, China

Elizabeth H Weinshel, Fritz Francois, Department of Medicine, New York University School of Medicine, New York, NY 10016, United States

Peng Lee, NYU Cancer Institute, New York University School of Medicine, and New York Harbor Healthcare System, New York, NY 10016, United States

Author contributions: Jain S, Singhal S, Francis F, Hajdu C, Wang JH, Suriawinata A, Wang YQ, Zhang M, Lee P and Xu RL performed the study and wrote the manuscript; Weinshel EH, Francois F, Pei ZH participated in manuscript preparation and/or research design.

Supported by Department of Pathology Research Fund, NYU School of Medicine, New York, NY 10016, United States

Correspondence to: Ru-Liang Xu, MD, PhD, Department of Pathology, New York University School of Medicine, 560 First Avenue, New York, NY 10593,

United States. ruliang.xu@nyumc.org

Telephone: +1-212-2630728 Fax: +1-212-2637916

Received: December 16, 2010 Revised: January 11, 2011

Accepted: January 18, 2011

Published online: September 21, 2011

cer (CRC) development.

METHODS: Tissue microarrays were prepared from archival paraffin embedded tissue, including 51 colorectal carcinomas, 25 tubular adenomas (TA) and 26 HPs, each with matched normal colonic epithelium. Immunohistochemistry was performed using antibodies against TIF1 γ , Smad4 and TGF β R II. The levels of expression were scored semi-quantitatively (score 0-3 or loss and retention for Smad4).

RESULTS: Overexpression of TIF1 γ was detected in 5/26 (19%) HP; however, it was seen in a significantly higher proportion of neoplasms, 15/25 (60%) TAs and 24/51 (47%) CRCs ($P < 0.05$). Normal colonic mucosa, HP, and TAs showed strong Smad4 expression, while its expression was absent in 22/51 (43%) CRCs. Overexpression of TGF β R II was more commonly seen in neoplasms, 13/25 (52%) TAs and 29/51 (57%) CRCs compared to 9/26 (35%) HP ($P < 0.05$). Furthermore, there was a correlation between TIF1 γ overexpression and Smad4 loss in CRC (Kendall tau rank correlation value = 0.35, $P < 0.05$). The levels of TIF1 γ overexpression were significantly higher in stage III than in stage I and II CRC ($P < 0.05$).

CONCLUSION: The findings suggest that over-expression of TIF1 γ occurs in early stages of colorectal carcinogenesis, is inversely related with Smad4 loss, and may be a prognostic indicator for poor outcome.

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Key words: Colorectal cancer; Transcriptional intermediary factor 1 gamma; Transforming growth factor-beta signaling pathway; Smad4

Peer reviewer: Finlay A Macrae, MD, Professor, Department of Colorectal Medicine and Genetics, Royal Melbourne Hospital, Po Box 2010, Victoria 3050, Australia

Abstract

AIM: To determine the expression and clinical significance of transcriptional intermediary factor 1 gamma (TIF1 γ), Smad4 and transforming growth factor-beta (TGF β R) across a spectrum representing colorectal can-

Jain S, Singhal S, Francis F, Hajdu C, Wang JH, Suriawinata A, Wang YQ, Zhang M, Weinschel EH, Francois F, Pei ZH, Lee P, Xu RL. Association of overexpression of TIF1 γ with colorectal carcinogenesis and advanced colorectal adenocarcinoma. *World J Gastroenterol* 2011; 17(35): 3994-4000 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/3994.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.3994>

INTRODUCTION

Colorectal cancer (CRC) continues to be a significant cause of morbidity and mortality worldwide with over 1 million new cases diagnosed each year^[1]. Recent advances in treatment include both cytotoxic chemotherapies and novel biologic agents targeting specific cellular signaling pathways that regulate cell proliferation, apoptosis, and angiogenesis. The transforming growth factor-beta (TGF- β) signaling pathway is important in colorectal carcinogenesis and provides potential therapeutic molecular targets^[2].

The TGF- β signaling pathway is composed of TGF- β receptor type I (TGF β R I) and type II (TGF β R II) and Smad proteins. TGF- β ligands bind directly to membranous TGF β R II, trans-phosphorylating TGF β R I and enabling the TGF β R I kinase domain to act on cytoplasmic receptor-regulated Smad proteins (R-Smads)^[3,4]. TGF- β plays a unique dual role in growth suppression and cellular proliferation. In the normal colon, TGF- β may act as a tumor suppressor, inhibiting cellular proliferation and inducing apoptosis. Conversely in late stages of CRC, TGF- β acts as a tumor promoter, stimulating invasion and angiogenesis^[5,6]. The specificity in TGF- β activity is determined by R-Smads that propel downstream signaling through nuclear translocation and activation of target transcription genes. Development of hyperplasia, adenoma, CRC, and finally metastasis often involve inactivation of tumor suppressor genes and activation of oncogenes resulting in alterations in cell proliferation, apoptosis, migration, and invasion at the cellular level.

Transcriptional intermediary factor 1 gamma (also termed TIF1 γ /TRIM33/RFG7/PTC7/Ectoderm) functions as a cofactor of the TGF- β signaling pathway. However, TIF1 γ 's precise functional role is not clear. It is proposed to have functions both dependent and independent of R-Smads. TIF1 γ acts as a regulator of the TGF- β pathway through multiple R-Smad dependent mechanisms including by (1) targeting cytoplasmic Smad4 for degradation^[7]; (2) disrupting Smad-Smad complex formation; and (3) targeting nuclear Smad4 for degradation^[8]. In an R-Smad independent model, TIF1 γ is reported to act as a new co-Smad required for TGF- β signaling during human erythropoiesis^[9]. Thus TIF1 γ can function as either a negative regulator or a complementary agonist of TGF- β signaling.

Prior studies have shown that Smad4 deletion occurs in 16%-25% of CRCs and low levels of Smad4 suggest a poor response to therapy and short survival^[10,11]. Both TGF β R II and Smad4 mutations are reported to occur at

the early stage of transition of adenoma to carcinoma^[11]. An inverse relationship between the expression of Smad4 and TIF1 γ has been proposed based on *in vitro* studies^[7], whereas studies on pancreatic cystic tumors suggest that the two factors complement each other in tumorigenesis^[12]. The expression and the role of TIF1 γ in colorectal carcinogenesis remain unknown. This study is designed to analyze the expression of TIF1 γ in CRC and its pre-cancerous lesion (i.e., adenoma) in comparison with non-neoplastic lesions [hyperplastic polyps (HP)] and normal epithelium by immunohistochemical methods, and to assess the prognostic significance of abnormal TIF1 γ expression for CRC.

MATERIALS AND METHODS

Specimens

Formalin-fixed paraffin embedded archival tissue blocks of 51 colorectal carcinomas, 25 tubular adenomas (TA), 26 HPs, excluding sessile serrated polyp/adenoma morphologically with their normal epithelium were retrieved from the Department of Pathology, Tisch Hospital, New York University Medical Center between 2007 and 2009. Normal control tissue from each patient was taken from the margin of the resection specimen and from different parts or adjacent normal (non-neoplastic) tissue in the same patient. The study was approved by Institutional Review Board. Clinical and pathological data of each patient were obtained including age, sex, tumor size, tumor location, grade, type, stage and the status of *Kras* mutation. The type and differentiation of all neoplasms was evaluated by two independent pathologists (Cristina Hajdu and Ru-Liang Xu). Tissue microarrays (TMA) were prepared by using a 3-mm biopsy punch needle. Two representative cores from each lesion and 1 core from normal epithelium were taken from archival paraffin-embedded tissue blocks. Hematoxylin-eosin staining was performed for histological characterization.

Immunohistochemical staining for TIF1 γ , Smad4 and TGF β R II

Immunohistochemistry (IHC) was performed using single label technique by the NexES automated immunostainer and detection system (Ventana Medical Systems, Tucson, AZ, United States). Four micron-thick sections were deparaffinized in xylene, rehydrated through graded alcohols, and rinsed in distilled water. All incubations were carried out at 37 °C unless otherwise noted. After deparaffinization, heat induced epitope retrieval was performed by microwaving sections with 0.01 M, pH 6.0 citrate buffer for 20 min in a 1200 watt microwave oven. Endogenous peroxidase was blocked by application of hydrogen peroxide for 4 min. Monoclonal antibodies against TIF1 γ (TIF1gamma: sc-101179, Santa Cruz), Smad4 (Smad4: sc-7966, Santa Cruz, Biotechnology Inc, Santa Cruz, California, United States) and TGF β R II (TGFbeta2 receptor: ab28382, Abcam) at (1:50), (1:200) and (1:300) dilution respectively were applied to the TMAs followed by adding

Table 1 Correlation of transcriptional intermediary factor 1 gamma overexpression, Smad4 inactivation, and transforming growth factor-beta receptor type II overexpression with various clinicopathological features (%)

	<i>n</i>	TIF1 γ overexpression	Smad4 loss	TGF β R II overexpression
Stage				
I + II	30	10 (33.3)	11 (36.6)	15 (50.0)
III	21	14 (66.7) ^a	11 (52.4)	14 (66.6)
Grades of differentiation				
Well-mod	37	18 (48.6)	18 (48.6)	27 (73.0)
Poor	8	4 (50.0)	2 (25.0)	2 (25.0)
Mucinous	6	2 (33.3)	2 (33.3)	0 (0.0)
Site				
Right	27	10 (37.0)	10 (37.0)	13 (48.2)
Left	16	10 (62.5)	7 (43.8)	10 (62.5)
Rectal	8	4 (50.0)	5 (62.5)	6 (75.0)
<i>Kras</i> mutation				
Present	11	6 (54.5)	9 (81.8) ^a	8 (72.7)
Absent	26	10 (38.5)	11 (42.3)	14 (53.8)

^a*P* < 0.05. TIF1 γ : Transcriptional intermediary factor 1 gamma; TGF β R II: Transforming growth factor-beta receptor type II.

a biotinylated goat anti-rabbit for 8 min, and subsequently by the application of streptavidin-horseradish peroxidase for 8 min. The reaction was visualized by applying chromogen, 3,3'-diaminobenzidine/hydrogen peroxide mix for 8 min and copper sulfate for enhancement for an additional 4 min. Slides were then counterstained with hematoxylin, dehydrated, and mounted in permanent media. Primary antibody was omitted in negative controls.

Immunohistochemical labeling for TIF1 γ , Smad4 and TGF β R II was evaluated by three independent authors with the agreement in all cases examined. Cytoplasmic staining of TGF β R II and nuclear staining of TIF1 γ was considered positive. The immunolabeling pattern of each case was scored based on the percentage of cells with positive staining and intensity. Intensity was scored semi-quantitatively as: strong (3+), moderate (2+), weak (1+) and negative (0). Final score was determined as the highest intensity score obtained by > 20% of positively stained cells. Final expression was determined by adjusting for the staining score on corresponding normal epithelium. Overexpression of TIF1 γ and TGF β R II in the neoplasm was considered if the adjusted final score was \geq 1.

For Smad4, the immunostaining pattern of each case was scored as "no loss" (positive) or "loss" (negative). Normal colonic epithelium served as a positive control showing strong nuclear staining. Neoplasms and HP were scored as "no loss" if the neoplastic or hyperplastic epithelium showed any nuclear labeling and as "loss" if they showed no or only faint cytoplasmic staining with total absence of nuclear Smad4 protein.

Kras mutation

Out of 51 CRCs cases, 37 cases had *Kras* mutation data. *Kras* genotyping was performed using polymerase chain reaction with exon 2 flanking primers followed by capil-

lary gel electrophoresis fluorescence detection in the Molecular Diagnostic Laboratory, Genzyme, Inc. This assay analyzes codons 12 and 13 in exon 2 of the *Kras* gene. However, mutations in codon 61 and other sites were not tested. The analytical sensitivity of the assay is approximately 10%, thus mutations present in a low percentage of cells may not be detected.

Statistical analysis

Summary data were expressed as proportions and percentages. Comparisons among groups were performed using χ^2 test. Correlation between the different protein expression and clinical variables was performed by Kendall tau rank correlation test. Probability values of 0.05 or less were considered significant. Linear predictive module was used to predict the stage of the cancer according to different variables.

RESULTS

We studied the expression of TIF1 γ in association with Smad4 and TGF β R II in TA (*n* = 25) and CRC (*n* = 51) in comparison with matched normal control and non-neoplastic lesions or HP (*n* = 26). Of the 51 CRCs 37 (72%) were well-to-moderately differentiated, 8 (15%) poorly differentiated, and 6 (11%) mucinous adenocarcinoma. According to the TNM staging system by the American Joint Committee on Cancer, 16 (31.3%) were stage I, 14 (27.4%) stage II, and the remaining 21 (41.2%) stage III. Lymph node metastases were present in 21 (41.2%) of the CRCs at the time of diagnosis. The clinical and pathologic characteristics of the 51 patients with CRC were collected. The median age of the 51 individuals diagnosed with CRC was 70 \pm 10.8 years (42-85 years), and 27 of 51 (52.9%) were men. Patients showed no sex predilection. Twenty-seven (41%) of the lesions diagnosed as CRCs were located in the proximal colon (right side), 16 (45%) were in the distal colon (left side), while 8 (13%) were in the rectum. None of the patients diagnosed with CRC received neoadjuvant therapy. The mean size of the 51 tumors was 4.3 cm \pm 1.7 cm. The levels of nuclear or cytoplasmic expression of TIF1 γ , TGF β R II, and Smad4 were correlated with patient's clinical and *Kras* mutation status (Table 1).

Increased TGF β R II expression in TA and CRCs

TGF β R II expression was observed in the cytoplasm of the normal epithelium and its intensity varied from 1+ to 2+ in all the cases (Figure 1A, B and C). Increased expression was more commonly seen in neoplastic lesions, TA (13/25 or 52%) and CRC (29/51 or 57%), as compared to HP (9/26 or 35%) (*P* < 0.05) (Figure 2). However, no statistical difference was found in overexpression between CRC and TA. Increased expression of TGF β R II was more frequent in well-to-moderately differentiated carcinomas compared to poorly differentiated lesions (73% vs 25%). Overexpression of TGF β R II was not observed in mucinous type carcinomas. TGF β R II expression in CRC

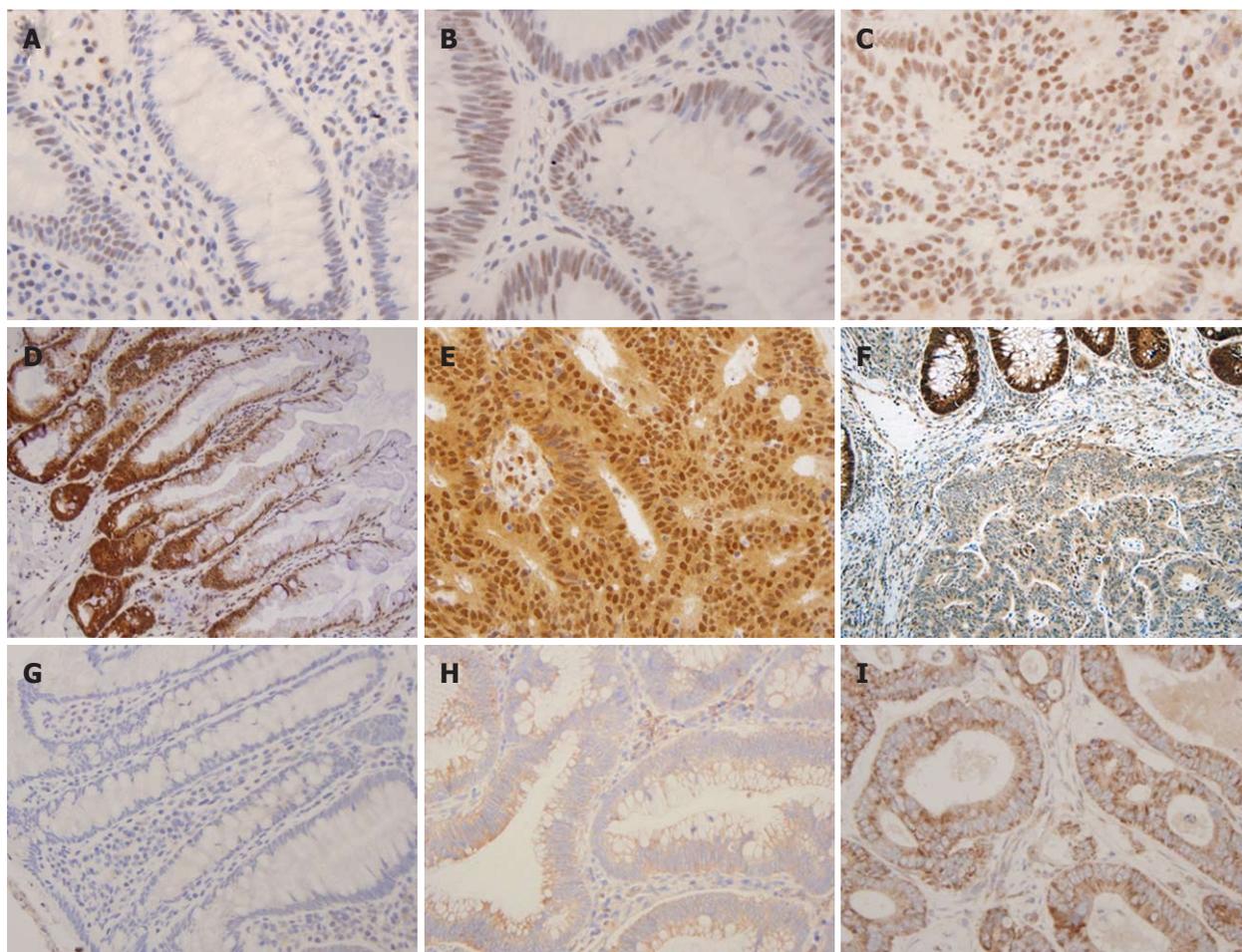


Figure 1 Semi-quantitative immunohistochemistry scoring of transcriptional intermediary factor 1 gamma, Smad4 and transforming growth factor-beta receptor type II overexpression. Immunohistochemistry (IHC) staining for transforming growth factor-beta receptor type II shown in A, B, C: Weak cytoplasmic (0-1+) staining seen in normal colonic mucosa (A). Moderate (2+) cytoplasmic and weak (0-1+) membranous staining in the tubular adenoma (TA) (B). Strong cytoplasmic and focal membranous staining in the cancer cells (C). IHC staining for Smad4 shown in D, E, F: Strong (3+) Smad4 staining seen in normal colonic mucosa (D). Tumour cells showing strong (3+) expression of Smad4 protein in the nucleus and cytoplasm (E). Adenocarcinoma with loss of Smad4 in the nuclei (F). IHC staining for transcriptional intermediary factor 1 gamma (TIF1 γ) shown in G, H, I: Weak (1+) nuclear staining for TIF1 γ seen in normal colonic mucosa (G), moderate (2+) nuclear staining seen in TA (H), strong nuclear staining (3+) in cancer cells (I) (400 \times).

lesions did not correlate with patient age, sex, site, size, stage or *Kras* mutation.

Loss of Smad4 expression in CRC

Normal colonic epithelium displayed uniformly distributed strong (3+) nuclear Smad4 staining but in occasional cases staining was stronger at the bottom of the crypts (Figure 1D, E and F). While none of the HP or TAs showed loss of expression of Smad4 (Table 1), a significant proportion of CRC cases (22/51 or 43%) completely lacked the nuclear expression of Smad4. The lack of Smad4 expression was more frequent in primary CRC with lymph node metastasis (11/21 or 52.4%) than without lymph node metastasis (11/30 or 36.6%). Of 37 cases of CRC with available data of *Kras* mutation status, 11 cases had mutations in codon 12 or 13. The loss of Smad4 expression was significantly higher in CRCs with *Kras* mutations (9/11 or 81.8%) than the cases without (11/26 or 42.3%) ($P < 0.05$). There was no significant difference in the expression loss of Smad4 related to the

age, sex, site, size and grade of CRC.

Overexpression of TIF1 γ in hyperplastic and neoplastic lesions

TIF1 γ protein was located in the nucleus of normal colonic epithelium on IHC with intensity of 1+ to 2+ in the majority (96%) of cases, and 3+ nuclear staining in 4% normal epithelium. The staining was seen predominantly in the lower part of the crypts in comparison to the luminal (Figure 1G, H and I). Increased expression of TIF1 γ was more frequently seen in neoplasms, TA (15/25 or 60%) and CRC (24/51 or 47%) than HPs (5/26 or 19%) ($P < 0.05$). There was no statistical difference in TIF1 γ overexpression between TA and CRC ($P < 0.05$) (Figure 2).

Association of overexpression of TIF1 γ with higher stage CRC

The frequency of TIF1 γ overexpression was significantly higher in stage III CRC in comparison to stage I and II CRC ($P < 0.05$). Using a linear predictive module for

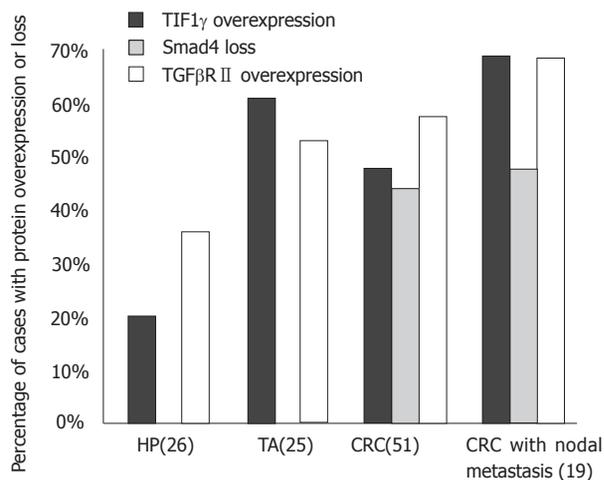


Figure 2 Comparison of transcriptional intermediary factor 1 gamma overexpression, Smad4 loss, and transforming growth factor-beta receptor type II overexpression among hyperplastic polyps, tubular adenomas, and colorectal cancer. HP: Hyperplastic polyps; TA: Tubular adenomas; CRC: Colorectal cancer; TIF1 γ : Transcriptional intermediary factor 1 gamma; TGF β R II: Transforming growth factor-beta receptor type II.

the higher stage (III), the predictivity was 67% by TIF1 γ overexpression in comparison to the marginal predictivity (58%) by loss of Smad4. However, there was no statistical difference in TIF1 γ overexpression between the primary tumors and matched lymph node metastasis ($P > 0.05$). There was no significant difference in the expression of TIF1 γ related to the age, sex, site, size, grade and *Kras* mutation of CRCs ($P > 0.05$) (Table 1).

Correlation between overexpression of TIF1 γ and abnormal expression of Smad4 and TGF β R II in CRC

To determine the relationship among the levels of expression for TIF1 γ , Smad4 and TGF β R II, we performed Kendall tau rank correlation test. In CRC a significant correlation was seen between TIF1 γ overexpression and Smad4 loss (Kendall tau value 0.35, $P < 0.05$). There was no significant correlation between Smad4 loss and TGF β R II overexpression in CRC, or between TIF1 γ and TGF β R II overexpression in CRC, TA or HP.

DISCUSSION

Our study has evaluated the expression of important mediators, TIF1 γ , Smad4 and TGF β R II, in TGF- β signaling pathway in normal epithelium, HP (benign nondysplastic lesion), TA (precursor lesion) and CRC by semi-quantitative IHC analysis. TGF- β , belonging to a ligand-receptor family that also includes bone morphogenetic protein and activin, is often excessively produced in CRCs, presumably owing to loss of feedback inhibition with disruption of its intracellular Smad signaling pathway. The autocrine activity from elevated secretion of TGF- β ligand increases expression of TGF β R II to unmask the interruption of Smad-dependent signaling to suppress tumor growth. A similar phenomenon has been reported previously *in*

in vitro in prostate cancer cells^[13]. Our study also showed increased expression of TGF β R II in TA (52%) and well-to-moderately differentiated CRCs (73%). Functional mutations in TGF β R II have been reported in approximately 30% of CRCs in later stage^[14,15]. Low TGF β R II expression was found in poorly differentiated CRC (25%), similar to previous studies. The finding represents failure of TGF- β antiproliferative and apoptotic effects in advanced CRC and other cancers^[16-18]. Smad4 protein expression was absent in a significant proportion (43%) of CRC cases, which is consistent with literature^[10]. None of normal epithelium, HP and TAs displayed Smad4 loss, supporting that Smad4 loss occurs at a later stage in colorectal carcinogenesis^[19,20].

This is the first report attempting to elucidate the role of TIF1 γ in colorectal carcinogenesis and its interaction with Smad4, a key regulator of TGF- β signaling pathway. TIF1 γ is the third member of the TIF1 gene family observed in the nucleus of normal epithelium and cancer cells and has been shown to selectively bind to receptor-activated Smads 2 and 3^[9]. In the present study TIF1 γ overexpression was seen in non-dysplastic or non-precursor lesion HP (19%), and more frequently found in neoplasms, TA (60%) and CRC (47%) ($P < 0.05$ respectively). This suggests that TIF1 γ is involved in abnormal cell proliferation and early stages of colorectal carcinogenesis, as compared to Smad4 loss which was only seen in later adenoma stage and CRC. This finding is, however, different from the results in human pancreatic ductal adenocarcinoma, which conversely showed down-regulation of TIF1 γ ^[12].

In a mouse model of pancreatic carcinogenesis, TIF1 γ was shown to be in cooperation with *Kras* activation to induce pancreatic tumors, reminiscent of human Intraductal Papillary Mucinous Neoplasms^[12]. In our study, we did not find a positive relationship between the expression of TIF1 γ and common mutations (codon 12 or 13 of exon 2) of *Kras* in CRC, but showed an inverse relationship between Smad4 loss and *Kras* mutation. This may suggest that interaction between TGF- β tumor suppressing pathway and Ras-MAPK pathways is different in colorectal carcinogenesis from in its pancreatic counterpart. However, this observation may be biased because of a relatively small number of samples. A large sample size study and/or an animal model is needed to provide a more definite answer.

A significant correlation between TIF1 γ overexpression and Smad4 loss suggests a mutual interaction between the two molecules similar to the result demonstrated *in vitro* in breast, colon and pancreatic Smad4-defective cancer cell lines^[9]. Recently it has been shown that TIF1 γ acts as a general inhibitor of TGF- β and bone morphogenetic proteins (BMP) signaling pathways by acting as an E3 ubiquitin ligase causing Smad4 ubiquitination and degradation^[7]. Consistent with this hypothesis, our findings suggest that TIF1 γ is overexpressed in early stages, and possibly acts by degradation of Smad4 disrupting its tumor suppressor activity leading to progression of

adenoma-carcinoma sequence. Our results, however, are only based upon immunohistochemical study, and further conformational study is needed to support the above conclusions.

In addition to its role in abnormal proliferation and early carcinogenesis, we found a significantly high expression of TIF1 γ in stage III CRC with nodal metastasis in comparison to stage I and II CRC. Using a linear predictive module for advanced stage III in comparison to stage I/II, Smad4 loss had marginal predictivity (58%) in comparison to 67% by TIF1 γ overexpression. The findings suggest that TIF1 γ might also be involved in tumor progression. Consistent with earlier reports, the frequency of Smad4 loss was also higher in stage III CRC in comparison to stage I/II CRC^[10]. This further indicates that TIF1 γ and Smad4 could possibly act in collaboration with each other during colorectal carcinogenesis. Statistical differences in TIF1 γ and Smad4 expression based on tumor size, site and grade cannot be concluded from the present study. Combined TIF1 γ and Smad4 expression may serve as a marker for high stage disease in colon biopsy specimen. Since a large proportion of CRCs in our patients are located in the left side, further study is needed to clarify the relationship between the microsatellite stability of CRC and TIF1 γ and/or Smad4 expression.

In summary, overexpression of TIF1 γ occurs in association with abnormal proliferation and in early stages of colorectal carcinogenesis. It shows an inverse relationship with Smad4 loss, suggesting that TIF1 γ may have a collaborative effect with Smad4 in colorectal carcinogenesis. Our study also found that overexpression of TIF1 γ is associated with high tumor stage, indicating that it is a poor prognostic factor. Further molecular studies are needed to evaluate the role of TIF1 γ in colorectal carcinogenesis, its interaction with other factors in TGF β /BMP pathways, and utility as a prognostic marker for CRC.

COMMENTS

Background

Colorectal cancer (CRC) continues to be a significant cause of morbidity and mortality worldwide despite recent development of new therapy, mainly because the molecular mechanisms underlining the colorectal carcinogenesis are not completely understood. Transcriptional intermediary factor 1 gamma (TIF1 γ) is a recently identified cofactor of Smad4, a key part of transforming growth factor-beta (TGF- β) signaling pathway. It has been shown that TIF1 γ plays an important role in early embryonic development and potentially is involved in carcinogenesis in some organs or systems. Hitherto, the role of this factor in colorectal carcinogenesis and the significance of the abnormal expression in the gastrointestinal tract are unknown.

Research frontiers

TIF1 γ functions as a cofactor of the TGF- β signaling pathway, acting on Smad4 or Smads complex by ubiquitination. Smad4 as a tumor suppressor has been shown to be a prognostic factor in a subset of patients with CRC. In a mouse model the inactivation of this protein appeared to cooperate with Kras mutation (G12D) to induce cystic tumors of the pancreas, and the down-regulation of this protein was seen in pancreatic ductal carcinoma. This study is designed to analyze the expression of TIF1 γ in CRC and its precancerous lesion (i.e., adenoma) in comparison with a non-neoplastic lesion (hyperplastic polyps)

and normal epithelium by an immunohistochemical method, and to explore the prognostic significance of abnormal TIF1 γ expression in CRC. Correlation with abnormal expression of Smad4, TGF β R II in TGF- β pathway and Kras mutation in CRC has also been investigated.

Innovations and breakthroughs

This is the first study attempting to elucidate the role of TIF1 γ in colorectal carcinogenesis and to determine its interaction with Smad4, a key regulator of TGF- β signaling pathway and Kras mutation in CRC. The findings suggest that TIF1 γ is overexpressed in early stages, independently from the inactivation of Smad4 protein and Kras mutation. This study further demonstrates that overexpression of TIF1 γ is associated with high stage of CRC, indicating that it is a poor prognostic factor.

Applications

The results will open an avenue for further research to evaluate the role of TIF1 γ in colorectal carcinogenesis, its interaction with other factors in TGF β /bone morphogenetic proteins pathways, and to elucidate its utility as prognostic marker for CRC.

Terminology

The TGF- β signaling pathway is involved in regulating cell proliferation, apoptosis, and angiogenesis. TIF1 γ (also termed TIF1 γ /TRIM33/RF7/PTC7/Ectodermin) functions as a cofactor of the TGF- β signaling pathway, proposed to have functions both dependent and independent from receptor-regulated Smad proteins. Understanding the pathways involved in carcinogenesis provides potential prognostic value and facilitates novel therapeutic molecular targets.

Peer review

This is a new insight into colorectal carcinogenesis, which deserves publishing. One would like to see the results confirmed with alternate techniques e.g., other expression studies. The English is good. The number of samples is adequate.

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S- Editor Tian L L- Editor O'Neill M E- Editor Xiong L

Role of high definition colonoscopy in colorectal adenomatous polyp detection

Tolga Erim, John M Rivas, Evelio Velis, Fernando Castro

Tolga Erim, Department of Gastroenterology, Cleveland Clinic Florida, Weston, FL 33331, United States

John M Rivas, Department of Internal Medicine, Cleveland Clinic Florida, Weston, FL 33331, United States

Evelio Velis, Health Services Administration Master Program, Barry University, Miami Shores, Florida 33161, United States

Fernando Castro, Department of Gastroenterology, Cleveland Clinic Florida, Weston, FL 33331, United States

Author contributions: Erim T, Rivas JM and Castro F contributed equally to this work; Erim T and Castro F designed the research; Erim T and Rivas J performed the research; Velis E contributed new reagents/analytic tools; Erim T, Rivas J, Velis E and Castro F analyzed the data; Erim T, Rivas JM and Castro F wrote the paper.

Supported by Cleveland Clinic Florida Institution Review Committee

Correspondence to: Dr. Tolga Erim, Department of Gastroenterology, Cleveland Clinic Florida, 2950 Cleveland Clinic Blvd, Weston, FL 33331, United States. erimt@ccf.org

Telephone: +1-954-6595000 Fax: +1-954-6595480

Received: December 17, 2010 Revised: March 11, 2011

Accepted: March 18, 2011

Published online: September 21, 2011

Abstract

AIM: To investigate the rates of polyp detection in a mixed risk population using standard definition (SDC) vs high definition colonoscopes (HDC).

METHODS: This was a retrospective cohort comparative study of 3 colonoscopists who each consecutively performed 150 SDC (307, 200 pixel) and 150 HDC (792, 576 pixels) in a community teaching hospital.

RESULTS: A total of 900 colonoscopies were evaluated (mean age 56, 46.8% men), 450 with each resolution. Polyps of any type were detected in 46.0% of patients using SDC and 43.3% with HDC ($P = 0.42$). There was no significant difference between the overall number of polyps, HDC (397) and SDC (410), detected among

all patients examined, ($P = 0.73$). One or more adenomatous polyps were detected in 24.2% of patients with HDC and 24.9% of patients with SDC colonoscopy ($P = 0.82$). There was no significant difference between HDC (M = 0.41) and SDC (M = 0.42) regarding adenomatous polyp ($P = 0.88$) or advanced adenoma ($P = 0.56$) detection rate among all patients examined.

CONCLUSION: HDC did not improve yield of adenomatous polyp, advanced adenoma or overall polyp detection in a population of individuals with mixed risk for colorectal cancer.

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Key words: High definition colonoscopy; Colon cancer screening; Adenomatous polyps

Peer reviewer: Dr. Shinji Tanaka, Director, Department of Endoscopy, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.

Erim T, Rivas JM, Velis E, Castro F. Role of high definition colonoscopy in colorectal adenomatous polyp detection. *World J Gastroenterol* 2011; 17(35): 4001-4006 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/4001.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.4001>

INTRODUCTION

It is estimated that up to 15 million colonoscopies are performed annually in the United States^[1,2]. Colonoscopy and polypectomy have been estimated to prevent 50%-80% of colorectal cancers^[3-5]. However, recent trials have implied a lower level of protection and even a failure of colonoscopy to prevent right sided colon cancer^[6,7]. With adenoma miss rates of up to 20% demonstrated for moderate sized polyps, the potential of improved polyp detection in preventing colon cancer

deaths could be substantial^[8-10].

A considerable effort is being spent on optimizing the yield of colonoscopy with respect to polyp detection. Several technologies such as wide-angle, cap-fitted, retroflexion colonoscopy and Third Eye Retroscope colonoscopy have been used in an attempt to increase mucosa exposure. Various optical enhancing technologies such as chromoendoscopy, narrow-band (NBI) and multi-band imaging, high definition, and autofluorescence have been studied as well. While some have been found to be effective in expert hands in tertiary care centers, many techniques suffer from issues of practicality. The rising demand of colon cancer screening and the advent of several different modalities for this purpose, such as computerized tomography colonoscopy, have stressed the importance of improved efficiency in colonoscopy.

At present, the only technical developments that are readily available and in use in routine practice settings in the United States are wide angle, high definition and NBI/multi-band imaging. High-definition endoscopes have been touted by manufacturers to show markedly clearer images in hopes that this would translate into higher polyp detection rates. In the current study, we present a comparison of polyp detection rate of endoscopists using standard definition and high definition endoscopy systems.

MATERIALS AND METHODS

Patients

Nine hundred consecutive patients who had colonoscopy between May 2007 and May 2008 by three experienced endoscopists (> 6000 colonoscopies each) were selected for analysis retrospectively. Patients were mixed risk and all colonoscopies were performed at the same endoscopy center of a community teaching hospital in Florida, United States. Colonoscopies performed by gastroenterology fellows were excluded from the study. The study was approved by the Institutional Review Board at Cleveland Clinic Florida.

Endoscopy equipment

The standard definition colonoscopies (SDC) were performed with an EPK-1000 processor (Pentax), EC-3430LK, EC-3830LK, EC-3470LK, and EC-3870LK model colonoscopes (Pentax), a 19-inch CRT monitor at a resolution of 640 × 480 producing a 307, 200 pixel image at distance of approximately 2.8 m from the endoscopists. The high definition colonoscopies (HDC) were performed with an EPX-4400 digital processor (Fujinon), EC-450HL5 and EC-450LS5 model colonoscopes (Fujinon), a 32-inch LCD monitor at a resolution of 1032 × 768 producing a 792, 576 pixel image at a distance of approximately 2.8 m from the endoscopists. Both standard definition and high definition colonoscopes had a 140° field of view.

The Fujinon system has the capability of multi-band imaging that produces images similar to the NBI endo-

scopes, commercially termed Fuji Intelligent Chromo Endoscopy (FICE). The difference lies in that the Fujinon system uses software to construct images based on preset RGB wavelength combinations. The NBI systems use optical filters that restrict the bandwidth of a transmitted light signal. Currently available NBI systems utilize 2 narrow-band filters that provide tissue illumination in the blue (415 nm) and green (540 nm) spectrums of light^[11]. The Fujinon equipment has ten factory-determined wavelength preset combinations.

Endoscopic procedures

Data from one hundred fifty consecutive patients who had colonoscopy with standard definition (SD) equipment were collected for three endoscopists from May - October 2007. Following the installation of the HDC system, all endoscopic procedures in our unit were performed exclusively using the high definition (HD) scopes and data was collected from 150 consecutive patients who had colonoscopy by the same three endoscopists from October 2007 - March 2008. The endoscopists were not aware of the study. Bowel preparation agents used were predominantly sodium phosphate and polyethylene glycol based regimens. The procedures were performed under a nurse administered standard sedation with Meperidine and Midazolam or anesthesiologist administered Propofol. Colonoscopy withdrawal times were recorded by the nursing staff.

Endoscopists were free to use the multi-band feature on the HDC system as needed. The system was initially set on the factory default preset of 0, which produced an image restricted to the following wavelengths: R 500 nm, G 445 nm, and B 415 nm. A push-button on the handle of the colonoscope was programmed to enable switching between conventional white-light image and the preset multi-band image. Endoscopists were also free to change to a different factory preset according to their preferences. The study was designed prior to arrival of the high definition system; however, data collection was started afterwards.

Data collection

The data was collected from electronic medical records, procedure nursing notes, procedure reports, and pathology reports. The numbers of detected polyps recorded on the procedure reports were corroborated with the pathology reports and the nursing notes. The main outcome parameter was the polyp detection rate in the two groups. Secondary outcome measures included: detection rates of adenomatous polyps, advanced adenomas, and cancer. Advanced adenoma was defined as adenomatous polyps having one or more features of: > 1 cm in diameter, high-grade dysplasia, and villous histology. Additional data was collected with regards to patient age, gender, race, indication for colonoscopy, polyp location, procedure time, withdrawal time, type of sedation, and prep quality.

Table 1 Patient Characteristics *n* (%)

Parameters	HD group (<i>n</i> = 450)	SD ² group (<i>n</i> = 450)	<i>P</i> value
Patients			
Mean age, years (SD ¹)	55 (± 12.5)	56 (± 11.4)	0.21
Men	213 (47.3)	208 (46)	0.86
Race			
White	233 (51.8)	281 (62.4)	0.10
African American	49 (10.9)	53 (11.8)	0.75
Hispanic	139 (30.9)	95 (21.1)	0.01
Others	29 (6.4)	21 (4.7)	0.31
Indication			
Screening	216 (48.0)	173 (38.4)	0.07
Non-screening	234 (52.0)	277 (61.6)	0.13
Cecal intubation	433 (96.2)	438 (97.3)	0.92
Poor prepare	14 (3.1)	17 (3.8)	0.72
Withdrawal all procedures, min (SD ¹)	11.3 (± 6.1)	10.8 (± 5.6)	0.20
Withdrawal non-polypectomy, min (SD ¹)	10.0 (± 5.9)	9.2 (± 4.2)	0.02

¹Standard deviation; ²Standard definition; HD: high definition; min: Minutes.

Table 2 Detection of all polyps, adenomas, and cancer *n* (%)

Parameters	HD group (<i>n</i> = 450)	SD ² group (<i>n</i> = 450)	<i>P</i> value
Total polyps detected	397	410	0.73
Non-adenomas	196 (51.3)	209 (52.0)	0.81
Non-advanced adenomas	150 (39.3)	150 (37.3)	0.84
Advanced adenomas	34 (8.9)	40 (10.0)	0.60
Cancer	2 (0.5)	3 (0.7)	1.00
Pathology not identified	15 (3.8)	8 (2.0)	0.14
< 6 mm	325 (81.9)	340 (82.9)	0.96
6-10 mm	50 (12.6)	44 (10.7)	0.45
> 10 mm	22 (5.5)	24 (5.9)	1.00
Size not specified	0	2	0.50
All patients			
With non-adenomas	84 (18.7)	92 (20.4)	0.56
With non-advanced adenomas	84 (18.7)	83 (18.4)	1.00
With advanced adenomas	25 (5.6)	29 (6.4)	0.67
With cancer	2 (0.4)	3 (0.7)	1.00
Polyps per pt, mean (SD ¹)	0.88 (± 1.63)	0.91 (± 1.41)	0.77
Adenomas per pt, mean (SD ¹)	0.41 (± 1.04)	0.42 (± 0.94)	0.88
Advanced adenoma per pt, mean (SD ¹)	0.076 (± 0.35)	0.089 (± 0.38)	0.56
Adenocarcinoma per pt (mean)	0.004	0.006	
Screening patients, <i>n</i>	216	173	0.07
With non-adenomas	49 (22.7)	36 (20.8)	0.81
With non-advanced adenomas	41 (19.0)	32 (18.5)	1.00
With advanced adenomas	13 (6.0)	14 (8.1)	0.55
With cancer	1 (0.5)	0 (0.0)	1.00
Non-screening patients, <i>n</i>	234	277	0.13
With non-adenomas	35 (15.0)	56 (20.2)	0.59
With non-advanced adenomas	43 (18.4)	51 (18.4)	0.21
With advanced adenomas	12 (5.1)	15 (5.4)	1.00
With cancer	1 (0.4)	3 (1.1)	0.63

¹Standard deviation; ²Standard definition; HD: high definition; Per pt: Per patient.

Statistical analysis

The Statistical Package for Social Sciences (SPSS 16.0) was used in order to organize, validate and analyze the collected data. Quantitative data were summarized using mean values (M) and standard deviation; Student's *t* test were performed in order to detect significant differences between colonoscope types; equality of variances was inspected using Levene's tests. We examined associations between categorical variables, performing χ^2 tests or Fisher's exact test when appropriate.

RESULTS

A total of 900 colonoscopies were evaluated, comparing 450 patients each who had colonoscopy with SDC equipment and HDC equipment. Each endoscopist performed 300 colonoscopies equally divided between standard and high definition procedures. The mean age of the study population was 56, and 46.8% were men. There were no statistically significant differences in patient characteristics between the two groups with the exception of a higher number of Hispanic patients and those that had screening colonoscopy in the HDC group (Table 1). However, there was no overall difference in adenomatous polyp detection rate in Hispanics (23.9%) *vs* Non-Hispanics (24.6%) ($P = 0.86$) and the screening (25.7%) and non-screening (21.7%) groups ($P = 0.18$).

Cecal intubation, bowel prep quality and withdrawal times were also not statistically significantly different between the HDC and SDC groups. The cecum was reached in 96.7% of all cases. Average withdrawal time was 11.1 min, which included polypectomy time.

Polyps of any type were detected in 46.0% of patients with SDC and 43.3% of those patients who had HDC ($P = 0.42$). There was no significant difference between the overall number of polyps, HDC (397) and SDC (410), detected among all patients examined ($P = 0.73$). One or more adenomatous polyps were detected in 24.2% of patients with HDC and 24.9% of patients with SDC ($P = 0.82$). There was no significant difference between HDC (M = 0.41) and SDC (M = 0.42) regarding adenomatous polyp detection rate among all patients examined ($P = 0.88$). In addition, there was no significant difference between the study groups regarding advanced adenoma polyp detection rates ($P = 0.60$) or cancer detection rate among all patients examined ($P > 0.05$) (Table 2). There was no difference in polyp detection rates when each individual endoscopist's HDC and SDC detection rates were compared (data not shown).

Polyps detected during the procedures were also analyzed according to size. There was no significant difference between the detected number of polyps of sizes < 6 mm, 6-10 mm, and >10 mm in the HDC and SDC groups.

Gender was shown to be a significant variable as men in this study were found to have a higher incidence of all polyps ($P < 0.01$), adenomatous polyps ($P < 0.01$) and

Table 3 Detection of all polyps, adenomas and cancer, with respect to gender *n* (%)

Parameters	HD group (<i>n</i> = 450)	SD group (<i>n</i> = 450)	<i>P</i> value
Male			
Total polyps detected	264	245	0.74
Patients	213	208	0.86
With non-adenomas	42 (19.7)	46 (22.1)	0.48
With non-advanced adenomas	52 (24.4)	43 (20.7)	0.50
With advanced adenomas	17 (8.0)	21 (10.1)	0.50
With cancer	1 (0.5)	2 (1.0)	0.62
Female			
Total polyps detected	133	165	0.21
Patients	237	242	0.87
With non-adenomas	42 (17.7)	46 (19.0)	0.82
With non-advanced adenomas	32 (13.5)	40 (16.5)	0.45
With advanced adenomas	8 (3.4)	8 (3.3)	1.00
With cancer	1 (0.4)	1 (0.4)	1.00

SD: Standard definition; HD: High definition.

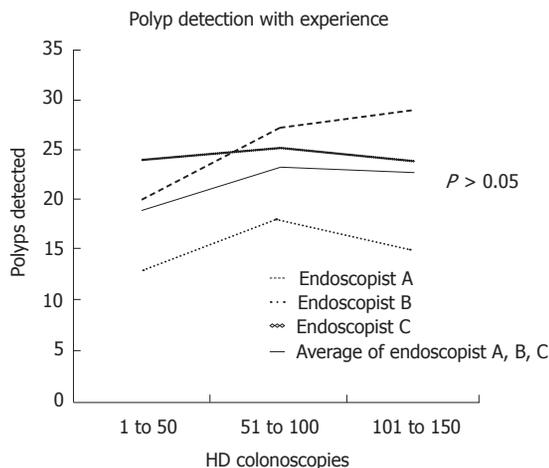


Figure 1 Comparison of polyp detection per endoscopist with gained high definition experience. HD: High definition.

Table 4 Comparison of standard vs high definition adenomatous polyp detection studies to date

Study	Date	Method	<i>n</i>	Colonoscope		<i>P</i> value for adenoma detection rates
				(resolution)	(Angle) (NBI capable)	
				Group 1	Group 2	
East <i>et al</i> ^[13]	2008	Prospective nonrandomized	130	Olympus SD 140	Olympus HD 140	0.200
Pellise <i>et al</i> ^[12]	2008	Prospective randomized	620	Olympus SD 140	Olympus HD 170	0.850
Tribonias <i>et al</i> ^[14]	2010	Prospective randomized	390	Olympus SD 140	Olympus HD 170	0.160
Burke <i>et al</i> ^[16]	2010	Retrospective	852	Olympus SD 140	Olympus HD 170	0.360
Buchner <i>et al</i> ^[15]	2010	Retrospective	2430	Olympus SD 140	Olympus HD 170 NBI	0.012

NBI: Narrow-band imaging; SD: Standard definition; HD: High definition.

advanced adenomas ($P < 0.01$). However, this disparity was consistent in both cohorts with no statistically significant difference between the HDC and SDC groups (Table 3).

We found that the overall polyp and adenoma detection rates did not change significantly between the first, second or third 50 HDC performed by our endoscopists when the three were coned ($P > 0.05$). Therefore, there does not seem to be a learning effect associated with use of HDC with on demand multi-band imaging capability by endoscopists who had not used them before (Figure 1).

DISCUSSION

Our goal in this study was to assess the performance of HDC with regards to polyp detection. Five prior studies have compared adenoma detection rate between standard and high definition white light colonoscopy with conflicting results. There have been methodological and technical differences between the studies (Table 4) with all using Olympus colonoscopes, whereas our study is the first performed using Fujinon HDC with FICE. Among the three prospective studies, Pellisé *et al*^[12] had the largest patient population. It was a prospective randomized controlled trial of 620 patients conducted in

Spain involving seven colonoscopists. Patients were randomized to either HDC with wide angle (170°) field or SDC with 140° view, with the investigators finding no difference in adenoma detection rate between the study groups (HDC 26% vs SDC 25%, $P = 0.85$). The study by East *et al*^[13] was not randomized, consisting of 130 patients who underwent either HDC with 140° view or SC 140° view by a single colonoscopist. Although HDC did not improve the yield of adenomatous polyp detection, there was a trend in this direction (71% vs 60%). The Tribonias *et al*^[14] study randomized 390 patients prospectively into HDC with wide angle vs SDC groups and, although there was a significant difference between the two groups with regards to overall rate of polyp detection, (HDC 63% vs SDC 53%, $P = 0.03$), there was no significant difference demonstrated in the detection of rate of adenomas (HDC 58% vs SDC 50%, $P = 0.16$).

The largest patient population study on this topic, by Buchner *et al*^[15], was retrospective involving 2430 patients in two arms: HDC and SDC. The HRC were 170° wide-angle and NBI was used as needed. The SDC in the study had a 140° view and did not have NBI. The study found that the HDC were able to detect a statistically significant higher number of adenomatous polyps compared with SDC (28.8% vs 24.3%, $P = 0.012$). The most recently

published study was by Burke *et al.*^{16]} and consisted of 426 individuals in each group and found no advantage of HDC in overall polyp detection rate, adenomas or advanced adenomas.

In our study, we found no difference in detection rates of overall polyps, adenomas, advanced adenomas, and cancer between the HD and SD groups. There was no difference in polyp detection rates when each individual endoscopist's HD and SD detection rates were compared despite having used 32-inch LCD high-resolution monitors with the Fujinon system whereas 19-inch CRT monitors were used with the standard definition colonoscopes. Although there are considerable methodological differences between the Pellisé *et al.*^{12]} and our study, both studies show very similar results and conclusions. In fact, their adenomatous polyp detection rates are nearly identical to ours in the SD and HD arms: 0.45 ± 1.07 *vs* our 0.41 ± 1.04 adenomas per patient in SD and 0.43 ± 0.87 *vs* our 0.42 ± 0.94 adenomas per patient in HD. Our polyp detection rates are well in line with several other studies of white light colonoscopy with regards to prevalence of adenomas, advanced adenomas, cancer, and gender differences^[17-19]. We were also able to demonstrate that polyp detection rate did not improve as the endoscopist experience with HDC increased by comparing adenoma detection in consecutive groups of 50 colonoscopies ($P > 0.05$). This lack of learning effect was also demonstrated by Adler *et al.*^{20]} in 2008 in a prospective randomized study of NBI *vs* conventional colonoscopy for adenoma detection. Although prior studies were meant to compare HD and SD, the HD equipment used in these reports also had a wide angle field of view and the study by East is the only one that used 140° scopes in both arms, but it was underpowered for detecting small differences in polyp detection rate. Similar to East, our study design eliminates the confounding factor of the wide angle field of view by using 140° scopes in the HD and SD groups.

There is significant variability amongst endoscopists in adenoma detection rates, making the endoscopist probably the most important variable in adenoma detection rate^[21]. We tried to minimize the impact factor of the endoscopist by assigning an equal number of overall cases per endoscopist (300) and dividing these equally amongst the study groups. Our study has a significant advantage in this.

It can be argued that our study's retrospective design was a limitation, but it may have also served to reduce endoscopist bias. Endoscopist bias is an inherent limitation of nearly all prospective colonoscopy study designs since the equipment cannot be hidden from the performer of the examination. A second limitation is that the population was a mixed-risk sample and there were slight differences with respect to Hispanics and screening patients. However, there was no statistically significant difference in the detection of adenoma, advanced adenoma or cancer between the Hispanic *vs* Non-Hispanic and screening *vs* non-screening groups. In fact, there was a slightly higher prevalence of adenomas in the populations that were overrepresented in the HD group. This would have

worked to bias the results in favor of HDC had it been a significant difference. In summary, the results of our study are relevant to most practices as the majority of the new colonoscopy equipment purchased in the future will have HD and NBI or multi-band imaging capabilities. Until recently, evidence regarding the potential of this new technology in improving yield of polyp detection was lacking. Complementing the results of Pellisé, and Burke, our study concludes that HDC with multi-band imaging capability does not detect more total polyps, adenomas, advanced adenomas or cancer. For now at least, the endoscopist and not the equipment used, continues to be the major factor in polyp detection.

COMMENTS

Background

It is estimated that up to 15 million colonoscopies are performed annually in the United States. Colonoscopy and polypectomy have been estimated to prevent 50%-80% of colorectal cancers. A considerable effort is being spent on optimizing the yield of colonoscopy with respect to polyp detection. At present, high definition colonoscopy (HDC) is being widely adapted but whether or not it impacts detection of colon polyps is debatable.

Research frontiers

HDC is widely touted by manufacturers to improve polyp detection. Yet, several studies have compared detection of polyps with standard definition colonoscopy (SDC) *vs* HDC with variation in results.

Innovations and breakthroughs

A major confounding factor in previous studies on this subject is that the endoscopists are aware of the high definition equipment and this may have led to bias in polyp detection rates. This study uniquely eliminates the issue of endoscopist bias by using a retrospective model of consecutive colonoscopies.

Applications

By providing added evidence of HDC's role in polyp detection, this study may shift opinion further to the side of lack of benefit in improving yield.

Terminology

Standard colonoscopes typically use 640×480 resolution monitors producing a 307, 200 pixel image. The HDC with high resolution monitors can produce a 1032×768 resolution and a 792, 576 pixel image.

Peer review

The authors examined whether or not HDC resulted in detection of more polyps. The results show no significant difference in polyp detection between SDC and HDC. The results complement the conclusion of other recent studies in this field and suggest that high definition by itself may not improve yield of polyp detection.

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S- Editor Sun H L- Editor Rutherford A E- Editor Xiong L

Does N ratio affect survival in D1 and D2 lymph node dissection for gastric cancer?

Ibrahim Sakcak, Barış Doğu Yıldız, Fatih Mehmet Avşar, Saadet Akturan, Kemal Kilic, Erdal Cosgun, Enver O Hamamci

Ibrahim Sakcak, Barış Doğu Yıldız, Enver O Hamamci, Department of 6th General Surgery, Numune Teaching and Research Hospital, 06100 Sıhhiye, Ankara, Turkey

Fatih Mehmet Avşar, Department of 6th General Surgery, Numune Teaching and Research Hospital, 06100 Sıhhiye, Ankara, Turkey

Fatih Mehmet Avşar, Kafkas University Faculty of Medicine, Chief of Surgery, 36000 Kars, Turkey

Saadet Akturan, Department of General Surgery, Etlik Teaching and Research Hospital, 06010 Ankara, Turkey

Kemal Kilic, Kafkas University Faculty of Medicine, General Surgery Department, 36000 Kars, Turkey

Erdal Cosgun, Department of Statistics, Hacettepe University Faculty of Medicine, 06100 Sıhhiye, Ankara, Turkey

Author contributions: Sakcak I, Yıldız B, Kilic K, Cosgun E and Akturan S provided data and analytical tools and were also involved in editing the manuscript; Sakcak I and Avşar FM coordinated and provided the collection of all the human material in addition to providing financial support for this work; Sakcak I and Yıldız BD designed the study and wrote the manuscript. Hamamci EO provide to revising it critically for important intellectual content.

Correspondence to: Ibrahim Sakcak, MD, Cukurambar Mah. 42. Cad. Sancak Apt. No. 11/7, 06600 Cankaya, Ankara, Turkey. ibrahimsakcak@yahoo.com

Telephone: +90-312-5085252 Fax: +90-312-5085252

Received: December 29, 2010 Revised: March 2, 2011

Accepted: March 9, 2011

Published online: September 21, 2011

SPSS 15.0 software was used for statistical analysis.

RESULTS: Ninety-six (44.4%) patients underwent D1 dissection and 120 (55.6%) had D2 dissection. When groups were evaluated, 23 (24.0%) patients in D1 and 21 (17.5%) in D2 had stage migration ($P = 0.001$). When both D1 and D2 groups were evaluated for number of pathological lymph nodes, despite the fact that there was no difference in N ratio between D1 and D2 groups, a statistically significant difference was found between them with regard to pN1 and pN2 groups ($P = 0.047$, $P = 0.044$ respectively). In D1, pN0 had the longest survival while pN3 had the shortest. In D2, pN0 had the longest survival whereas pN3 had the shortest survival.

CONCLUSION: N ratio is an accurate staging system for defining prognosis and treatment plan, thus decreasing methodological errors in gastric cancer staging.

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Key words: Gastric cancer; Lymph node dissection; Node ratio; Tumor nodule metastasis

Peer reviewer: Shogo Kikuchi, MD, PhD, Professor, Department of Public Health, Aichi Medical University School of Medicine, 21 Karimata, Yazako, Nagakute-cho, Aichi-gun, Aichi 480-1195, Japan

Abstract

AIM: To identify whether there could have been changes in survival if lymph node ratio (N ratio) had been used.

METHODS: We assessed 334 gastric adenocarcinoma cases retrospectively between 2001 and 2009. Two hundred and sixteen patients out of 334 were included in the study. Patients were grouped according to dissection1 (D1) or dissection 2 (D2) dissection. We compared the estimated survival and actual survival determined by Pathologic nodes (pN) class and N ratio, and

Sakcak I, Yıldız BD, Avşar FM, Akturan S, Kilic K, Cosgun E, Hamamci EO. Does N ratio affect survival in D1 and D2 lymph node dissection for gastric cancer? *World J Gastroenterol* 2011; 17(35): 4007-4012 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/4007.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.4007>

INTRODUCTION

Staging in gastric cancer is usually carried out according to

Japanese Research Society for Gastric Cancer (JRS GC) or Union for International Cancer Control (UICC)/American Joint Committee on Cancer (AJCC) systems. In JRS GC, staging depends on anatomical localization of the involved lymph nodes, whereas the UICC/AJCC system uses number of metastatic lymph nodes. The UICC/AJCC system requires removal of at least 15 lymph nodes^[1-3]. One shortcoming of the UICC/AJCC system is presence of less than 15 nodes in the surgical specimen, which might cause inadequate staging and over-optimistic prediction of prognosis. Only 29%-31% of curative gastric resections include ≥ 15 nodes^[4,5]. Low number of lymph node removal increases risk of shift migration.

In the Japanese system, lymph nodes are classified in stations according to their localization. Lymph node dissections performed according to these stations are expressed as D0, D1, D2, D3. Japanese surgeons generally recommend D2 dissection as gold standard. Experts from the USA and Europe state that there is not a survival difference between D1 and D2 dissections and that there is high postoperative morbidity and mortality rate in D2^[6,7]. However, there are some surgeons in the Western world who support D2 dissection^[8,9].

Lymph node ratio (N ratio) is defined as the ratio of positive lymph nodes to total number of lymph nodes examined^[10]. In the latest publications it is shown that N ratio compared to UICC/AJCC pN class is a more independent prognostic factor and predicts survival more accurately^[11,12]. Tumor nodule metastasis (TNM) classification stages gastric cancers 10%-15% lower than they should be, which causes errors in survival expectations and treatment planning^[13-15]. When N ratio is used instead of pN, there is 10% upgrade in staging which results in a 8.14% decrease in 5 year survival^[16]. When nodal staging in TNM classification is changed to N ratio, pN1 in TNM might become N ratio 2. This alters treatment planning as N ratio 2 requires adjuvant chemoradiation while pN1 does not. Accurate staging is necessary for an accurate adjuvant therapy planning, which can increase survival and quality of life.

In this study, we re-staged patients who previously had either D1 or D2 dissection according to N ratio and investigated whether there could have been a change in treatment and survival if N ratio had been used for staging.

MATERIALS AND METHODS

We retrospectively assessed 334 gastric adenocarcinoma cases who underwent either D1 or D2 lymph node dissection between May 2001 and October 2009. Exclusion criteria were distant metastasis (including macroscopically significant paraaortic, superior mesenteric artery and mid-colic artery lymph node metastasis), D3 and D0 dissections, previous history of gastric surgery, postoperative mortality (death within 30 d postoperatively) and palliative surgery. Two hundred and sixteen patients out of 334 were included in the study. Ninety-six (44.4%) of these patients had D1 dissection and 120 (55.6%) had D2

dissection. Forty-seven patients who were lost in follow up, 17 with insufficient pathology reports, 5 with gastric stump recurrence, 12 who died within 30 d postoperatively, 7 who were inoperable, 15 who had palliative surgery, 12 with D0 dissections, and 3 with D3 dissections made up the excluded 118 patients.

In all patients, dissection was carried out according to JRS GC criteria, taking into account the anatomical localization of primary tumor and lymph nodes (n0 = no lymph node metastasis, n1 = metastasis to N1 lymph nodes, n2 = N2 lymph node metastasis, n3 = N3 lymph node metastasis)^[17]. Metastatic lymph nodes were classified according to UICC/AJCC 2002 criteria. According to these criteria: N0 = no metastasis, N1 = 1-6 lymph node metastasis, N2 = 7-15 lymph node metastasis, N3 = more than 15 lymph nodes metastasis. N ratio was classified according to previously published studies as: N ratio 0 = 0%, N ratio 1 = 1%-10%, N ratio 2 = 11%-25%, N ratio 3 $\geq 25\%$. Patients with adjacent organ involvement or lymph node positivity in histopathological examination were referred to the oncology department.

Patients were grouped according to D1 or D2 dissection. Groups were analyzed for the significance of age (< 70, ≥ 70 years), gender, type of resection (total, subtotal), tumor localization (diffuse, upper 1/3, middle 1/3, lower 1/3), number of lymph nodes removed, number of metastatic lymph nodes, depth of invasion, N class, TNM stage, pathological diagnosis, N ratio and Lauren classification.

Statistical analysis

SPSS 15.0 (SPSS Inc. Chicago, IL, United States) was used for statistical analysis. A *P* value < 0.05 was accepted as statistically significant. Independent two sample *t* test was used for quantitative variables (age, number of lymph nodes, number of pathological lymph nodes). χ^2 test was used for categorical variables (type of surgery, gender, anatomical location of tumor, N ratio, TNM stage, pathological diagnosis).

For survival analysis in the D1 and D2 groups, Kaplan-Meier method was used. Log rank test was used to analyze differences between statistical significances. Log rank test was also used to assess each N class and N ratio crossed with D1 and D2 groups for 5 year survival analysis.

RESULTS

The characteristics of D1 and D2 groups are shown in Table 1. Groups were compared with univariate analysis for significant prognostic factors. There were statistically significant differences in age, localization of primary tumor, depth of invasion and TNM stage (*P* = 0.009, 0.007, 0.001, 0.001, respectively). In 72 (33.3%) cases, more than 15 lymph nodes were identified in pathology specimens. There was a statistically significant difference in D1 and D2 groups regarding > 15 lymph node removal [27 (28.1%), 45 (37.5%), respectively] (*P* < 0.001).

The total number of lymph nodes obtained in all cases in the D1 group was 1381 (14.4 \pm 6.1) and in the D2

Table 1 Case characteristics *n* (%)

	D1	D2	<i>P</i> value
Gender			
M	61 (63.5)	71 (59.2)	0.512
F	35 (36.5)	49 (40.8)	
Age (yr)			
< 70	70 (72.9)	86 (71.7)	0.142
≥ 70	26 (27.1)	34 (28.3)	
Surgical Procedure			
TG	79 (82.3)	55 (45.8)	0.001
DSG	17 (17.7)	65 (54.2)	
Anatomical localization of primary tumor			
Proximal	9 (9.4)	9 (7.5)	0.087
Middle	39 (40.6)	43 (35.8)	
Distal	33 (34.4)	59 (49.2)	
Diffuse	15 (15.6)	9 (7.5)	
Number of lymph nodes removed (mean ± SD)	14.4 ± 6.1	23.5 ± 9.3	0.022
Number of metastatic lymph nodes (mean ± SD)	3.8 ± 2.2	6.1 ± 4.6	0.034
T (Depth of invasion)			
T1	8 (8.3)	9 (7.5)	0.056
T2	9 (9.4)	15 (12.5)	
T3	59 (61.5)	75 (62.5)	
T4	20 (20.8)	21 (17.5)	
N (According to AJCC)			
0	20 (20.8)	26 (21.7)	0.296
1	32 (33.3)	47 (39.2)	
2	40 (41.7)	37 (30.8)	
3	4 (4.2)	10 (8.3)	
TNM stage			
IA	2 (2.1)	6 (5.0)	0.001
IB	12 (12.5)	7 (5.8)	
II	19 (19.8)	30 (25)	
IIIA	42 (43.8)	58 (48.3)	
IIIB	21 (21.9)	19 (15.8)	
V	-	-	
Pathology			
Adenocarcinoma	77 (80.2)	97 (80.8)	NA
Signet ring cell	4 (4.2)	18 (15.0)	
MAC	10 (10.4)	2 (1.7)	
Carcinoid tumor	2 (2.1)	-	
Lymphoma	3 (3.1)	3 (2.5)	
N ratio			
0	12 (12.5)	19 (15.8)	0.001
1	29 (30.2)	34 (28.3)	
2	39 (40.6)	23 (19.2)	
3	16 (16.7)	44 (36.7)	
Lauren classification			
Diffuse	47 (49.0)	52 (53.3)	0.424
Intestinal	49 (51.0)	68 (56.7)	

D1: D1 lymphadenectomy; D2: D2 lymphadenectomy; M: Male; F: Female; N ratio: Node ratio; TG: Total gastrectomy; DSG: Distal subtotal gastrectomy; MAC: Mucinous adenocarcinoma; NA: Not available; AJCC: American Joint Committee on Cancer; TNM: Tumor nodule metastasis.

group this figure was 2823 (23.5 ± 9.3) (*P* = 0.022). Pathological lymph nodes in the D1 group numbered 374 (3.8 ± 2.2) whereas in the D2 group there were 732 (6.1 ± 4.6) (*P* = 0.034). There was no difference in pN0, N ratio 0, pN3 and N ratio 3 between groups. In the D1 group, N2 class had the highest number of cases while the same was true for pN1 class in the D2 group (*P* = 0.003, *P* = 0.011, respectively). In Table 2, five year survival rates of D1 and D2 dissections in the pN and N ratio groups are com-

Table 2 Comparison of 5 year survivals in dissection 1 and dissection 2 dissection groups depending on N and N ratio *n* (%)

N	D	<i>n</i>	5 YS	<i>P</i> value	N ratio	<i>n</i>	5 YS	<i>P</i> value
N0	D1	20	6 (30.0%)	0.172	0	20	6 (30.0%)	0.389
	D2	26	13 (50.0%)			26	13 (50.0%)	
N1	D1	32	4 (12.5%)	0.047	1	29	4 (13.8%)	0.070
	D2	47	15 (31.9%)			44	13 (29.5%)	
N2	D1	40	6 (15.0%)	0.044	2	39	5 (12.8%)	0.192
	D2	37	1 (2.7%)			32	3 (9.4%)	
N3	D1	4	NA	NA	3	16	1 (6.3%)	0.267
	D2	10	NA			44	0	

D1: D1 lymphadenectomy; D2: D2 lymphadenectomy; N ratio: Node ratio; 5 YS: Five years survival; NA: Not available.

Table 3 Node ratios vs node classes *n* (%)

	N ratio (metastatic/total number of lymph nodes removed)				Total
	0 (0)	1 (0-9)	2 (10-25)	3 (> 25)	
D1 group					
N0 (0)	20 (100)				20
N1 (1-6)	21 (65.6)	11 (34.4)			32
N2 (7-15)		28 (70.0)	12 (30.0)		40
N3 (> 15)				4 (100)	4
D2 group					
N0 (0)	26 (100)				26
N1 (1-6)		37 (78.7)	6 (12.8)	4 (8.5)	47
N2 (7-15)			26 (70.3)	11 (29.7)	37
N3 (> 15)				10 (100)	10

D1: D1 lymphadenectomy; D2: D2 lymphadenectomy; N: Groups with respect to number of lymph nodes removed.

pared. None of the N ratio subgroups (N ratio 0, 1, 2, 3) demonstrated statistically significant differences in 5 year survival after D1 or D2 dissections (*P* = 0.389, *P* = 0.070, *P* = 0.192, *P* = 0.267, respectively). In the pN0 and pN3 subgroups, D1 or D2 dissection did not cause a statistically significant change in 5 year survival (*P* = 0.172, not available) while pN1 and pN2 did (*P* = 0.047, *P* = 0.044, respectively).

In the D1 group, 20 patients who were deemed pN0 (no metastasis) were found to be N ratio 0. Twenty-one out of 32 cases (65.6%) with 1-6 lymph node metastasis (pN1) were found to be N ratio 1, while the remaining 11 (34.4%) were classed as N ratio 2. Twenty-eight (70%) of pN2 patients (*n* = 40) were classified as N ratio 2; 12 were classified as N ratio 3. Four patients who had ≥ 16 positive nodes were found to be N ratio 3.

In the D2 group, 26 patients who were classified as pN0 were N ratio 0. Thirty-seven (78.7%) out of 47 pN1 patients were N ratio 1, six of them (12.8%) were N ratio 2 and 4 of them (8.5%) were N ratio 3. Thirty-seven cases were pN2; of which 26 (70.3%) were N ratio 2, and 11 were (29.7%) N ratio 3. All pN3 cases (*n* = 10) were found to be N ratio 3 (Table 3).

When both D1 and D2 groups were evaluated, 23 (24.0%) patients in D1 and 21 (17.5%) in D2 had stage migration (*P* = 0.001).

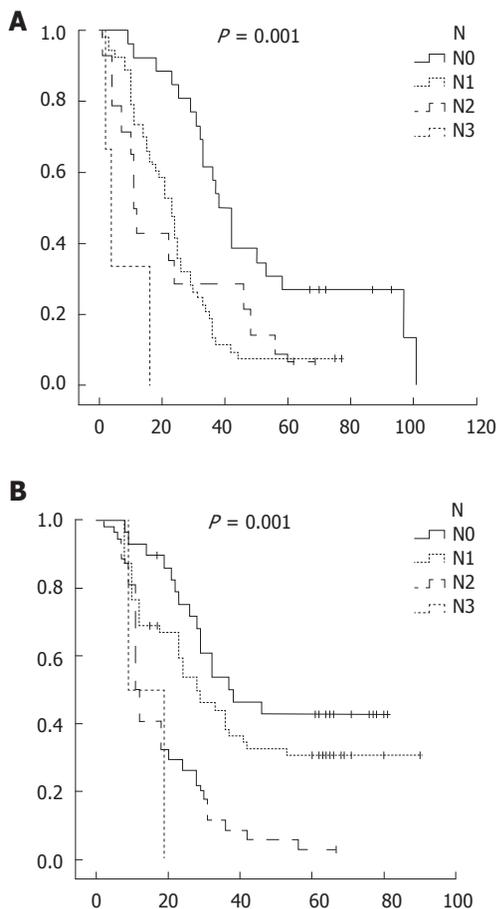


Figure 1 Kaplan meier survival analysis of dissection 1 (A) and dissection 2 (B) group with N class.

In D1, pN0 had the longest survival while pN3 had the shortest. In D2, pN0 had the longest survival whereas pN3 had the shortest survival (Figure 1). In D1, N ratio 0 had the longest survival while N ratio 2 had the shortest (24.3 mo for N ratio 2, 24.8 mo for N ratio 3). In D2, N ratio 1 had the longest survival whereas N ratio 3 had the shortest survival. Overall, 5 year survival was 20.8% ($n = 45$). Survival in the D2 (24.2%) group was longer than in the D1 group (13.3%) ($P < 0.001$) (Figure 2).

DISCUSSION

Staging is of paramount importance in decision making for treatment of cancer. This staging should be universally accepted and standardized. Currently, the most commonly used staging system for gastric cancer is the AJCC/UICC system. For an accurate staging in this system at least 15 nodes are required, thus at least a D2 dissection should be performed (pN3 class requires at least 16 lymph nodes in the specimen). A lesser lymph node dissection is inadequate for predicting survival and making a treatment plan. Sun *et al*^[18], in 2159 curative resections, showed a correlation between total number of lymph nodes and metastatic lymph nodes. In our study, total lymph node number dissected in the D1 group was 1 381 (14.4 ± 6.1) and 2823 (23.5 ± 9.3) in the D2 group. Pathological lymph nodes

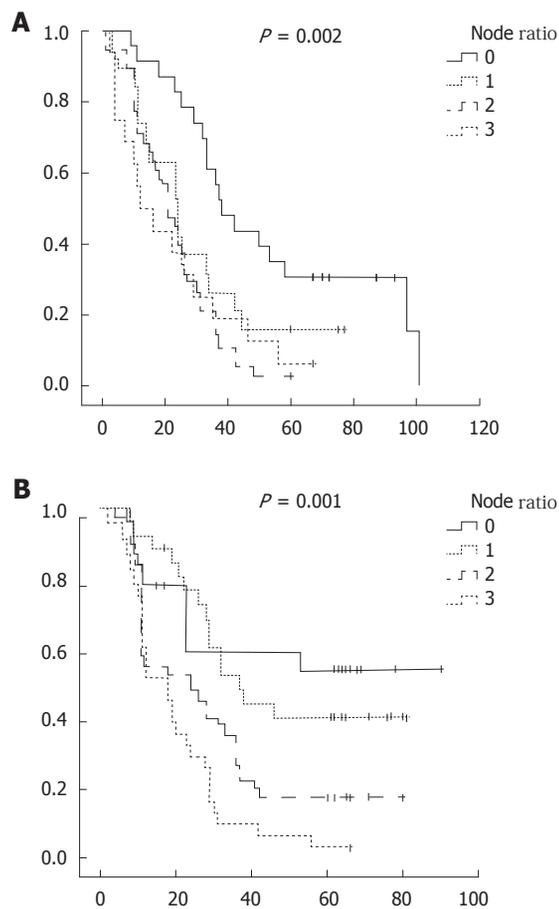


Figure 2 Kaplan meier survival analysis of dissection 1 (A) and dissection 2 (B) group with N ratio.

in the D1 and D2 groups were 374 (3.8 ± 2.2) and 732 (6.1 ± 4.6), respectively. This might mean that in D1 dissection an average of 2.3 lymph nodes are not included in specimens, which could end up in recurrence.

Multivariate analysis shows that N ratio is independent of the number of lymph nodes dissected (even less than 10 lymph nodes is enough for classification)^[11]. Thus, there was no change in N ratio or stage regardless of overall number of dissected lymph nodes. It was Okusa *et al*^[19] who first showed that N ratio in addition to number of positive lymph nodes is a prognostic factor affecting survival in gastric cancer. In contrast to previous study, Bilici *et al*^[20] concluded that in 202 curative gastric resections N ratio and pN were independent prognosticators and did not have superiority over each other. Pedrazzani *et al*^[21], in their study of 526 gastric cancer patients, showed that survival was not different between pN1 and pN2 patients while patients classified as N ratio 1 and 2 had different mortality and survivals.

The aim of lymphadenectomy in gastric adenocarcinoma is to prolong survival. All metastatic lymph nodes should be removed for this purpose. The wider the lymphatic dissection, the higher the chance of removing all metastatic lymph nodes. However, this must be achieved with low mortality and morbidity rates. In their multi-center study, Marchet *et al*^[22] found that in 1853 cases

when D2 dissection was performed, patients who had D1 dissection and were staged as pN1 would turn out to be pN2 or pN3. Bando *et al.*^[15] stated in their study that if their 228 patients with lymph node metastasis (who actually had D2 gastrectomy) had D1 gastrectomy, 103 of them would have been understaged. The authors also stated that using the N ratio would have decreased this shift in staging. Nitti *et al.*^[11] suggested that D2 dissection should be performed to define number of metastatic, reactional or normal lymph nodes and to also decrease stage migration. In our study, in the D1 group, N ratio 0 had the longest survival while N ratio 2 had the shortest. In the D2 group, N ratio 1 had the longest survival despite shortest survival in the N ratio 3 group. There was a statistically significant difference between both N1 and N2 in both the D1 and D2 groups ($P = 0.047$, $P = 0.044$, respectively). N ratio groups did not show any significant difference for either D group.

Another target in gastric cancer treatment is maintaining locoregional control. Wide lymph node dissection is necessary for this. In one study, it was shown that higher number of lymph nodes provides better staging and more accurate prediction of prognosis^[23]. Marchet *et al.*^[22] stated that 15 lymph nodes was the cut off point for statistical significance for survival in corresponding N ratio groups. In 257 patients with D1 gastrectomy, Maduekwe *et al.*^[24] obtained an average of 14 lymph nodes and showed that cases with > 15 lymph node removal had better overall survival than cases with < 15 nodes in pathology specimen. Karpeh *et al.*^[25] showed in 1038 gastric cancer patients that there was an increase in median survival in patients with ≥ 15 lymph node removal. When the same patients were staged with N ratio independent from number of lymph nodes, there was no difference in survival. In our study, we resected ≥ 15 nodes in 27 (28.1%) patients in the D1 group and 45 (32.5%) patients in the D2 group ($P < 0.001$). When each group was analyzed separately, stage migration was less in D2 when compared to D1 (17.5% and 24.0%, respectively).

Curative gastric resections include total gastrectomy or subtotal gastrectomy. Total gastrectomy should be performed for patients with proximal, middle or diffusely located cancers. Prospective, randomized studies have proven that in distally-located tumors, total gastrectomy does not have an advantage over distal subtotal gastrectomy^[26]. The crucial point is achieving tumor-free distal and proximal margins. Extent of gastric resection does not affect the predictive power of N ratio. Huang *et al.*^[10] showed that in 634 distal gastrectomy patients, N ratio was more accurate in predicting survival than pN stage, thus better for treatment planning.

It might not be radical to say that until a reliable staging with less stage migration is proposed, N ratio could be used for staging. Regardless of the lymph node staging used, a wide lymph node dissection should be done to prevent errors in staging. The shortcomings of our study were mainly the number of cases involved and the retrospective design. Prospective, multicenter trials are needed to better define whether N ratio should replace pN stag-

ing in gastric cancer.

In conclusion, N ratio is an accurate and up-to-date staging system for defining prognosis and treatment plan, thus decreasing methodological errors in gastric cancer staging. We believe that, in order to better evaluate prognosis and define a treatment plan, D2 dissection should be the preferred option instead of D1 dissection.

COMMENTS

Background

Staging in gastric cancer is usually carried out according to Japanese Research Society for Gastric Cancer or Union for International Cancer Control /American Joint Committee on Cancer systems. The authors tried to identify whether there could have been change in survival if Node ratio had been used for classification of gastric cancer.

Research frontiers

In this study, the authors re-staged patients who previously had either dissection 1 and dissection 2 according to N ratio and investigated whether there could have been a change in treatment and survival if N ratio had been used for staging.

Innovations and breakthroughs

N ratio is an accurate and up-to-date staging system for defining prognosis and treatment plan, thus decreasing methodological errors in gastric cancer staging. In order to better evaluate prognosis and define a treatment plan, D2 dissection should be the preferred option instead of D1 dissection.

Peer review

This study retrospectively analyzed relationships between n-factors and prognosis among gastric cancer patients who underwent D1 or D2 resection, and concluded that N ratio, which is defined as the ratio of positive lymph nodes to total number of lymph nodes examined, is an accurate staging system for defining prognosis and treatment plan.

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S- Editor Tian L L- Editor Logan S E- Editor Xiong L

Practical approaches to effective management of intestinal radiation injury: Benefit of resectional surgery

Nikolaos Perrakis, Evangelos Athanassiou, Dimitra Vamvakopoulou, Maria Kyriazi, Haris Kappos, Nikolaos C Vamvakopoulos, Iakovos Nomikos

Nikolaos Perrakis, Maria Kyriazi, Haris Kappos, Iakovos Nomikos, Department of Surgery, Metaxa Cancer Memorial Hospital, Piraeus 11522, Greece

Evangelos Athanassiou, Department of Surgery, University of Thessalia Medical School, Larissa 41110, Greece

Dimitra Vamvakopoulou, Nikolaos C Vamvakopoulos, Department of Biology and Genetics, University of Thessalia Medical School, Larissa 41110, Greece

Author contributions: Nomikos I, Athanassiou E, Vamvakopoulos NC contributed to study concept and design; Perrakis N, Kyriazi M, Kappos H, Vamvakopoulou D contributed to acquisition of data; Athanassiou E, Vamvakopoulos NC, Nomikos I drafted the manuscript; Athanassiou E, Vamvakopoulou D critically reviewed the manuscript for important intellectual content. Supported by The University Hospital of Larissa

Correspondence to: Evangelos Athanassiou, Associate Professor of Surgery, University Hospital of Larissa, Larissa 41110, Greece. evangelosathanassiou@yahoo.com

Telephone: +30-241-3501560 Fax: +30-241-3501560

Received: November 24, 2010 Revised: December 26, 2010

Accepted: January 2, 2011

Published online: September 21, 2011

Abstract

AIM: To study the outcome of patients undergoing surgical resection of the bowel for sustained radiation-induced damage intractable to conservative management.

METHODS: During a 7-year period we operated on 17 cases (5 male, 12 female) admitted to our surgical department with intestinal radiation injury (IRI). They were originally treated for a pelvic malignancy by surgical resection followed by postoperative radiotherapy. During follow-up, they developed radiation enteritis requiring surgical treatment due to failure of conservative management.

RESULTS: IRI was located in the terminal ileum in 12 patients, in the rectum in 2 patients, in the descending

colon in 2 patients, and in the cecum in one patient. All patients had resection of the affected region(s). There were no postoperative deaths, while 3 cases presented with postoperative complications (17.7%). All patients remained free of symptoms without evidence of recurrence of IRI for a median follow-up period of 42 mo (range, 6-96 mo).

CONCLUSION: We report a favorable outcome without IRI recurrence of 17 patients treated by resection of the diseased bowel segment.

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Key words: Pelvic neoplasms; Bowel; Radiation injuries; Surgery

Peer reviewers: Antonio Basoli, Professor, General Surgery "Paride Stefanini", Università di Roma-Sapienza, Viale del Policlinico 155, Roma 00161, Italy; Dr. Benjamin Perakath, Professor, Department of Surgery Unit 5, Christian Medical College, Vellore 632004, Tamil Nadu, India; Michael Leitman, MD, FACS, Chief of General Surgery, Beth Israel Medical Center, 10 Union Square East, Suite 2M, New York, NY 10003, United States

Perrakis N, Athanassiou E, Vamvakopoulou D, Kyriazi M, Kappos H, Vamvakopoulos NC, Nomikos I. Practical approaches to effective management of intestinal radiation injury: Benefit of resectional surgery. *World J Gastroenterol* 2011; 17(35): 4013-4016 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/4013.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.4013>

INTRODUCTION

Intestinal radiation injury (IRI) is a common complication of radiation therapy for rectal, gynecologic and urologic malignancies. Despite improvements in radiation

technology, the incidence of IRI is increasing mainly as a consequence of the rapidly expanding application of radiation plus chemotherapy in the management of pelvic malignancies^[1,2]. It usually develops either early as acute radiation enteropathy, during or shortly after radiation therapy and resolving within 2-6 wk, or late, from 6 mo to as long as 30 years post radiotherapy as a chronic injury^[3-9]. Although the true incidence of IRI has not been well defined, it varies from 1.2% to as high as 37% especially in patients with rectal cancer^[5,6,8].

Surgical management of IRI patients adopts either a conservative approach with adhesiolysis, and/or creation of diverting stomas, or a more radical operation with resection of the diseased segment of the bowel. Morbidity, mortality and recurrence rate after these two surgical options in the management of radiation enteropathy have not been well documented.

We report on the clinical presentation, pathology and outcome of 17 consecutive IRI cases managed by surgical removal of the diseased segment.

MATERIALS AND METHODS

Of the 1261 patients irradiated for pelvic malignancies in the radiotherapy department of Metaxa Cancer Memorial Hospital during a 7-year period, 83 were hospitalized for IRI and 17 (5 male, 12 female) required surgery. Their mean age was 69.3 years (range, 46-88). They were irradiated with a mean dose of 48.6 Gy (range, 40-55Gy) for pelvic visceral organ malignancies. Their main presenting symptoms were: intractable abdominal pain in 10 patients, rectal bleeding in 5 patients, intractable diarrhea in 5, constipation in 3 and vomiting in 3. Eleven of the 17 patients had obstructive ileus and malabsorption before surgery. Table 1 provides comprehensive data of the operated patients.

Upon admission, all patients submitted to routine laboratory tests, endoscopic examination with biopsies, barium contrast studies of the small intestine and colon as required and computed tomography scans of the abdomen and pelvis. On admission they were placed on supportive measures to deal with the acute clinical situation and finally surgery was decided for failure of the conservative management approach.

RESULTS

Of the 17 patents, 12 had right hemicolectomy, 3 had sigmoidectomy and 2 underwent low anterior resection. We treated all patients by resection of a variable length of bowel and primary anastomosis and only 2 required an isolated stoma after resection. Figure 1 shows characteristic histological manifestations of the broad range of IRIs observed in our patient group (Figure 1B-D) relative to normal tissue (Figure 1A).

No postoperative death was recorded while morbidity was seen in 3/17 patients. Two patients developed abdominal wound infection and one an enterocutaneous

fistula that was treated conservatively and required prolonged hospital stay (Table 1). No evidence of recurrent IRI disease was observed during a median follow-up period of 42 mo (range, 6-96 mo).

DISCUSSION

IRI symptoms appeared 6 mo to several years after radiation therapy. Their severity was disproportional to the extent of mucosal damage projected from the histology of the resected bowel (Figure 1). The shortened bowel and its mesentery along with marked compromise of the vasculature were common operative findings.

It is well recognized today that radiation-induced intestinal fibrosis seems to be the unifying underlying cause for most symptoms in patients who undergo postoperative radiotherapy for intra-abdominal malignancies. Abdominal pain, the most frequent symptom, is commonly due to intestinal obstruction or colonic loading and spasm, both treatable either by conservative measures or surgery^[10].

The exact incidence of enteropathy lesions from radiotherapy varies considerably^[11]. When a total radiation dose of 45 Gy is delivered, chronic radiation lesions will be observed in 5% of patients. With 65 Gy of radiation, as many as 50% of patients are likely to be affected. Late injuries develop in 2%-8% of patients within 12 to 30 mo after treatment^[12-14]. A decreasing daily radiation dose increases the number of required sessions and minimizes radiation injury to normal tissue^[15]. Certain medical conditions, such as diabetes mellitus, hypertension and cardiovascular disease, that affect blood supply and compromise the splanchnic circulation are associated with a higher incidence of bowel injury^[16].

In our series no postoperative death occurred and the patient morbidity was 17.7% (Table 1). Reappraisal of the surgical treatment on 48 IRI patients reported 21.7% morbidity and 4.1% mortality following small bowel resection of the radiation-damaged bowel and restorative proctectomy for rectal disease^[17]. Similar findings of 4.5% mortality and 30.2% morbidity after bowel resection on 109 operated patients with radiation enteritis and good life expectancy without recurrence of previous neoplastic disease have previously been reported^[18].

The total dose of radiation therapy delivered to our patients never exceeded 55 Gy. In all patients adhesiolysis was performed before the definitive surgical procedure. All patients were followed for a median time of 42 mo and showed no evidence of IRI recurrence.

It is well known that acute infections secondary to mucositis of the oral cavity appear as complications after systemic chemotherapy and local radiation therapy for head and neck cancers^[19]. Potential causes of increased incidence of infections are vascular damage impairing oxygen delivery and immunologic function, lymphatic vascular injury leading to lymphedema and soft tissue necrosis. Similarly, the radiation-induced bowel injury in patients who have also received chemotherapy renders them susceptible

Table 1 Clinical presentation and surgical management of radiation enteritis patients

Sex/age (yr)	Primary site of tumor	Radiation dose/period delivered (Gy/wk)	Dominant symptoms	Localization of radiation lesions	Procedure	Morbidity/mortality	Follow-up (mo)
M/46	Prostate	70/7	Blood PR, pain	Rectosigmoid	Hartman Procedure	-/-	31
M/74	Prostate	72/6	Blood PR	Terminal Ileum	RC3	SWI ⁶ /-	25
F/71	Rectum	52/4	Pain, diarrhea	Terminal Ileum	RC	-/-	42
F/69	Rectum	50/4	Pain, vomiting	Terminal Ileum	RC	-/-	47
F/69	Rectum	52/4	Intestinal obstruction vomiting	Cecum	RC, ileostomy	-/-	56
F/83	Rectum	50/4	Intestinal obstruction vomiting	Terminal Ileum	RC	-/-	16
F/66	Urethra	52/5	Pain, diarrhea	Terminal Ileum	RC	Fecal fistula/-	72
M/74	SML	70/6	Pain, diarrhea	Descending colon	LC	-/-	8
M/70	Prostate	70/7	Intestinal obstruction vomiting	Terminal Ileum	RC	-/-	72
M/76	Prostate	70/6	Blood PR	Rectosigmoid	LAR	-/-	69
F/88	Cervix	54/4	Blood PR	Descending colon	LC	-/-	84
F/63	Cervix	54/4	Pain	Terminal Ileum	RC	-/-	79
F/54	Uterus	50/8	Pain, diarrhea	Terminal Ileum	RC	-/-	96
F/76	Uterus	50/5	Pain	Terminal Ileum	RC	-/-	12
F/51	Uterus	48/4	Pain	Terminal Ileum	RC	-/-	27
F/76	Ovaries	50/4	Blood PR	Terminal Ileum	RC	SWI/-	6
F/73	Uterus	54/4	Pain, diarrhea	Terminal Ileum	RC	-/-	14

M: Male; F: Female; SML: Spinal metastasis from lung cancer; PR: Per rectum; RC: Right colectomy; LC: Left colectomy; LAR: Low anterior resection; SWI: Surgical wound infection.

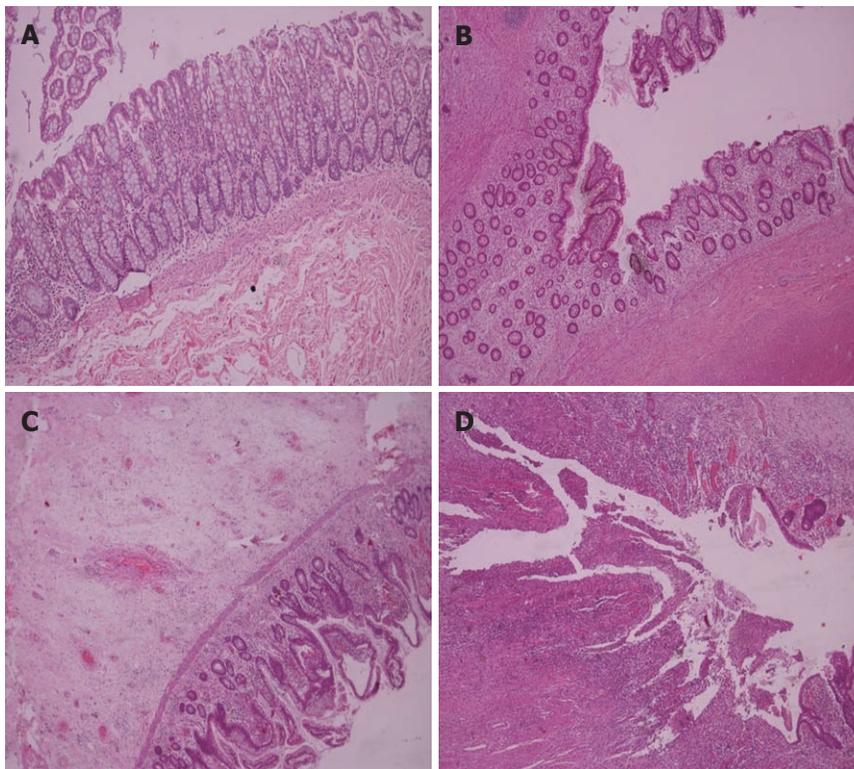


Figure 1 Representative histologic findings of normal (A) and radiation enteritis associated (B-D) intestinal mucosa. A: Normal; B: Mild lesions with edema and increased number of fibroblasts; C: Moderate lesions with sub-mucosal edema and disturbed mucosal architecture; D: Severe lesions with mucosal ulceration. Tissue sections were stained with standard hematoxylin-eosin (40 x magnification).

to septic complications. Thus, when surgery is mandatory and resection is feasible, it should be performed, since removal of the diseased bowel segment acting as a possible source of sepsis diminishes the risk of recurrence of IRI and improves patient outcome.

Use of preventive surgical measures to avoid the harmful consequences of IRI is the most preferable choice^[20]. The 2 most common techniques utilized for IRI preven-

tion have been the omental flap used as a sling across the pelvis^[21] and an absorbable mesh to suspend the small intestine up out of the true pelvis. Another surgical technique involves peritoneal reconstruction using the posterior rectus sheath to form a tissue shelf just at the level of the umbilicus, suturing this to the sacral promontory^[22,23]. Although the long-term efficacy of the above techniques is unproven, the poly-glycolic acid mesh sling appears to

be the most reliable and reasonable method of keeping the small intestine out of the pelvis when postoperative pelvic radiation therapy is contemplated^[24,25].

In conclusion, functional staging of IRI^[26-28] may reveal either acute radiation enteritis or chronic radiation injury that should be managed conservatively. If that fails and resection of the diseased bowel is feasible^[29], it should be adopted in all cases without exception.

COMMENTS

Background

Surgical resection of the radiation damaged bowel segment and primary anastomosis whenever possible is accompanied by a better outcome in patients who failed to respond to the original conservative management due probably to the removal of a septic area following surgical resection of the original pelvic malignancy and postoperative radiotherapy.

Research frontiers

The reasoning of this study is the development of an area in the radiation-damaged bowel that serves as a potential cause of sepsis not responding to conservative management and requiring radical measures for effective treatment.

Innovations and breakthroughs

Surgical removal of a sepsis inside the abdomen is always more effective resulting in a better outcome.

Applications

It is always desirable to be able to treat patients suffering from serious complications of radiation enteritis with radical resection if they fail conservative management.

Peer review

The article is an important topic. The problem of radiation-induced intestinal injury causes important morbidity. The number of patients in this series is small. However, the results are impressive.

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S- Editor Tian L L- Editor Cant MR E- Editor Zhang DN

Corticotropin-releasing factor secretion from dendritic cells stimulated by commensal bacteria

Mariko Hojo, Toshifumi Ohkusa, Harumi Tomeoku, Shigeo Koido, Daisuke Asaoka, Akihito Nagahara, Sumio Watanabe

Mariko Hojo, Daisuke Asaoka, Akihito Nagahara, Sumio Watanabe, Department of Gastroenterology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

Toshifumi Ohkusa, Harumi Tomeoku, Shigeo Koido, Department of Gastroenterology and Hepatology, The Jikei University Kashiwa Hospital, Chiba 277-8567, Japan

Author contributions: Hojo M and Tomeoku H contributed equally to this work; Hojo M, Ohkusa T and Watanabe S designed the research; Hojo M, Tomeoku H, Koido S, Asaoka D and Nagahara A performed the research; and Hojo M and Ohkusa T wrote the paper.

Supported by Grants in Aid for Scientific Research (C) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, No. 17590679

Correspondence to: Toshifumi Ohkusa, Professor, Department of Gastroenterology and Hepatology, The Jikei University Kashiwa Hospital, Chiba 277-8567, Japan. ohkusa@jikei.ac.jp

Telephone: +81-4-71641111 Fax: +81-4-71669374

Received: December 24, 2010 Revised: February 11, 2011

Accepted: February 18, 2011

Published online: September 21, 2011

Abstract

AIM: To study the production and secretion of corticotropin-releasing factor (CRF) by dendritic cells and the influence of commensal bacteria.

METHODS: JAWSII cells (ATCC CRL-11904), a mouse dendritic cell line, were seeded into 24-well culture plates and grown for 3 d. Commensal bacterial strains of *Clostridium clostridioforme* (JCM1291), *Bacteroides vulgatus* (*B. vulgatus*) (JCM5856), *Escherichia coli* (JCM1649), or *Fusobacterium varium* (*F. varium*) (ATCC8501) were added to the cells except for the control well, and incubated for 2 h. After incubation, we performed enzyme-linked immunosorbent assay for the cultured medium and reverse transcription polymerase chain reaction for the dendritic cells, and compared these values with controls.

RESULTS: The level of CRF secretion by control dendritic cells was 40.4 ± 6.2 pg/mL. The CRF levels for cells incubated with *F. varium* and *B. vulgatus* were significantly higher than that of the control ($P < 0.0001$). CRF mRNA was present in the control sample without bacteria, and CRF mRNA levels in all samples treated with bacteria were above that of the control sample. *F. varium* caused the greatest increase in CRF mRNA expression.

CONCLUSION: Our results suggest that dendritic cells produce CRF, a process augmented by commensal bacteria.

Key words: Commensal bacteria; Corticotrophin-releasing factor; Dendritic cell; *Fusobacterium varium*; Irritable bowel syndrome

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Peer reviewer: Jorgen Rask-Madsen, MD, FRCP, Professor of Gastroenterology, Department of Gastroenterology, Herlev Hospital, Skodsborg Strandvej 280A, 2942, Herlev DK-2730, Denmark

Hojo M, Ohkusa T, Tomeoku H, Koido S, Asaoka D, Nagahara A, Watanabe S. Corticotropin-releasing factor secretion from dendritic cells stimulated by commensal bacteria. *World J Gastroenterol* 2011; 17(35): 4017-4022 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/4017.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.4017>

INTRODUCTION

Corticotropin-releasing factor (CRF), a 41-amino acid peptide, is produced mainly in the paraventricular nucleus of the hypothalamus. CRF is a key regulator of the hypothalamic-pituitary adrenal axis. Release of CRF leads to pituitary production of adrenocorticotrophic hormone,

followed by glucocorticoid secretion by the adrenal cortex^[1-5]. Stress induces the release of CRF by the hypothalamus^[6]. Stress is known to delay gastric emptying, accelerate colonic transit and evoke colonic motility^[7-9], and to decrease the visceral threshold^[10] resulting in the development of abdominal symptoms. These events can be triggered by intracerebroventricular or intravenous administration of CRF^[18,9,11-13], while stress-induced gastrointestinal dysmotility and visceral hypersensitivity can be improved by central or peripheral administration of a non-selective CRF receptor antagonist^[14-16]. Accordingly, stress can induce changes in gastrointestinal motility and visceral perception through CRF. Stressful events are more common in irritable bowel syndrome (IBS) patients^[17]. Gastrointestinal dysmotility and visceral hypersensitivity, which are often observed in IBS patients, play an important role in the pathogenesis of IBS^[18,19]. Therefore, it appears that CRF is involved in the pathogenesis of IBS.

The risk of developing IBS is increased after an episode of bacterial gastroenteritis. Rodríguez *et al.*^[20] reported that the risk of developing IBS after bacterial gastroenteritis increased by 14.4-fold compared to the general population. Three months after development of bacterial gastroenteritis, patients with IBS symptoms and those without IBS symptoms appeared to be negative for macroscopic colitis, but only patients with IBS symptoms showed increases in the number of chronic inflammatory cells^[21]. Moreover, intraepithelial lymphocytosis is observed in some IBS patients without history of bacterial gastroenteritis^[22]. Accordingly, inflammation is also involved in the pathogenesis of IBS. The colitis that develops spontaneously in animal models^[23-26] requires the presence of intestinal flora, since the disease fails to develop under germ-free conditions. Thus, it appears that commensal bacteria affect the intestinal mucosa and may be required for chronic intestinal inflammation. Dendritic cells (DCs) recognize luminal antigens (such as bacteria or viruses) through toll-like receptors (TLRs), activate natural immunity, and act as antigen-presenting cells resulting in the activation of acquired immunity. Consequently, it is possible that DCs react to common intestinal microbes.

CRF is present not only in the brain but also in several other human tissues, such as the spinal cord, adrenal medulla, lung, liver, pancreas, placenta, and gastrointestinal tract^[27-31], and in arthritic joints of patients with rheumatoid arthritis^[32]; however, other tissues may also serve as a source of CRF. Lymphocytes appear to be an important source of immunoreactive CRF^[33-35], and normal human colonic mucosal enterochromaffin cells have also been reported to produce CRF^[36]. Moreover, CRF receptor expression in DCs was recently reported^[37]; however, the possibility that CRF is present in DCs has not yet been investigated.

We hypothesize that (1) DCs produce and secrete CRF; (2) that some commensal bacteria augment these processes; and (3) that secreted CRF influences gut motor function and visceral perception prior to the development of IBS. In the present study, we examined whether CRF

is produced or secreted by DCs, and whether production or secretion of CRF from DCs is augmented by commensal bacteria.

MATERIALS AND METHODS

Dendritic cell cultures

Mouse dendritic cells (JAWS II; ATCC CRL-11904) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, United States). JAWS II cells were grown in alpha minimum essential medium with ribonucleosides + deoxyribonucleosides (Gibco, Invitrogen Co., NY, United States) containing 20% fetal bovine serum (Thermo Electron Corp. Melbourne, AU) and 5 ng/mL murine GM-CSF (PeproTech EC, London, United Kingdom). 1×10^5 JAW II cells per well were added to 24-well culture plates (Corning Inc., Corning, NY, United States) and incubated for 72 h under 5% CO₂ at 37 °C in a humidified incubator.

Bacterial strains and culture conditions

We used 4 type strains: *Clostridium clostridioforme* (*C. clostridioforme*) (JCM1219; Japan Collection of Microorganisms, RIKEN, Wako, Japan), *Bacteroides vulgatus* (*B. vulgatus*) (JCM5826), *Escherichia coli* (*E. coli*) (JCM1649), and *Fusobacterium varium* (*F. varium*) (ATCC8501; ATCC, Rockville, MD, United States). These strains have been reported to be pathogens for inflammatory diseases such as ulcerative colitis^[38-40]. The bacterial strains were harvested in GAM agar plates (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) at 37 °C for 72 h. Aerobic bacteria (*E. coli*) were incubated under 5% CO₂ in a humidified incubator. Anaerobic bacteria (*C. clostridioforme*, *B. vulgatus*, and *F. varium*) were incubated in an anaerobic chamber (Forma Scientific, Marietta, Ohio) containing 10% CO₂, 10% H₂, and 80% N₂. After collection of colonies with a disposable plastic loop, they were suspended at 1×10^5 cells/mL in the same medium as used for dendritic cell culture and used immediately.

Sample preparation

JAWS II cells were grown to near confluence before infection with bacteria. Bacteria were added into each well, except the control well, at a cell to bacteria ratio of 1:1000 and incubated for 2 h under 5% CO₂ at 37 °C. Following incubation, conditioned medium and floating cells were collected using a pipette and attached cells were removed from the wells using a cell scraper. All cells with medium were pooled and centrifuged at $125 \times g$ for 7 min according to the manufacturer's instructions. The pelleted cells were resuspended in 200 µL of Isogen (Nippon Gene, Inc., Japan) and frozen at -80 °C. Conditioned medium was also stored at -80 °C.

Detection of CRF by ELISA

To examine whether dendritic cells secrete CRF, enzyme-linked immunosorbent assays (ELISAs) were conducted for the conditioned medium using commercial assay kits

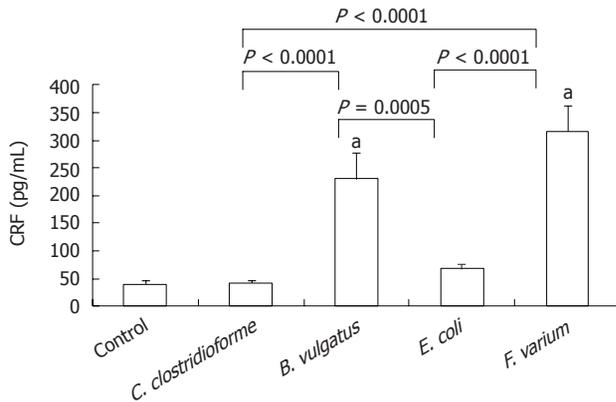


Figure 1 Detection of corticotropin-releasing factor by enzyme-linked immunosorbent assay. JAWS II cells were treated with indicated bacteria for 2 h and conditioned medium was examined by enzyme-linked immunosorbent assay. Data represent the mean \pm SEM of 8 independent experiments. Statistical analysis was carried out using analysis of variance and Fisher's protected least significant difference. ^a $P < 0.0001$ vs control. CRF: Corticotropin-releasing factor; *C. clostridioforme*: *Clostridium clostridioforme*; *B. vulgatus*: *Bacteroides vulgatus*; *E. coli*: *Escherichia coli*; *F. varium*: *Fusobacterium varium*.

(CRF EIA kit; Phoenix Pharmaceuticals Inc., CA, United States). Data represent the averages of 8 independent experiments.

Detection of CRF mRNA by real-time reverse transcription polymerase chain reaction

Total RNA was isolated from cells using an RNeasy Mini Kit (QIAGEN, Inc., Chatsworth, CA), and treated with an RNase-free DNase Set (QIAGEN) following the manufacturer's instructions. RT for synthesizing the first-strand cDNAs was carried out using SuperScript II RT (Invitrogen) according to the manufacturer's instructions. The resultant cDNA corresponding to 10-100 ng of total RNA in a 25 μ L final volume was examined by real time PCR using the ABI PRISM 7700 sequence detection system (Applied Biosystems, CA, United States) with Taqman Universal PCR Master Mix together with TaqMan gene expression assay products (Assay ID Mm01293920-s1; Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was examined as an internal reference and CRF gene expression was examined by means of the comparative C_T method. C_T values reflect the PCR cycle number for which the amount of amplified target reaches a fixed threshold. The C_T for the target gene and reference gene was calculated for each sample. ΔC_T is the difference in C_T between the target and reference genes. $\Delta\Delta C_T$ is the difference between the ΔC_T of the sample and the ΔC_T of the calibrator (the control sample). CRF mRNA expression normalized to an internal reference and expressed relative to the control sample treated without bacteria is given by $2^{-\Delta\Delta C_T}$. Data represent the averages of 4 independent experiments.

Statistical analysis

All the data were expressed as mean values \pm SEM. Sta-

tistical analysis was carried out with analysis of variance and Fisher's protected least significant difference. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Secretion of CRF and augmentation of secretion upon infection with commensal bacteria

The level of CRF secretion by DCs was 40.4 ± 6.2 pg/mL [95% confidence interval (CI), 25.7-55.2]. When commensal bacteria of the strains *C. clostridioforme* JCM1291, *B. vulgatus* JCM5826, *E. coli* JCM1649 and *F. varium* ATCC8501 were added, the level of CRF secretion by DCs was 41.9 ± 4.9 pg/mL (95% CI, 30.4-53.4), 230.7 ± 46.7 pg/mL (95% CI, 120.3-341.1), 67.9 ± 8.0 pg/mL (95% CI, 49.0-86.7) and 316.9 ± 46.4 pg/mL (95% CI, 207.1-426.6), respectively (Figure 1). The CRF levels for both *F. varium* and *B. vulgatus* were significantly higher than that of the control ($P < 0.0001$). When CRF levels for different bacterial strains were compared, the levels for both *F. varium* and *B. vulgatus* were significantly higher than for *E. coli* ($P < 0.0001$, $P = 0.0005$) and *C. clostridioforme* ($P < 0.0001$, $P < 0.0001$), respectively.

Induction of CRF mRNA upon infection with commensal bacteria

CRF mRNA was present in all samples. Mean C_T values were as follows: control, 27.44; *C. clostridioforme*, 25.70; *B. vulgatus*, 25.96; *E. coli*, 26.27; *F. varium*, 24.63. *F. varium* caused the greatest increase in CRF mRNA expression (3.7 ± 0.2 fold increase; 95% CI, 3.1-4.3 fold), followed by *E. coli* (2.4 ± 0.4 ; 1.3-3.6), *B. vulgatus* (2.2 ± 0.3 ; 1.2-3.2), and *C. clostridioforme* (1.6 ± 0.1 ; 1.1-1.2). The 95% CI for CRF mRNA levels in all samples treated with the indicated bacteria were above that of the control sample without bacteria, indicating that the bacteria significantly stimulated CRF mRNA expression (Figure 2). A comparison of CRF mRNA levels in different bacterial strains showed that levels were significantly higher with *F. varium* than with the other bacteria ($P < 0.0001$ vs *C. clostridioforme*, $P < 0.0018$ vs *B. vulgatus*, $P < 0.006$ vs *E. coli*). The CRF mRNA level for *E. coli* was significantly higher than for *C. clostridioforme* ($P = 0.0398$).

DISCUSSION

The results from this study demonstrate that DCs can secrete CRF protein. Moreover, secretion of the CRF protein and expression of CRF mRNA were stimulated after challenge with commensal bacteria.

The term "commensal" generally refers to living in a relationship in which one organism derives food or other benefits from another organism without hurting or helping it; therefore, intestinal microorganisms have been referred to as commensal bacteria. Studies with animal models of mucosal inflammation, such as the interleukin-10 knockout mouse model^[24,26] and the T cell receptor alpha-chain-deficient mouse model^[25], suggested that

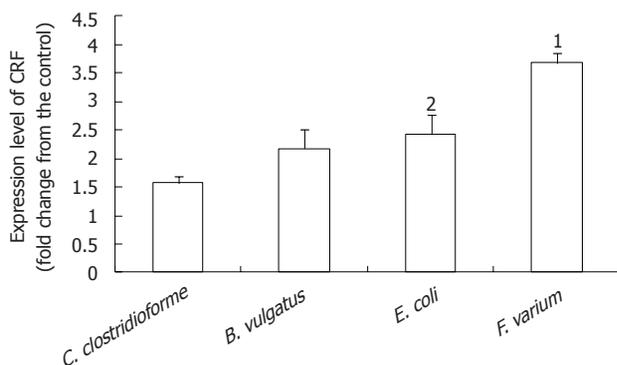


Figure 2 Corticotropin-releasing factor expression determined by comparative Ct method. JAWS II cells were treated with the indicated bacteria for 2 h. After treatment, total RNA was isolated from cells. Ct value reflects the polymerase chain reaction cycle number for which the amount of amplified target reached a fixed threshold. Data represent the mean \pm SEM of 4 independent experiments. Statistical analysis was carried out using analysis of variance and Fisher's protected least significant difference. ¹Significantly higher than other indicated bacteria [$P < 0.0001$ vs *Clostridium clostridioforme* (*C. clostridioforme*), $P < 0.0018$ vs *Bacteroides vulgatus*, $P < 0.006$ vs *Escherichia coli*]. ²significantly higher than *C. clostridioforme* ($P = 0.0398$). CRF: Corticotropin-releasing factor; *C. clostridioforme*: *Clostridium clostridioforme*; *B. vulgatus*: *Bacteroides vulgatus*; *E. coli*: *Escherichia coli*; *F. varium*: *Fusobacterium varium*.

the presence of intestinal flora was required in order for colitis to develop spontaneously, since the disease failed to develop under germ-free conditions. Bacterial overgrowth in the small intestine is associated with the severity of symptoms of IBS associated with increased intestinal gas and immune responses, and antibiotic therapy has been shown to attenuate IBS symptoms in human patients^[41]. Thus, intestinal flora that have been described as commensal flora may actually have several harmful effects on the intestinal mucosa.

DCs recognize luminal antigens, and consequently trigger mucosal inflammation. In such instances, DCs may react to common intestinal microbes and may increase the output of CRF. In the present study, the commensal bacteria *F. varium* and *B. vulgatus* resulted in a significant increase in CRF protein levels as compared with control samples that were not exposed to bacteria. All 4 bacterial strains caused an increase in the expression of CRF mRNA as compared with control samples. Thus, all of the bacterial strains examined in this study appear to have the ability to increase the output of CRF from DCs. *F. varium* appeared to have the strongest effect, since CRF mRNA levels in the presence of *F. varium* were significantly higher than in the presence of the other bacteria. *C. clostridioforme* had the lowest ability to increase both CRF protein and CRF mRNA from DCs. *C. clostridioforme* are the only gram-positive bacteria of the 4 strains examined in this study. Gram-negative bacteria activate immune cells through TLR4 of DCs, while gram-positive bacteria initiate their effect through TLR2^[42]. Differences in such pathways may lead to different results. In further work, another intestinal microbe such as probiotic bacteria should be examined. As probiotic bacteria should have a potentially beneficial effect for the host^[43], it would be of great interest to similarly examine the response of DCs

with regard to CRF levels.

JAWS II cells, a type of DC, are derived from the bone marrow. In the murine Peyer's patch, myeloid DCs are present in the subepithelial dome region. While little is known about the phenotype of colonic DCs, it was recently reported that myeloid DCs made up the largest population^[44]. Thus, JAWS II cells are thought to have the character of colonic DCs. Additional studies with human colonic DCs would be valuable, as would further studies to delineate the processes by which commensal bacteria increase the amount of CRF from DCs.

In conclusion, DCs produce and secrete CRF, and commensal bacteria augment production or secretion of CRF from DCs. The results of the present study suggest that the secretion of CRF from DCs is stimulated by commensal bacteria in the gut, and that CRF derived from DCs may play a role in the pathogenesis of IBS.

COMMENTS

Background

Corticotropin-releasing factor (CRF) influencing gut motor function and visceral perception appears to be involved in the pathogenesis of irritable bowel syndrome (IBS). Intestinal inflammation is associated with post-infectious IBS, and also observed in some IBS patients without history of bacterial gastroenteritis. Intestinal inflammation may be triggered by dendritic cell-mediated immune responses to commensal bacteria, which may involve CRF generation by dendritic cells (DCs).

Research frontiers

Spontaneously developing colitis requires intestinal flora, since colitis fails to develop under germ-free conditions. DCs recognize luminal exogenous antigens and activate natural and acquired immunity. It is possible that DCs react to common intestinal microbes. CRF is present in several human tissues, and CRF receptor expression in DCs was recently reported. There has been no report on the production and secretion of CRF from DCs.

Innovation and breakthroughs

This is the first report that DCs produce and secrete CRF and that commensal bacteria augment production or secretion of CRF from DCs.

Applications

DCs produce and secrete CRF and some commensal bacteria augment these processes. Secreted CRF may influence gut motor function and visceral perception prior to the development of IBS. Accordingly, the results of this study may shed light on the pathogenesis of irritable bowel syndrome.

Terminology

CRF is produced mainly in the paraventricular nucleus of the hypothalamus, but recently has been reported to be present not only in the brain but also in several other human tissues. CRF is a key regulator of the hypothalamic-pituitary-adrenal axis, and it appears that CRF is involved in the pathogenesis of IBS.

Peer review

The manuscript reports the effect on CRF secretion in short term cultures (3 d) by a murine dendritic cell line following incubation with three different commensal bacterial strains. The methodology appears generally well conceived and the manuscript is well written. The conclusions that dendritic cells produce and secrete CRF and that commensal bacteria augment production or secretion of CRF from dendritic cells are straightforward and justified by the results obtained, although the biological significance of the observations remains obscure.

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S- Editor Tian L L- Editor Logan S E- Editor Xiong L

Clinicopathological significance of altered Notch signaling in extrahepatic cholangiocarcinoma and gallbladder carcinoma

Hyun Ah Yoon, Myung Hwan Noh, Byung Geun Kim, Ji Sun Han, Jin Seok Jang, Seok Ryeol Choi, Jin Sook Jeong, Jin Ho Chun

Hyun Ah Yoon, Myung Hwan Noh, Byung Geun Kim, Ji Sun Han, Jin Seok Jang, Seok Ryeol Choi, Department of Gastroenterology, Dong-A University Hospital, 3-1, Dong dae shin-dong, Seo-gu, Busan 602-715, South Korea

Jin Sook Jeong, Department of Pathology, Dong-A University Hospital, 3-1, Dong dae shin-dong, Seo-gu, Busan 602-715, South Korea

Jin Ho Chun, Department of Preventive Medicine, Inje University Hospital, Gae gum 2-dong, Busanjin-gu, Busan 614-735, South Korea

Author contributions: Yoon HA, Noh MH and Jeong JS designed the study and collected the data; Kim BG, Han JS, Jang JS and Choi SR contributed to the analysis of the clinical data; Chun JH assisted in interpretation of statistical analysis; Jeong JS was involved in analysis and interpretation of histological data, and critical revision of the manuscript; Yoon HA wrote the manuscript; Noh MH was involved in study supervision and drafting the manuscript.

Correspondence to: Myung Hwan Noh, MD, PhD, Department of Gastroenterology, College of Medicine, Dong-A University, 3-1, Dong Dae Shin-dong, Seo-gu, Busan 602-715, South Korea. mhnoh@dau.ac.kr

Telephone: +82-51-2402862 Fax: +82-51-2425852

Received: November 12, 2010 Revised: January 11, 2011

Accepted: January 18, 2011

Published online: September 21, 2011

Abstract

AIM: To investigate the role and clinicopathological significance of aberrant expression of Notch receptors and Delta-like ligand-4 (DLL4) in extrahepatic cholangiocarcinoma and gallbladder carcinoma.

METHODS: One hundred and ten patients had surgically resected extrahepatic cholangiocarcinoma (CC) and gallbladder carcinoma specimens examined by immunohistochemistry of available paraffin blocks. Immunohistochemistry was performed using anti-Notch receptors 1-4 and anti-DLL4 antibodies. We scored the immunopositivity of Notch receptors and DLL4 expres-

sion by percentage of positive tumor cells with cytoplasmic expression and intensity of immunostaining. Coexistent nuclear localization was evaluated. Clinicopathological parameters and survival data were compared with the expression of Notch receptors 1-4 and DLL4.

RESULTS: Notch receptor proteins showed in the cytoplasm with or without nuclear expression in cancer cells, as well as showing weak cytoplasmic expression in non-neoplastic cells. By semiquantitative evaluation, positive immunostaining of Notch receptor 1 was detected in 96 cases (87.3%), Notch receptor 2 in 97 (88.2%), Notch receptor 3 in 97 (88.2%), Notch receptor 4 in 103 (93.6), and DLL4 in 84 (76.4%). In addition, coexistent nuclear localization was noted [Notch receptor 1; 18 cases (18.8%), Notch receptor 2; 40 (41.2%), Notch receptor 3; 32 (33.0%), Notch receptor 4; 99 (96.1%), DLL4; 48 (57.1%)]. Notch receptor 1 expression was correlated with advanced tumor, node, metastasis (TNM) stage ($P = 0.043$), Notch receptor 3 with advanced T stage ($P = 0.017$), tendency to express in cases with nodal metastasis ($P = 0.065$) and advanced TNM stage ($P = 0.052$). DLL4 expression tended to be related to less histological differentiation ($P = 0.095$). Coexistent nuclear localization of Notch receptor 3 was related to no nodal metastasis ($P = 0.027$) and Notch receptor 4 with less histological differentiation ($P = 0.036$), while DLL4 tended to be related inversely with T stage ($P = 0.053$). Coexistent nuclear localization of DLL4 was related to poor survival ($P = 0.002$).

CONCLUSION: Aberrant expression of Notch receptors 1 and 3 play a role during cancer progression, and cytoplasmic nuclear coexistence of DLL4 expression correlates with poor survival in extrahepatic CC and gallbladder carcinoma.

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Key words: Notch receptors; Delta-like ligand-4; Cholangiocarcinoma; Gallbladder carcinoma; Immunohistochemistry

Peer reviewer: Dr. Neil L Julie, MD, Gastroenterologist, 1 5225 Shady Grove Road Suite 103, Rockville, MD 20850, United States

Yoon HA, Noh MH, Kim BG, Han JS, Jang JS, Choi SR, Jeong JS, Chun JH. Clinicopathological significance of altered Notch signaling in extrahepatic cholangiocarcinoma and gallbladder carcinoma. *World J Gastroenterol* 2011; 17(35): 4023-4030 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/4023.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.4023>

INTRODUCTION

Cholangiocarcinoma (CC) is a highly malignant neoplasm that generally is diagnosed at advanced stage and is associated with a fatal outcome^[1]. CC includes cancers originating by malignant transformation of biliary epithelial cells, histologically occurring in the intrahepatic and extrahepatic biliary trees with gallbladder. The extrahepatic CC accounts for 80% to 90% of CC and intrahepatic CC comprises the remainder^[1]. The incidence of intrahepatic CC is increasing, in comparison with the stagnant incidence of extrahepatic CC, especially in Western countries^[2], although the cause of rising incidence of intrahepatic CC has not been clarified. Intrahepatic and extrahepatic CCs differ in biologic and clinical variables, including not only histopathologic distinction and clinical characteristics, but also carcinogenesis and molecular profiling, even management. In order to define the carcinogenesis of CC, dysregulated genes and pathways involved in proliferation, evasion from apoptosis and senescence, cell cycle dysregulation, invasion, metastasis and angiogenesis, have all been investigated, suggesting a complex network of variable factors and pathways^[3,4]. The development of new therapeutic modalities for molecular targeting resulting from defining of the molecular carcinogenesis of CC has been growing recently, mainly as a consequence of preclinical *in vitro* studies. Till now, complete surgical resection has been regarded as the only curative therapy.

Since the Notch gene was originally discovered in *Drosophila*, Notch signaling has been investigated in varied organisms from *C. elegans* to humans. Thus, Notch signaling has been noted to be an evolutionally conserved pathway which regulates physiological processes and is involved in pathological conditions^[5]. There are four Notch receptors (Notch 1-4) and five ligands [Jagged 1, Jagged 2, Delta-like ligand-1, -3 and -4 (DLL1, DLL3, DLL4)] in mammals. Ligand-receptor interaction between two neighboring cells activates two sequential proteolytic cleavages of Notch receptor, mediated by the metalloprotease tumor necrosis factor- α -converting enzyme, and by γ -secretase^[6]. The liberated Notch intracellular domain translocates into the nucleus, activating target genes, such as *Hex*, *Hey*, etc. Physiologically, Notch signaling regulates cellular differentiation, proliferation, apoptosis and stem cell maintenance, and participates in cell-fate specification during development of multicellular organisms^[7]. Also, Notch signaling regulates biological events in adult tissue. Recently, the disruption of Notch gene was reported to

be implicated in hematological malignancies^[8,9]. In addition, aberrant Notch signaling has been reported in a variety of solid cancers, including breast, kidney, pancreas, prostate, cervix, endometrium, brain, lung, liver and skin^[10-12]. According to the type of cancer, Notch receptors may have a role as an oncogene or a tumor suppressor gene, though the majority of studies reveal that Notch signaling promotes tumorigenesis^[13]. In both physiological and pathological angiogenesis including tumor angiogenesis, the role of Notch signaling has been recognized^[8].

DLL4 is an endothelial-specific ligand of the Notch signaling pathway, expressed at areas of vasculogenesis and angiogenesis^[14,15]. DLL4 is induced by vascular endothelial growth factor (VEGF) and acts to the downstream of VEGF, as an autoregulatory negative feedback network for inactivation of VEGF, resulting in maturation and stabilization of microvessels^[14]. Recently, in addition to endothelium, DLL4 has been shown to be expressed in epithelium, stromal cells of inflammatory cells in normal or reactive tissues, and in tumor cells of solid cancers in humans^[16], raising possibilities for a role in tumorigenesis.

In this study, we investigated the role of Notch receptors and DLL4 in progression of human extrahepatic CC and gallbladder carcinoma by analysis of expression with immunohistochemistry (IHC), during carcinogenesis. Furthermore, we investigated the correlations between aberrant expression of Notch receptors and DLL4, and clinicopathological parameters with survival.

MATERIALS AND METHODS

Patient selection and specimens

One hundred and ten patients with surgically resected extrahepatic CC and gallbladder carcinoma were included in this study, from January 1999 till December 2008 at Dong-A University Hospital, Busan, South Korea. None of the patients recruited in this study received chemotherapy or radiotherapy before surgery. After review of pathological information and medical records, available cases for IHC with paraffin blocks were collected. The pathologic reviews were performed by two pathologists who are experienced in biliary cancer pathology. Outcomes were determined from the date of surgery until death or 31 December 2008, which resulted in a follow-up period of from 1 to 88 mo (mean, 28 mo). Cases lost to follow-up or who died from problems other than extrahepatic CC and gallbladder carcinoma were censored during the survival analysis. The sites of extrahepatic biliary tract consisted of common bile duct and gallbladder. All cases were adenocarcinomas. Clinicopathological parameters such as age, gender, site, tumor differentiation and tumor, node, metastasis (TNM) with staging according to American Joint Committee on Cancer classification were evaluated by reviewing medical and pathological records. The study was approved by the Institutional Review Board of Dong-A University Hospital, Busan, South Korea (10-10-6).

Preparation of tissue array

Core tissue biopsy specimens (diameter 2 mm) were obtained from individual paraffin-embedded extrahe-

Table 1 Antibodies used for immunohistochemistry

Antibody	Dilution	Company	Non-neoplastic tissue	Aberrant expression in cancer
Notch 1	0.111	Santa Cruz	Variable	Cytoplasm/nucleus
Notch 2	0.111	Santa Cruz	Variable	Cytoplasm/nucleus
Notch 3	0.111	Santa Cruz	Variable	Cytoplasm/nucleus
Notch 4	1:50	Santa Cruz	Variable	Cytoplasm/nucleus
DLL4	1:50	Sigma	Variable	Cytoplasm/nucleus

DLL4: Delta-like ligand-4.

Table 2 Expression rates of Notch receptors 1-4 and delta-like ligand-4 in extrahepatic cholangiocarcinoma and gallbladder carcinoma *n* (%)

	Negativity	Positivity		Total
		Low grade	High grade	
Notch 1	14 (12.7)	55 (50.0)	41 (37.3)	110 (100)
Notch 2	13 (11.8)	60 (54.6)	37 (33.6)	110 (100)
Notch 3	13 (11.8)	56 (50.9)	41 (37.3)	110 (100)
Notch 4	7 (6.4)	61 (55.4)	42 (38.2)	110 (100)
DLL4	26 (23.6)	63 (57.3)	21 (19.1)	110 (100)

DLL4: Delta-like ligand-4.

patic CC and gallbladder carcinoma (donor blocks) and arranged in new recipient paraffin blocks (tissue array blocks) using a trephine apparatus (Superbiochips Laboratories, Seoul, South Korea). Non-neoplastic biliary mucosa specimens were included in each of the array blocks. Each tissue array block contained up to 60 cores.

Immunohistochemistry

Immunohistochemistry was performed using tissue array paraffin blocks. Utilized antibodies are summarized in Table 1. With antibodies to Notch receptors 1-4, avidin-biotin-peroxidase complex (ABC) method was applied and with anti-DLL4, Ventana Autostainer System was used. Four to six micron thick sections from array blocks were dewaxed in xylene, rehydrated using a graded alcohol series and placed in an endogenous peroxidase blocker for 15 min and washed with buffer. The slides were then placed in citrate buffer (10% citrate buffer stock in distilled water, pH 6.0) and microwaved for 10 min for antigen retrieval. Non-reactive staining was blocked using 1% horse serum in Tris buffered saline (pH 6.0) for 3 min. Primary antibodies to Notch receptors 1-4 were applied and antibody binding was detected using avidin-biotin-peroxidase complex (Universal Elite ABC kit PK-6200; Vectastain, Burlingame, CA, United States) for 10 min and diaminobenzidine tetrahydrochloride solution (Kit HK153-5K; Biogenex, San Ramon, CA, United States). For DLL4, antigen retrieval was performed using CC1 antigen retrieval buffer (Ventana Medical Systems, Tucson, AZ, United States). Next, the sections were incubated with anti-DLL4, and stained on the Ventana automated slide stainer (NEXES) using the Ventana diaminobenzidine detection kit (Ventana Medical Systems, Tucson, AZ, United States).

Interpretation after IHC

There are few analysis criteria for the immunopositivity of Notch receptors and DLL4 expression. The scoring was based on distribution and intensity according to a previous report^[12]. Briefly, the percentage of positive tumor cells with cytoplasmic expression was determined semi-quantitatively and each sample was scored on a scale of 0-4, in which 0: negative, 1: positive staining in 1%-25% of cells, 2: in 26%-50%, 3: 51%-75%, and 4: 76%-100%. The intensity of immunostaining was determined as 0: negative staining, 1: weakly positive staining, 2: moderately positive staining, and 3: strongly positive staining. The immunoreactive score of each section was calculated by the sum of these two parameters. The total sum score was transformed into a three tier system and graded as negative (sum: 0), low (sum: 2-4) and high (sum: 5-7).

Statistical analysis

The two-tailed χ^2 test was performed to determine the significance of the difference between the covariates. Survival durations were calculated using the Kaplan-Meier method. The log-rank test was used to compare cumulative survival in the patient groups. The SPSS software program (version 12.0; SPSS Inc., Chicago, IL) was used in the analyses.

RESULTS

Clinicopathologic characteristics of patients

One hundred and ten patients comprised 47 males (42.7%) and 63 females (57.3%), with a range from 37 to 81 years. The mean patient age was 63 years. Carcinomas from the extrahepatic common bile duct were 47 cases (42.7%) and from the gallbladder, 63 cases (57.3%). Well differentiated adenocarcinomas were 53 cases (48.2%); moderately differentiated, 46 cases (41.8%); and poorly differentiated, 11 cases (10.0%). Seventeen cases were T1 (15.5%), T2: 55 cases (50.0%), T3: 32 cases (29.1%), and T4: 6 cases (5.4%). Seventy-eight patients showed no evidence of lymph node metastasis (70.9%) and 32 patients (29.1%) showed lymph node metastasis. Seven patients (6.4%) showed distant metastasis. Fifty-one cases were stage I (46.4%), stage II: 45 cases (40.9%), stage III: 6 cases (5.4%), and stage IV: 8 cases (7.3%).

Results of IHC

Expression of Notch receptors: Notch receptors 1, 2, 3 and 4 were expressed in non-neoplastic biliary epithe-

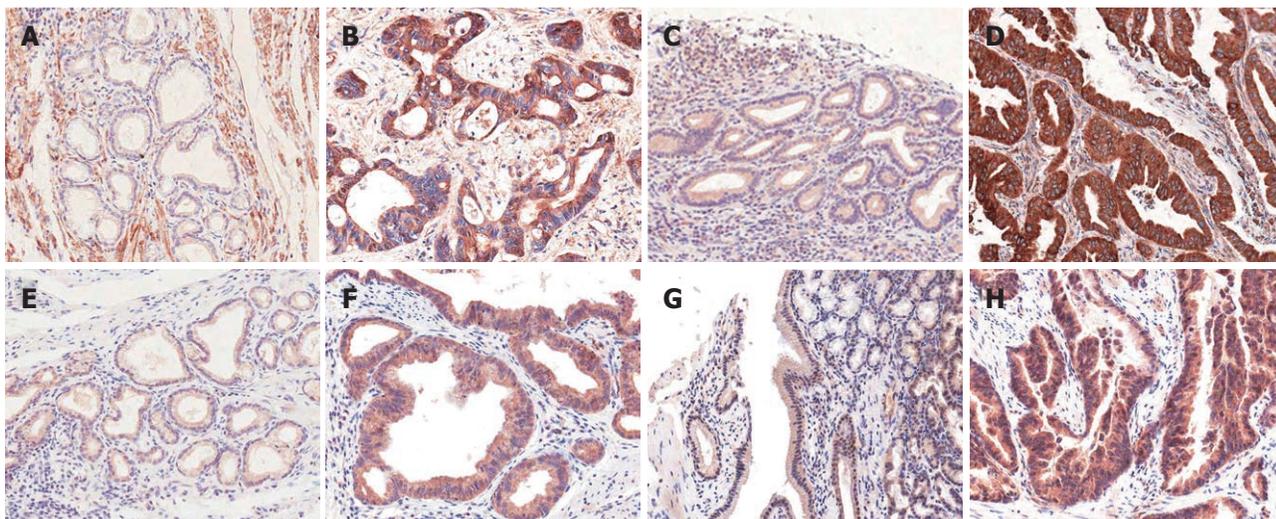


Figure 1 Notch receptor expression in cholangiocarcinomas (Immunohistochemistry). A, E: Notch receptor 1; B, F: Notch receptor 2; C, G: Notch receptor 3; D, H: Notch receptor 4; A-D: Non-neoplastic tissue (x 40); E-H: Cholangiocarcinomas (CCS) (x 100). Non-neoplastic biliary glandular or surface epithelial cells show weak cytoplasmic staining. High grade expression of Notch receptors is detected in cytoplasm of cancer cells of CCs.

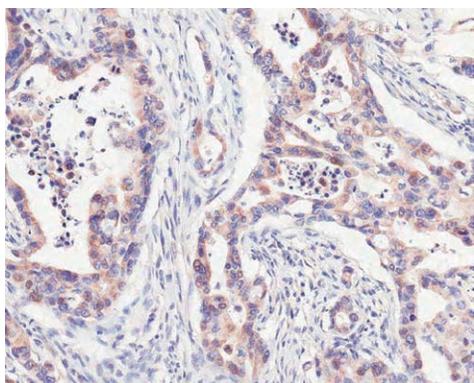


Figure 2 Cytoplasmic and nuclear localization of Notch receptor 3 in moderately differentiated cholangiocarcinoma (immunohistochemistry, × 200). Brown-colored positive immunostaining of cholangiocarcinoma cells in both cytoplasm and nuclei is frequently observed.

lial cells, mesenchymal cells and sometimes inflammatory cells with variable intensities, as well as in micro-vessels (Figure 1A, C, E and G). They were mainly expressed in the cytoplasm of CC cells (Figure 1B, D, F and H) and were evaluated semiquantitatively (Table 2). Notch receptor 1 showed 55 cases (50.0%) of low grade immunoreactivity and 41 cases (37.3%) of high grade immunoreactivity, Notch receptor 2: 60 cases (54.6%) of low grade and 37 cases (33.6%) of high grade, Notch receptor 3: 56 cases (50.9%) of low grade and 41 cases (37.3%) of high grade, and Notch receptor 4: 61 cases (55.4%) of low grade and 42 cases (38.2%) of high grade immunoreactivity.

In some cases with cytoplasmic positive immunoreaction, coexistent distinct nuclear staining was observed (Figure 2). The number of coexistent cytoplasmic and nuclear staining in Notch receptor 1 (+) carcinomas were 18 cases (18.8%), Notch receptor 2: 40 cases (41.2%) and Notch receptor 3: 32 cases (32.7%), and most of the Notch receptor 4 cases (95.2%) showed cytoplasmic and nuclear staining

except 5 cases (Table 3). In addition to cytoplasm and nucleus, occasionally incomplete membranous staining along with staining of luminal borders of neoplastic glands was noted.

Expression of DLL4: DLL4 was expressed in endothelial cells and non-neoplastic biliary epithelial cells, mesenchymal cells and sometimes inflammatory cells with variable intensities (Figure 3A). The cancer cells expressed DLL4 mainly in cytoplasm (Figure 3B), showing low grade: 63 cases (57.3%) and high grade: 21 cases (19.1%) (Table 2). As with Notch receptor protein expression, 48 cases (57.1%) out of DLL4 (+) 84 cases showed cytoplasmic and nuclear coexistent immunostaining (Figure 3C, Table 4). Occasionally, luminal borders of neoplastic glands showed distinct membranous immunostaining (Figure 3D).

Correlation between expression of Notch receptors 1-4 and DLL4, and clinicopathological factors with survival

Table 4 summarizes the correlations between expression of Notch receptors and DLL4, and clinicopathological parameters, including statistical analyses. Notch receptor 1 was expressed at advanced TNM stage, representing a statistically significant correlation ($P = 0.043$). Notch receptor 2 expression was positively correlated with female gender ($P = 0.005$). Notch receptor 3 was expressed at advanced T stage ($P = 0.017$) and tended to express in cases with lymph node metastasis ($P = 0.065$) and at advanced TNM stage ($P = 0.052$). The expression of Notch receptor 4 was not correlated with clinicopathological parameters. High DLL4 expression tended to be related to less histological differentiation ($P = 0.095$). The median survival of 110 extrahepatic CC and gallbladder carcinoma patients was 34.1 mo (Figure 4). There was no significant correlation between the expression of Notch receptors 1-4 and DLL4, and survival (Notch receptor 1;

Table 3 Correlation between cytoplasmic/nuclear coexistent localization of Notch receptors 1-4 and delta-like ligand-4 expression, and clinicopathological parameters

Nuclear localization	Notch1			Notch 2			Notch 3			Notch 4			DLL4		
	-	+	<i>P</i> value	-	+	<i>P</i> value	-	+	<i>P</i> value	-	+	<i>P</i> value	-	+	<i>P</i> value
Total	78	18		57	40		65	32		4	99		36	48	
Differentiation			0.545			0.418			0.556			0.036			0.175
Well	37	11		29	18		32	17		2	47		17	20	
Moderate	34	6		22	20		27	14		0	43		14	26	
Poorly	7	1		6	2		6	1		2	9		5	2	
T stage			0.846			0.594			0.269			0.365			0.053
T1	12	4		11	5		8	9		0	16		5	9	
T2	43	8		25	23		33	13		1	51		15	26	
T3	18	5		18	10		20	8		3	26		11	13	
T4	5	1		3	2		4	2		0	6		5	0	
N stage			0.100			0.408			0.027			0.34			0.558
N0	52	16		44	27		41	28		2	70		27	32	
N1	26	2		13	13		24	4		2	29		9	16	

P by χ^2 test. DLL4: Delta-like ligand-4.

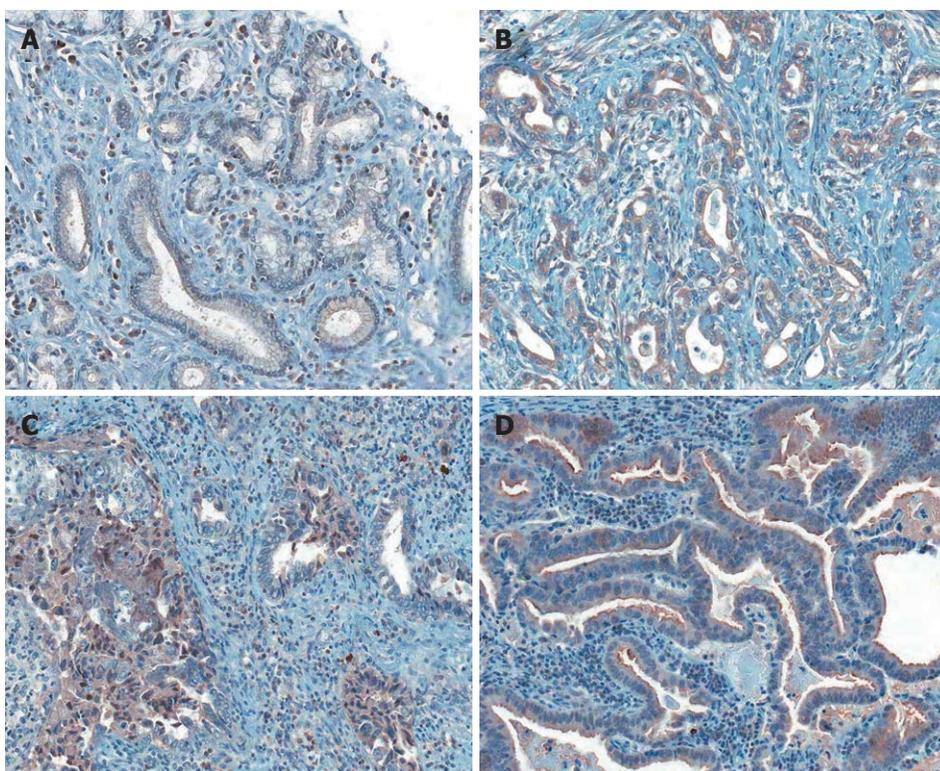


Figure 3 Delta-like ligand-4 expression in cholangiocarcinomas (immunohistochemistry, $\times 100$). A: Non-neoplastic biliary tissue; B-D: Cholangiocarcinomas. Non-neoplastic biliary epithelial cells (weak) and stromal inflammatory cells (strong) show cytoplasmic staining. Brown-colored expression of delta-like ligand-4 in cholangiocarcinoma cells is located at cytoplasm (B-D), coexisting nuclei (C), and luminal border (D).

$P = 0.487$, Notch receptor 2; $P = 0.922$, Notch receptor 3; $P = 0.391$, Notch receptor 4; $P = 0.474$, DLL4; $P = 0.441$.

Correlation between cytoplasmic/nuclear coexistent localization of Notch receptors 1-4 and DLL4, and clinicopathological factors with survival

Cytoplasmic/nuclear coexistent localization of Notch receptor 3 was correlated with no lymph node metastasis ($P = 0.027$), Notch receptor 4 correlated with less histological differentiation ($P = 0.036$), and DLL 4 tended to be

inversely related to advanced T stage ($P = 0.053$) (Table 4). The other clinicopathological parameters were not correlated with cytoplasmic/nuclear coexistent localization of Notch receptors 1-4 and DLL4 expression.

There was no significant correlation between the cytoplasmic/nuclear coexistent localization of Notch receptor 1-4 expression and survival (Notch receptor 1: $P = 0.280$, Notch receptor 2: $P = 0.204$, Notch receptor 3: $P = 0.768$, Notch receptor 4: $P = 0.425$). Cytoplasmic/nuclear coexistent localization of DLL4 expression was related to poor

Table 4 Correlation between expression of Notch receptors 1-4 and Delta-like ligand-4, and clinicopathological parameters

Total (n = 110)	Notch 1				Notch 2				Notch 3				Notch 4				DLL4			
	-	+	++	P value	-	+	++	P value	-	+	++	P value	-	+	++	P value	-	+	++	P value
Gender				0.207				0.005				0.2				0.144				0.517
Male	47	9	21	17	11	22	14		8	20	19		5	22	20		13	24	10	
Female	63	5	34	24	2	38	23		5	36	22		2	39	22		13	39	11	
Age				0.251				0.34				0.107				0.761				0.873
< 60	34	3	21	10	5	15	14		1	21	12		1	20	13		7	20	7	
≥ 60	76	11	34	31	8	45	23		12	35	29		6	41	29		19	43	14	
Differentiation				0.391				0.266				0.101				0.414				0.095
Well	53	5	30	18	6	29	18		4	28	21		4	29	20		16	31	6	
Moderate	46	6	20	20	4	24	18		5	23	18		3	23	20		6	27	13	
Poorly	11	3	5	3	3	7	1		4	5	2		0	9	2		4	5	2	
T stage				0.103				0.285				0.017				0.294				0.614
T1	17	1	8	8	1	11	5		0	13	4		1	12	4		3	12	2	
T2	55	4	32	19	7	31	17		9	26	20		3	25	27		14	31	10	
T3	32	9	11	12	4	13	15		4	11	17		3	19	10		8	15	9	
T4	6	0	4	2	1	5	0		0	6	0		0	5	1		1	5	0	
N stage				0.999				0.223				0.065				0.795				0.301
N0	78	10	39	29	7	46	25		9	45	24		6	42	30		19	47	12	
N1	32	4	16	12	6	14	12		4	11	17		1	19	12		7	16	9	
M stage				0.467				0.443				0.999				0.999				0.221
M0	103	13	53	37	13	57	33		13	52	38		7	57	39		25	60	18	
M1	7	1	2	4	0	3	4		0	4	3		0	4	3		1	3	3	
TNM stage				0.043				0.144				0.052				0.898				0.36
I	51	2	29	20	4	31	16		6	30	15		3	27	21		11	33	7	
II	45	11	20	14	8	21	16		7	16	22		4	24	17		12	22	11	
III	6	0	4	2	1	5	0		0	6	0		0	5	1		1	5	0	
IV	8	1	2	5	0	3	5		0	4	4		0	5	3		2	3	3	

P by χ^2 test. TNM: Tumor, node, metastasis.

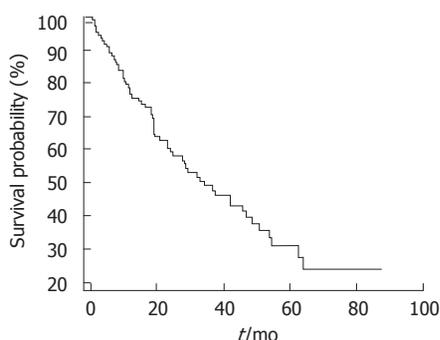


Figure 4 Overall survival curve using the Kaplan-Meier method by log rank test. Median survival is 34.1 mo.

survival in a statistically significant manner ($P = 0.002$, Figure 5).

DISCUSSION

The Notch signaling pathway has been shown to be expressed in a variety of adult tissues in mammals, and furthermore is involved in tumorigenesis with neoangiogenesis of many malignant tumors. In addition, it participates in the development of multicellular organisms by its self renewal potential and induction of differentiation^[10]. Few studies in the field of Notch signaling involving biliary epithelial cells have been reported with regard to developmental biology and neoplastic transformation. Notch receptor 2 signaling has been reported to be related to

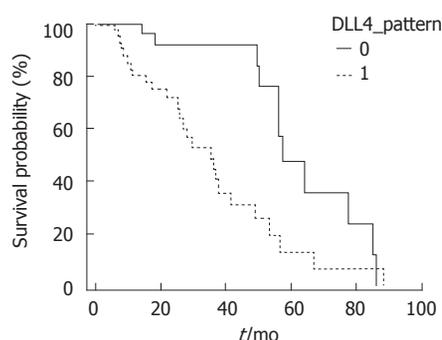


Figure 5 Survival curves of Cholangiocarcinomas with Delta-like ligand-4 expression with or without coexistent cytoplasmic and nuclear localization using the Kaplan-Meier method by log rank test. 0: Without nuclear localization; 1: With coexistent cytoplasmic and nuclear localization. Cases of coexistent cytoplasmic and nuclear localization of Delta-like ligand-4 expression show poor survival ($P = 0.002$).

the regulation of biliary epithelial cell differentiation, induction of tubulogenesis during early intrahepatic bile duct development in mice, and mutations of Jagged 1 and Notch receptor 2 resulting in Alagille syndrome, a rare hereditary multi-organ disorder involving impaired intrahepatic bile ducts^[5,17]. Ishimura *et al*^[18] reported that Notch receptor 1, but not other Notch receptors, was up-regulated in cholangiocytes when induced by inflammatory mediator nitric oxide synthase, and associated with malignant transformation. However, controversies about the role of Notch receptors in tumorigenesis as an oncogene or a tumor suppressor have arisen; Notch receptors

are reported generally as an oncogene in most human cancers^[10]. As a well-studied example, Notch receptor 1 functioned as an oncogene in T-cell leukemia through t(7; 9) chromosomal translocation of Notch receptor 1 and T-cell receptor J β locus^[8,9]. Activation of Notch signaling is reported in human non-hematopoietic solid tumors originating from mammary duct, colon, kidney, pancreas, and liver, etc^[16]. Others have shown Notch signaling as tumor suppressor in skin cancer and small cell lung cancer, and two faces of Notch as oncogene or tumor suppressor in cervical cancer^[10].

In this study, aberrantly high expression of Notch receptors 1-4 in extrahepatic CC and gallbladder carcinoma was observed by immunohistochemical study. In human breast cancer, the high expression of Notch receptor 1, Notch receptor 3 and Jagged 1 was reported to be correlated with poor predicted mortality, and the high-level co-expression of Notch receptor 1 and Jagged 1 was associated with poor overall survival^[19]. We found that the high expression of Notch receptor 1 related to advanced TNM stage ($P = 0.043$), and the high expression of Notch receptor 3 related to advanced T stage ($P = 0.017$) and tended to be related to nodal metastasis ($P = 0.065$) and advanced TNM stage ($P = 0.052$) in extrahepatic CC and gallbladder carcinoma. These results suggest that the up-regulation of Notch receptors 1 and 3 has a possible role in tumor progression of extrahepatic CC and gallbladder carcinoma, reflecting its role as an oncogene. However, there were inherent limitations in the number of studied cases for this study. One further limitation we must point out is that the studied cases were mostly surgically removed cancers, thus this study did not include late advanced or early cancers. In addition, there was no relation between high expression of Notch receptors and the overall survival.

Regarding the biologic roles of Notch signaling, liberated Notch intracellular domain after two sequential proteolytic cleavages translocates into the nucleus, and then activates transcription of target genes^[3]. In addition, the nuclear translocation of Notch receptor proteins is required for target gene activation, especially in malignant transformation^[20]. For the evaluation of gene expression in tissue, *in situ* IHC is valuable in morphologic identification of individual cell and intracellular localization of specific proteins. The reason why IHC should be applied in cases of human cancer tissue composed of neoplastic and non-neoplastic cells is because generally microvasculature cells, stromal cells and inflammatory cells express Notch signaling proteins, thus extracted samples contain non-neoplastic cells as well as cancer cells. We found that Notch receptors 1-4 were expressed in the cytoplasm of cancer cells, some of which were associated with nuclear co-localization. In hepatocellular carcinoma, the nuclear localization of Notch receptor 1 and Notch receptor 4 expression was reported to be involved in carcinoma development, representing target gene activation through translocated Notch receptor protein components^[12]. However, although coexistent cytoplasmic and nuclear localization of Notch receptors 1-4-expressing CC cells was found

with variable ratios, only the coexistent cytoplasmic and nuclear localization of Notch receptor 3 correlated with no nodal metastasis ($P = 0.027$) and Notch receptor 4 with less histologic differentiation ($P = 0.036$). Not many studies in human cancers have dealt with the cytoplasmic and/or nuclear expression of Notch receptor proteins, especially not in biliary epithelial carcinomas. Study of the mechanism of action regarding cytoplasmic high-expression of Notch receptors 1 and 3 in tumor progression during cholangiocarcinogenesis is needed through collection of more cases and application of biological tools.

As one of the five known Notch receptor ligands, the up-regulation of DLL4 expression was found to be involved in vasculogenesis and vessel maturation in human cancers^[21,22]. Recently, the expression of DLL4 was reported in human cancer cells as well as in normal epithelial cells, stromal cells, and endothelial cells of neo-angiogenesis^[16], thus interest was focused on possible roles in the development and progression of tumors, due to the nuclear or membranous localization. This study demonstrated that DLL4 is highly expressed in cancer cells, mainly in cytoplasm similar to Notch receptors, and that the up-regulation of DLL4 tended to be related to less histologic differentiation ($P = 0.095$) of extrahepatic CC and gallbladder carcinoma. Furthermore, the coexistent cytoplasmic and nuclear localization of DLL4 expression was observed and indicated poor survival ($P = 0.002$). Similar to Notch receptors, there are few reports concerning clinicopathological impact of DLL4 expression with intracellular localization. The up-regulation and cytoplasmic and nuclear localization of DLL4 expression is suggested to be involved in progression of cholangiocarcinogenesis, and has a probable role as a poor prognosticator. Overall, more cases should be studied for the identification of biological roles, according to intracellular compartmentalization in the expression of Notch receptors and DLL4.

In conclusion, these results imply that the up-regulation of Notch receptors 1 and 3 correlates with cancer progression, and that the coexistent cytoplasmic and nuclear localization of DLL4 expression correlates with poor survival in extrahepatic CC and gallbladder carcinoma. Further investigation on a large scale should be performed in order to understand the contribution of the involvement of Notch signaling in extrahepatic CC and gallbladder carcinoma.

COMMENTS

Background

Cholangiocarcinoma (CC) is the second most frequent primary liver cancer and the incidence is increasing. It has a poorer prognosis than hepatocellular carcinoma. Recently, in mammals, it was reported that the Notch signaling pathway is engaged in not only embryonic development, differentiation and specification of cell fate, but also in tumorigenesis with consequent possibilities of anti-cancer therapy targets. Basically, in human extrahepatic CC, including gallbladder cancer, the expression patterns of the Notch receptors and the representative ligand, Delta-like ligand-4, in cancer cells should be clarified.

Research frontiers

There are four Notch receptors (Notch 1-4) and five ligands [Jagged 1, Jagged 2, Delta-like ligand-1, -3 and -4 (DLL1, DLL3, DLL4)] in mammals. Recently, they have been reported to be involved in tumorigenesis as oncogenes or as tumor

suppressors, and proposed as prognostic factors or anti-cancer targets in aggressive or advanced cancers. In particular, antibodies to Notch receptor 4 and DLL4 have the possibility to be coupled with chemotherapeutic drugs as targeted therapy.

Innovations and breakthroughs

Abnormal Notch signaling has been reported in many human solid tumors, especially in breast cancer. With regard to cholangiocarcinoma, only its high expression has been reported with little study of the clinical impact. As with other solid tumors, the expression of Notch receptors in CC shows a possible role in cancer progression. The study of DLL4 expression in cancer cells has hardly been looked at. The coexistent cytoplasmic/nuclear localization of DLL4 expression has a novel value as a poor survival indicator.

Applications

The up-regulation and nuclear translocation of DLL4 has a probable role in the evaluation of survival as a poor prognosticator. These results of altered Notch signaling in CC can provide fundamentals for further investigation on an expanded scale of human extrahepatic CC and gallbladder carcinoma, mechanism of action through cross-talk of Notch receptors and their ligands, and other signaling networks.

Terminology

Four Notch receptors (Notch 1-4) and five ligands (Jagged 1, Jagged 2, DLL1, DLL3, DLL4) are found in mammals. Ligand-receptor interaction between two neighboring cells is involved in developmental, physiologic and pathologic processes.

Peer review

The paper is reasonably important and includes data on a large cohort of patient's with extrahepatic cholangiocarcinoma and gallbladder cancer.

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S- Editor Tian L L- Editor Logan S E- Editor Xiong L

Metabolic syndrome, lifestyle risk factors, and distal colon adenoma: A retrospective cohort study

Moon-Chan Kim, Chang-Sup Kim, Tae-Heum Chung, Hyoung-Ouk Park, Cheol-In Yoo

Moon-Chan Kim, Chang-Sup Kim, Tae-Heum Chung, Department of Family Medicine, Ulsan University Hospital, University of Ulsan College of Medicine, Ulsan 682-714, South Korea

Hyoung-Ouk Park, Cheol-In Yoo, Department of Occupational and Environmental Medicine, Ulsan University Hospital, University of Ulsan College of Medicine, Ulsan 682-714, South Korea

Author contributions: Kim MC contributed to the concept of the study, analyzed the data and wrote the paper; Kim CS and Chung TH collected the data and wrote the paper; Park HO analyzed the data and wrote the paper; Yoo CI was the principal investigator, designed the study, analyzed the data and wrote the paper.

Supported by the Biomedical Research Center Promotion Fund of the Ulsan University Hospital (UUH-2008-08)

Correspondence to: Cheol-In Yoo, Professor, MD, PhD, Department of Occupational and Environmental Medicine, Ulsan University Hospital, University of Ulsan College of Medicine, 290-3 Jeonha-dong, Dong-gu, Ulsan 682-714, South Korea. ciyoo@ulsan.ac.kr

Telephone: +82-52-2508819 Fax: +82-52-2507289

Received: December 26, 2010 Revised: May 19, 2011

Accepted: May 26, 2011

Published online: September 21, 2011

was significantly associated with the incidence of distal colon adenoma (Hazard ratio 1.66, 95% confidence interval 1.05-2.62).

CONCLUSION: Our results suggest that high BMI may increase the risk of colorectal adenoma in Korean adults.

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Key words: Body mass index; Distal colon adenoma; Korea; Lifestyle risk factor; Metabolic syndrome

Peer reviewer: Dr. Benjamin Perakath, Professor, Department of Surgery Unit 5, Christian Medical College, Vellore 632004, Tamil Nadu, India

Kim MC, Kim CS, Chung TH, Park HO, Yoo CI. Metabolic syndrome, lifestyle risk factors, and distal colon adenoma: A retrospective cohort study. *World J Gastroenterol* 2011; 17(35): 4031-4037 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/4031.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.4031>

Abstract

AIM: To investigate relationships between colorectal adenoma incidence, metabolic syndrome (MS) components and lifestyle factors.

METHODS: We conducted a retrospective cohort study using data from individuals who had multiple sigmoidoscopies for colon cancer at the Health Promotion Center of Ulsan University Hospital in Korea from 1998 to 2007.

RESULTS: By multivariate analysis, the incidence of distal colon adenoma was increased by more than 1.76 times in individuals with at least one component of MS compared to those without a component of MS. After adjustment for age, gender, smoking, drinking, and physical exercise, only high body mass index (BMI)

INTRODUCTION

Colorectal cancer is a major cause of cancer death in developed Western countries such as the United States and nations of Europe^[1]. However, the incidence of colorectal cancer has been increasing rapidly over the past two decades in Korea, which was previously known as a low-risk area. According to a report from the Ministry of Health and Welfare of Korea, colorectal cancer was the third most commonly diagnosed malignancy after stomach and lung cancer in 2005. From 1999 to 2005, the incidence of colorectal cancer rose from 26.2 to 39.6 cases per 100 000 population for men and from 16.4 to 22.2 per 100 000 for women^[2].

As colorectal adenomas are recognized as precursors

of colorectal cancer, identification of their risk factors would seem to be helpful in the prevention of colorectal cancer^[3]. One of the main explanations given for the increasing incidence of colorectal cancer in Korea is the growing adoption of the westernized diet consisting of high fat and sugar. The western diet is also known to increase the risk of metabolic syndrome (MS)^[4].

In several previously reported studies, MS or its individual components such as obesity, impaired glucose tolerance, hypertension, low high-density lipoprotein cholesterol, and hypertriglyceridemia were found to be associated with colorectal adenomas^[5-13]. In addition, lifestyle factors such as alcohol drinking, cigarette smoking, and lack of physical exercise also have been shown to be associated with the incidence of colorectal adenoma^[14-17]. However, most previous studies showed only a weak temporal relationship between exposure and disease occurrence.

To date, no cohort study has examined the association between the individual components of MS or lifestyle factors and the incidence of colorectal adenoma in Korea. In this study, we investigated the relationships between the incidence of distal colon adenoma and MS components and also lifestyle factors in a Korean population-based cohort.

MATERIALS AND METHODS

Study population

We conducted a retrospective cohort study using data from individuals who had multiple sigmoidoscopies at the Health Promotion Center of Ulsan University Hospital in Korea from 1998 to 2007. We recommend screening flexible sigmoidoscopy (SFS) to our patients according to guidelines for colorectal cancer screening issued by the American College of Gastroenterology^[18]. Nevertheless, some patients within our study population had undergone two or more surveillance SFS examinations at short intervals. Perhaps some of these study participants had misunderstood the recommended guidelines or had lower gastrointestinal tract symptoms or risk factors for colorectal cancer such as family history; or there may be other reasons for the multiple examinations at short intervals.

A total of 15 353 asymptomatic adults underwent a flexible sigmoidoscopy. Of these, 13 566 were excluded because they had undergone only one sigmoidoscopy or data were missing from their records. Among the remaining 1787 individuals who had had at least two sigmoidoscopies during a period of two years, 225 individuals who were specific for tubular adenoma ($n = 109$ adults) or hyperplastic polyps ($n = 116$ adults) at the first sigmoidoscopic examination were excluded. Thus, a cohort of 1562 individuals was included in this study (Figure 1).

The following parameters were collected from medical records and from self-administered questionnaires: height, body weight, systolic and diastolic blood pressures, serum biochemistry, flexible sigmoidoscopy re-

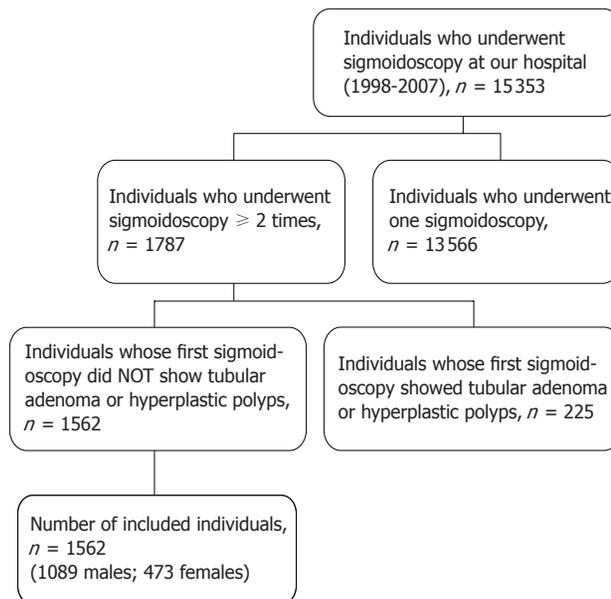


Figure 1 Case selection.

sults, and past medical history. The study protocol was approved by the Institutional Review Board of Ulsan University Hospital.

Questionnaire information

Using a structured, self-administered questionnaire, patients were asked about their smoking and drinking habits, physical activity, medical history and socioeconomic status.

Smoking was categorized as never, ex-smoker and current smoker. An individual who reported smoking within the past 30 d was classified as a current smoker. An ex-smoker was defined as an individual who had smoked at least one pack-year and was distinguished from someone who had never smoked. Current drinkers were defined as individuals who had consumed alcohol at least once per week over a period of at least 1 year. For alcohol consumption, individuals were classified as never or current drinkers.

Physical activity was classified as regular, irregular, and none based on the regularity, regardless of the type of exercise or intensity level. Individuals who reported having exercised regularly within the past year were classified into the regular exercise group. The irregular exercise group comprised individuals who had exercised irregularly within the past year, and were distinguished from those who had never exercised in the past year. The regular and irregular exercise groups were classified as the exercise “yes” group in the analysis.

Anthropometric and laboratory measurements

Anthropometric measurements were made by well-trained examiners in individuals wearing light clothing and without shoes. Height was measured to the nearest 0.1 cm and weight to the nearest 0.1 kg using Inbody 2.0 (Biospace, Seoul, Korea). Body mass index (BMI) values

Table 1 Age- and gender-specific prevalence of metabolic syndrome in the study population *n* (%)

Age group (yr)	Males							Females						
	<i>n</i>	MS criteria						<i>n</i>	MS criteria					
		BMI ≥ 25	FBS ≥ 110	HBP	TG ≥ 150	HDL < 40	MS ≥ 3		BMI ≥ 25	FBS ≥ 110	HBP	TG ≥ 150	HDL < 50	MS ≥ 3
20-29	4	0 (0)	0 (0)	1 (25)	0 (0)	1 (25)	0 (0)	2	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)
30-39	81	29 (36)	8 (10)	14 (17)	29 (36)	22 (27)	18 (22)	26	4 (15)	0 (0)	2 (8)	2 (8)	12 (46)	0 (0)
40-49	451	154 (34)	68 (15)	137 (30)	131 (29)	120 (27)	76 (17)	191	34 (18)	15 (8)	40 (21)	20 (10)	91 (48)	18 (9)
50-59	466	164 (35)	91 (20)	195 (42)	123 (26)	101 (22)	80 (17)	200	52 (26)	28 (14)	61 (31)	28 (14)	104 (52)	34 (17)
≥ 60	87	21 (24)	21 (24)	46 (53)	28 (32)	14 (16)	20 (23)	54	20 (37)	10 (19)	26 (48)	14 (26)	30 (56)	16 (30)
Sub-total	1089	368 (34)	188 (17)	393 (36)	311 (29)	258 (24)	194 (18)	473	110 (23)	53 (11)	129 (27)	64 (14)	239 (51)	68 (14)

BMI: Body mass index (kg/m²); FBS: Fasting blood sugar (mg/dL); HBP: High blood pressure (systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg); TG: Triglyceride (mg/dL); HDL: High density lipoprotein (mg/dL); MS: Metabolic syndrome.

were calculated by dividing weight (kg) by height squared (m²). Blood pressure was measured by well-trained nurses using a mercury sphygmomanometer in the sitting position after at least a 10-min rest period. Following an overnight fasting, blood samples were obtained and analyzed on a Hitachi Modular DPE system (Roche Diagnostics, Germany). The fasting plasma glucose (FPG) level was measured using a hexokinase UV method. Triglycerides (TG) were measured by an enzymatic calorimetric method. HDL-cholesterol (HDL-C) level was determined by the homogeneous enzymatic colorimetric method.

Diagnosis of distal colon adenoma

The sigmoidoscopies were performed by gastroenterologists who observed the entire procedure on a video monitor. The procedure was intended to screen the distal colon, including the descending and sigmoid colon, and the rectum. When fully inserted, the 60-cm sigmoidoscope reached to the mid-descending colon. Endoscopic findings were recorded in a computer database. All visualized lesions were biopsied and histologically assessed by an experienced pathologist. The size of each polyp was estimated by the use of 8-mm-diameter open-biopsy forceps. Histological assessment of the polyps was performed by a single pathologist blinded to each patient's status. In this study, the adenomas were classified as tubular, serrated, villo-tubular, high-grade dysplasia, and adenocarcinoma types according to the World Health Organization classification^[19]. For multiple primary adenomas in the distal colon at different times, the earliest diagnosis was applied, and for those occurring simultaneously, the most advanced and most invasive diagnosis was applied.

Definition of MS

MS was defined according to the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) criteria, with BMI used in place of waist circumference^[20]. BMI was calculated as described above (kg/m²). The definition of obesity was BMI ≥ 25 , as recommended by the Korean Ministry of Health and Welfare in 2006^[21]. The diagnosis of MS in this study was made when at least 3 of the following 5 characteristics were present: (1) BMI ≥ 25 kg/m²; (2) TG

≥ 150 mg/dL, or on drug treatment for elevated TG; (3) HDL-C < 40 mg/dL in males and < 50 mg/dL in females, or on drug treatment for reduced HDL-C; (4) blood pressure (BP) $\geq 130/85$ mmHg, or on drug treatment for hypertension; and (5) FPG ≥ 110 mg/dL, or on drug treatment for elevated glucose.

Statistical analysis

Multivariate Cox proportional hazards analysis was applied to assess the effect of prognostic variables in MS and non-MS individuals. Results of the Cox proportional hazards model were presented as the hazard ratio (HR) and the 95% confidence interval (95% CI). All *P* values were two-sided, and all statistical analyses were performed using SPSS version 14.0.

RESULTS

A total of 1562 individuals (1089 men and 473 women) were included in the study. Of these, 229 (14.7%) subjects (197 men and 32 women) were found to have distal colon adenomas.

Table 1 shows the age- and gender-specific prevalence of MS in the study population. The prevalence of MS in males was slightly higher than in females (18% and 14%, respectively). Hypertension was the most common metabolic abnormality in both sexes.

The associations between the incidence of distal colon adenomas and the number of MS components are shown in Table 2. In our cohorts, 263 (16.8%) of the individuals were diagnosed with MS according to NCEP-ATP III criteria. Among the MS subjects, 182 (69.2%) had three components, 64 (24.3%) had four components, and 17 (6.5%) had all five. After adjusting for age and gender, the presence of any component of MS showed a positive association with the incidence of distal colon adenoma. According to multivariate analysis, the risk of distal colon adenoma was increased in patients with even one component of MS compared to patients without any component of MS, but this result was not statistically significant.

Among the MS components, diastolic blood pressure, systolic blood pressure, BMI, HDL and FBS were posi-

Table 2 Hazard ratios for distal colon adenomas according to the number of metabolic syndrome components

Number of MS diagnostic criteria present	Cases	Person years	Age-, sex-adjusted hazard ratio	95% CI		Multivariate hazard ratio ¹	95% CI	
0	434	1446	1.00			1.00		
1	504	1697	1.18	0.83	1.67	1.76	0.94	3.29
2	361	1272	1.02	0.70	1.50	1.69	0.86	3.29
3	182	610	1.36	0.88	2.10	1.48	0.66	3.36
4	64	224	1.35	0.73	2.47	2.14	0.81	5.63
5	17	62	1.52	0.54	4.28	2.92	0.62	13.73

¹Adjusted for age, sex, smoking status, drinking status, exercise status. CI: Confidence Interval; MS: Metabolic syndrome.

Table 3 Hazard ratios for distal colon adenomas according to individual components of metabolic syndrome and lifestyle factors

Characteristics	Cases	Person years	Age-, sex-adjusted hazard ratio	95% CI		Multivariate hazard ratio ²	95% CI	
DBP								
< 85	1237	4106	1.00			1.00		
≥ 85	325	1206	0.75	0.54	1.04	0.68	0.35	1.29
SBP								
< 130	1085	3648	1.00			1.00		
≥ 130	477	1664	0.97	0.73	1.29	1.50	0.82	2.73
BMI								
< 25	1084	3688	1.00			1.00		
≥ 25	478	1623	1.39	1.07	1.82	1.66	1.05	2.62
HDL								
≥ 40 (≥ 50 ¹)	1065	3641	1.00			1.00		
< 40 (< 50 ¹)	497	1671	1.19	0.88	1.60	1.04	0.57	1.88
TG								
< 150	1187	4025	1.00			1.00		
≥ 150	375	1287	0.96	0.72	1.29	0.76	0.45	1.27
FBS ⁶								
< 110	1321	4457	1.00			1.00		
≥ 110	241	855	1.13	0.82	1.58	1.32	0.77	2.27
Smoking								
Never smoked	606	2048	1.00			1.00		
Ex-smoker	218	769	0.86	0.57	1.29	1.34	0.70	2.54
Current smoker	262	1072	0.81	0.56	1.17	1.39	0.79	2.44
Drinking								
No drinking	396	1307	1.00			1.00		
Current drinking	772	2695	1.59	1.02	2.47	1.82	0.92	3.60
Exercise								
Never	409	1587	1.00			1.00		
Irregular	398	1549	1.09	0.73	1.62	0.96	0.58	1.58
Regular (≥ 1 time/wk)	273	1077	1.22	0.78	1.90	0.88	0.49	1.60

¹Female; ²Adjusted for sex, age, systolic and diastolic blood pressure. DBP: Diastolic blood pressure; SBP: Systolic blood pressure; BMI: Body mass index; HDL: High density lipoprotein; TG: Triglyceride; FBS: Fasting blood sugar; HDL: High density lipoprotein; CI: Confidence interval.

tively associated with the risk of distal colon adenoma; however, only the association with BMI was statistically significant (Table 3). Among the lifestyle factors examined in our study, smoking and drinking were positively associated with the risk of distal colon adenoma whereas exercise showed a negative correlation, but these results were not statistically significant.

DISCUSSION

In this study, we confirmed a positive association between MS and distal colon adenoma in a Korean population. We found that, compared to persons without any

component of MS, the risk of distal colon adenoma positively increased with the presence of even one component of MS. These results are consistent with those of Lee *et al*^[8]. Moreover, we found only BMI to be an independent risk factor of distal colon adenoma among the five components of MS examined.

Although NCEP-ATP III requires waist circumference for diagnosis of MS, we used BMI as an indicator of obesity because the cut-off points of waist circumference in Koreans differ from those currently recommended by the NCEP-ATP III. The cut-off points of waist circumference for central obesity in Koreans are 90 cm for men and 85 cm for women^[22]. Moreover,

currently there is no equivalent method for the measurement of waist circumference, whereas BMI is an easy and accurate index. Waist circumference measurements show strong between-observer differences, and should, where possible, be carried out by one observer. Weight and height are the most precisely measured variables, and it is entirely appropriate that they continue to be the predominant measure of choice in the vast majority of nutritional anthropometric studies^[23]. Therefore, most medical institutions in Korea use BMI as an indicator of obesity, defined by The Korean Ministry of Health and Welfare as $\text{BMI} \geq 25$ ^[21].

Several studies have reported that MS is associated with colorectal adenoma and that obesity is a risk factor for the lesion, but the relationship between BMI and the prevalence of colorectal adenoma has remained controversial^[5-9,16,24,25]. Recently, a retrospective study of the effect of body weight changes on the development of new colorectal adenomas was reported by Yamaji *et al.*^[24]. In their cross-sectional study, they found that the prevalence of colorectal adenoma increased proportionally with increasing BMI. According to their results, the prevalence of colorectal adenoma was 15.4%, 20.6%, 22.7%, and 24.2%, respectively, in the first ($\text{BMI} < 21.350 \text{ kg/m}^2$), second ($\text{BMI} 21.350 \leq \text{BMI} < 23.199 \text{ kg/m}^2$), third ($\text{BMI} 23.199 \leq \text{BMI} < 25.156 \text{ kg/m}^2$), and fourth ($\text{BMI} \geq 25.156 \text{ kg/m}^2$) quartiles. Compared with the first quartile, the adjusted odds ratios (ORs) were 1.15 (95% CI, 0.97-1.37; $P = 0.10$) for the second quartile, 1.19 (95% CI, 1.01-1.41; $P = 0.04$) for the third quartile, and 1.32 (95% CI, 1.12-1.56; $P = 0.001$) for the fourth quartile. Their result is similar to our finding of a positive relationship between BMI and the prevalence of distal colon adenoma. In our study we confirmed that persons with high BMI (≥ 25) had a 66% higher incidence of distal colon adenoma compared to persons with $\text{BMI} < 25$. The study by Lee *et al.*^[8] also revealed that the occurrence of adenomatous colonic polyps was significantly associated with increased BMI levels. In contrast, Willett and colleagues found no significant association between BMI and colorectal adenoma^[16,25].

Another controversy remains regarding whether gender differences exist in the relationship between BMI and colorectal adenoma. Some authors reported that BMI was associated with a higher risk of colorectal adenomas among men but not among women^[26,27]. However, others reported an increased risk and prevalence for significant colorectal neoplasia in women as BMI increased, but not in men^[17,28].

In the present study, current and past smoking and current drinking behaviors all showed a positive association with the occurrence of distal colon adenoma, whereas physical activity showed a negative association. Our results are similar to those of other studies, but none of the associations in any of the studies achieved statistical significance^[29-32]. Prior to this study, we hypothesized that individuals with unhealthy lifestyle behaviors, such as cigarette smoking, alcohol drinking, and physical

inactivity, would be at greater risk of distal colon adenoma. The lack of statistical significance in the current study could be due to the short observation period and the interaction between MS components.

The strengths of our study include the cohort design and the relatively large sample size. Furthermore, we investigated the association of each component of MS, which provided the opportunity to examine the difference in incidence rate of distal colon adenoma between MS and non-MS individuals. Additionally, lifestyle factors related to MS, including cigarette smoking, alcohol consumption, and physical exercise, were examined. One other advantage of our study is that actual measurements of height and weight were made by trained individuals. This is preferred over self-reported data, which have been used frequently in a number of well-known, large-scale cohort studies, because heavier individuals tend to under-report their weight. Furthermore, our study simultaneously measured anthropometrics, FBS, TG, HDL-C, blood pressure, and adenoma incidence in a population of individuals at average adenoma risk undergoing sigmoidoscopy. Moreover, subjects in both the adenoma and normal groups were confirmed as having or not having the lesion through the same diagnostic procedure of sigmoidoscopy. Therefore, it is highly unlikely that the study results might be affected by misclassification bias, and this hospital-based retrospective study is internally valid because the normal and recurrent adenoma groups were selected from the same source population.

There were several limitations in our study which need to be addressed. Firstly, the information on cigarette smoking, alcohol drinking and physical activity was self-reported, thus allowing for recall bias. This may explain why a sedentary lifestyle and cigarette smoking were not associated with an increased risk of distal colon adenoma. Secondly, the study population may not be representative of the general population because the subjects were not randomly selected. Also, there may have been some heterogeneity in fasting status and the time of day that blood was collected. Finally, only the adenomas of the distal colon, not the entire colon, were screened using sigmoidoscopy. For the detection of colon adenoma, colonoscopy may be more accurate than sigmoidoscopy.

In conclusion, only BMI, but no other individual component of MS, was positively associated with distal colon adenoma risk. In contrast with previous cross-sectional studies, we found that other MS components did not have a synergistic effect on development of distal colon adenoma^[3,4]. However, this may be attributable to the limitations mentioned above. One possible explanation for our findings is that each component of MS may promote or prevent adenoma development *via* different mechanisms that do not act in an additive or synergistic manner. Further molecular biological research and epidemiological studies are needed to explore this topic.

To our knowledge, the present study is the first cohort study supporting a relationship between BMI and

new development of distal colon adenoma in a Korean population. BMI appears to be a better predictor than the MS cluster, and therefore BMI may be the only component needed.

ACKNOWLEDGMENTS

We thank the members of the Ulsan University Hospital Health Promotion Center and all subjects who participated in this study.

COMMENTS

Background

In Korea, as in the developed countries, the incidence of colorectal cancer has been increasing rapidly over the past two decades with the adoption of westernized lifestyles. Because the western diet increases the risk of metabolic syndrome (MS) and colorectal adenomas are precursors of colorectal cancer, some researchers have investigated the relationships between the incidence of colorectal adenoma and MS components and lifestyle factors.

Research frontiers

Several studies have reported that MS is associated with colorectal adenoma and that obesity is a risk factor for the lesion. However the relationship between body mass index (BMI) as a diagnostic tool of obesity and the prevalence of colorectal adenoma has remained controversial. Moreover, to date, no cohort study has examined the association between the individual components of MS or lifestyle factors and the incidence of colorectal adenoma in Korea.

Innovations and breakthroughs

In our study, we found that the incidence of distal colon adenoma was increased by more than 1.76 times in individuals with at least one component of MS compared to those without a component of MS. Among the five components of MS examined, only BMI was the independent risk factor of distal colon adenoma. Persons with high BMI (> 25) had a 66% higher incidence of distal colon adenoma compared to persons with BMI < 25. These results suggest that high BMI may increase the risk of colorectal adenoma in Korean adults. To our knowledge, this is the first cohort study with a relatively large sample size.

Applications

Reducing the BMI can be one of the best ways to prevent colorectal cancer in Korean.

Peer Review

This is the first cohort study to demonstrate an association between the individual components of metabolic syndrome and the incidence of colorectal adenoma in Korean. It revealed that only a high BMI was associated with a high risk of colorectal adenoma. The results are interesting and may contribute to reduce the risk of developing colorectal cancer.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Zhang DN

Neoplasm-like abdominal nonhematogenous disseminated tuberculous lymphadenopathy: CT evaluation of 12 cases and literature review

Ming Zhang, Min Li, Gui-Ping Xu, Hong-Juan Liu

Ming Zhang, Min Li, Gui-Ping Xu, Department of Medical Imaging, First Affiliated Hospital, Xi'an Jiaotong University School of Medicine, Xi'an 710061, Shaanxi Province, China

Hong-Juan Liu, Department of Intensive Care, First Affiliated Hospital, Xi'an Jiaotong University School of Medicine, Xi'an 710061, Shaanxi Province, China

Author contributions: Zhang M, Li M, Xu GP and Liu HJ contributed equally to this work; Liu HJ acquired the clinical material; Zhang M, Li M, Xu GP reviewed the CT findings; Zhang M, Li M and Liu HJ wrote the paper.

Correspondence to: Hong-Juan Liu, Associate Professor, Department of Intensive Care, First Affiliated Hospital, Xi'an Jiaotong University School of Medicine, Xi'an 710061, Shaanxi Province, China. lhj_xjtu@126.com

Telephone: +86-29-85324618 Fax: +86-29-85323248

Received: January 17, 2011 Revised: March 11, 2011

Accepted: March 18, 2011

Published online: September 21, 2011

Abstract

AIM: To assess the diagnostic value of computed tomography (CT) imaging in screening for abdominal nonhematogenous disseminated tuberculous lymphadenopathy (TL).

METHODS: The CT scans of 12 patients with abdominal nonhematogenous disseminated TL suggestive of neoplasm were retrospectively analyzed in this review. The final diagnoses were confirmed by lymph node pathology for seven patients and by laparoscopic surgery for five patients. All of the patients were treated at our institution between April 1995 and August 2009.

RESULTS: The sites of involvement were the periportal ($n = 6$), peripancreatic ($n = 3$), periaortic ($n = 3$), and mesenteric ($n = 2$) regions. On the plain CT scan, the lymphadenopathy showed a heterogeneous isodensity or hypodensity in 11 patients and a low density in one

patient. Peripheral enhancement was observed on the dynamic contrast-enhanced CT scans for all patients. In two cases, scans were more revealing during the portal venous and delayed phases.

CONCLUSION: Abdominal lymphadenopathy with predominant peripheral rim-like enhancement on the dynamic contrast-enhanced CT scan may suggest a diagnosis of TL.

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Key words: Abdomen; Lymph node; Tuberculosis; Tomography; X-ray computed

Peer reviewers: Dr. Andreas G Schreyer, Professor, Department of Radiology, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, Regensburg 93053, Germany; Vineet Ahuja, Associate Professor, Department of Gastroenterology and Human Nutrition, All India Institute of Medical Sciences, New Delhi 110029, India

Zhang M, Li M, Xu GP, Liu HJ. Neoplasm-like abdominal nonhematogenous disseminated tuberculous lymphadenopathy: CT evaluation of 12 cases and literature review. *World J Gastroenterol* 2011; 17(35): 4038-4043 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/4038.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.4038>

INTRODUCTION

The global health burden of tuberculosis (TB) has risen in recent years. In 2009, an estimated 9.4 million cases and 1.7 million deaths globally could be attributed to TB. More than one-third of these cases were found in South-east Asia^[1]. Although the respiratory system remains the primary disease site, TB also increasingly affects the gastrointestinal tract, peritoneum, lymph nodes, kidneys,

and other solid viscera. Intra-abdominal TB (1%-3%) constitutes up to 12% of extra-pulmonary TB, particularly in immunocompromised individuals^[2,5]. Tuberculous lymphadenopathy (TL) is one of the most common findings in patients with abdominal TB. The presence of active pulmonary tuberculosis may indicate the possibility of abdominal TB involvement; however, only 15% of patients with abdominal TB have any evidence of pulmonary involvement^[4]. Clinically, patients with abdominal nonhematogenous disseminated TL typically present with an isolated mass or a mass adhering to the surrounding organs. When abdominal neoplasm is suspected, computed tomography (CT) is used to examine the abdomen. With the widespread use of CT, physicians should be familiar with the features of CT images seen in these patients and be able to make differential diagnoses using CT findings. In this article, we present 12 patients with abdominal nonhematogenous disseminated TL and discuss the features observed on their CT scans.

MATERIALS AND METHODS

We collected and retrospectively reviewed the CT scans of 12 patients who were diagnosed with abdominal non-hematogenous disseminated TL and who were admitted and treated at our institution between April 1995 and August 2009. The patients included 9 women and 3 men, and their ages ranged from 24 to 56 years (median age 43 years). The mean duration of their symptoms was 46 d (range 29-65 d).

Clinical signs and symptoms among these patients included fever (ardent fever, $n = 2$) and weight loss, epigastric pain ($n = 8$), and night sweats ($n = 1$). An elevated blood sedimentation rate and positive tuberculin test were present in only two patients. The initial diagnostic sonography identified an intra-abdominal mass in all of the patients; three patients presented with pancreatic masses and one patient with a hepatic mass. The chest radiographic examinations found evidence of healed TB in 3 of the 12 patients. None of our patients had any evidence or history of opportunistic infection, drug abuse, or previously treated lung TB.

Neoplasms of the pancreas, liver, or periaortic area were pre-operatively diagnosed in seven patients and were removed surgically. The final histopathological examination of the resected masses showed caseous or liquefactive substances in the center of the enlarged lymph nodes surrounded by inflammatory lymphatic tissues and no evidence of malignant cells. TL was suspected in the other 5 patients based on CT findings and clinical presentations. A diagnostic laparoscopy to biopsy the mass was performed to rule out malignancy. The subsequent anti-TB therapy confirmed the diagnosis of TL.

We used the 9800 Quick CT Scanner (General Electric Medical Systems, Milwaukee, WI; $n = 5$), the Picker PQ 6000 CT Scanner (Picker International, Cleveland, Ohio; $n = 6$), and the Brilliance 64 CT Scanner (Philips Medical Systems, Best, Netherlands; $n = 1$). The scans were performed with conventional techniques. Diatrizoate solu-

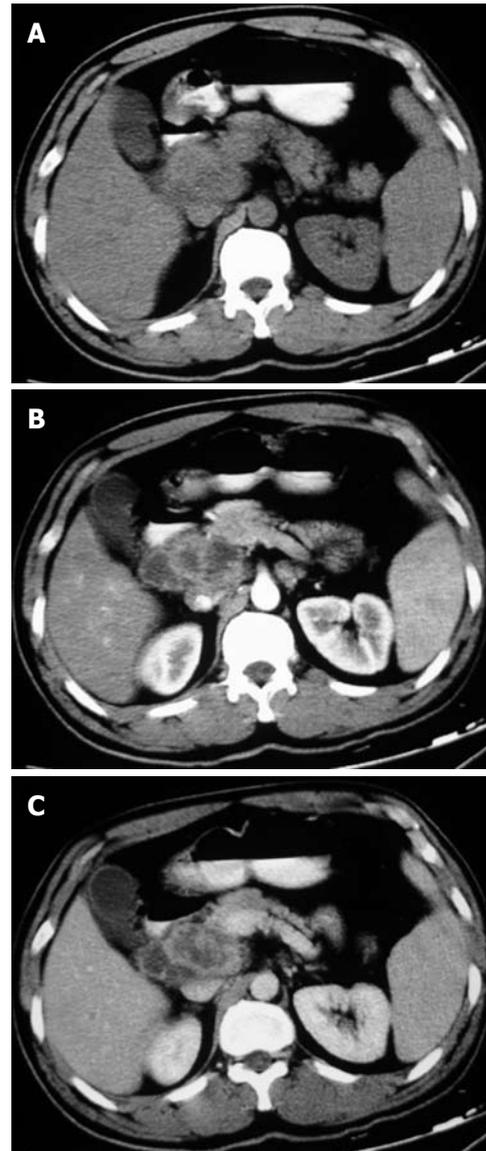


Figure 1 Peripancreatic tuberculous lymphadenopathy. A: Plain computed tomography (CT) showing peripancreatic, lobular, and slightly uneven density mass; B, C: Contrast-enhanced CT of the arterial phase (B) and portal venous phase (C) showing the mass with irregular peripheral enhancement.

tion (500-750 mL of 1.5%) or water was given orally to patients one hour prior to examination. Intravenous contrast medium (Omnipaque 300 mgI/mL, GE Healthcare) was administered at 1.0-1.2 mL/kg body weight with a flow rate of 3 mL/s. For enhanced scans, images were recorded for 25 s (arterial phase) and repeated for 60 s (portal venous phase) following the administration of the intravenous contrast using the single-slice spiral CT. The images of the delay phase (300 s after contrast administration) were recorded using the multi-slice spiral CT. Contiguous axial images of 7.5 mm or 10 mm sections were obtained from the epigastrium or from the dome of the diaphragm to the pubic symphysis.

RESULTS

The sites of lymph node adenopathy included the peri-

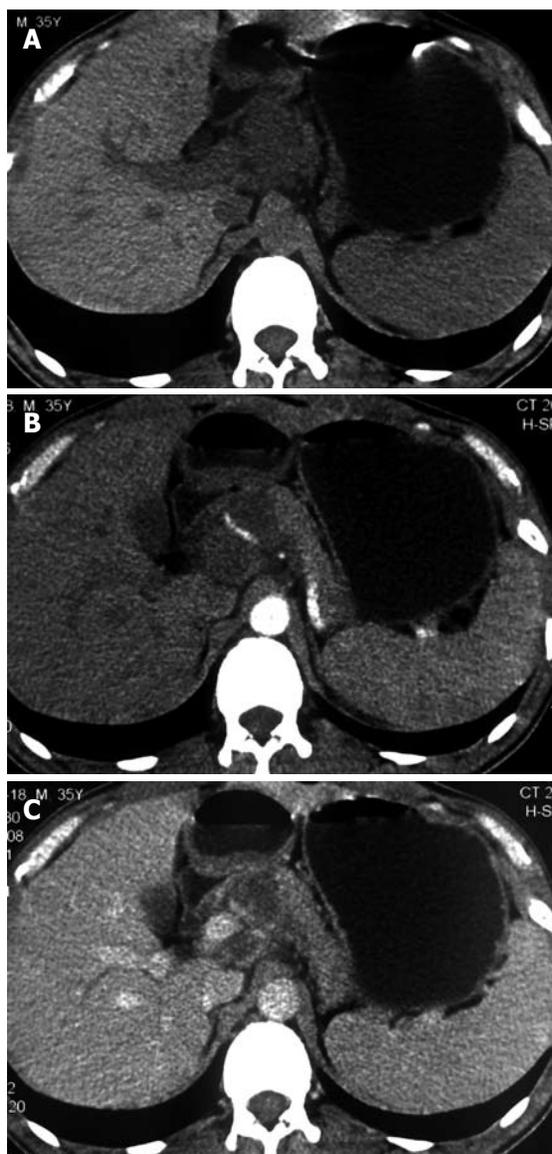


Figure 2 Peripancreatic tuberculous lymphadenopathy. A: Plain computed tomography (CT) showing peripancreatic, lobular, and low density mass; B, C: Contrast-enhanced CT of the arterial phase (B) and portal venous phase (C) showing the mass with slight peripheral enhancement and the encased common hepatic artery.

portal ($n = 6$), peripancreatic ($n = 3$), periaortic ($n = 3$), and small bowel mesenteric ($n = 2$) areas. The enlarged lymph nodes were 1.7-4.2 cm (mean 3.4 cm) in diameter. There was no calcification identified within the lesions. On the plain CT scans, the enlarged lymph nodes were of mostly heterogeneous isodensity or hypodensity, although one patient had lymph nodes of uniform low density. The margins of the involved lymph nodes were poorly defined. The lymph nodes could not be distinguished from the mass of the pancreas in three patients. After the administration of the contrast material, the lesions showed peripheral or ring-like enhancement with an expanded low-density central area in all the cases (Figures 1-4). Some ($n = 5$) had a conglomerated and multilocular appearance instead of single node involvement (Figures 1-3). The peripheral enhancement of the nodes was low in the

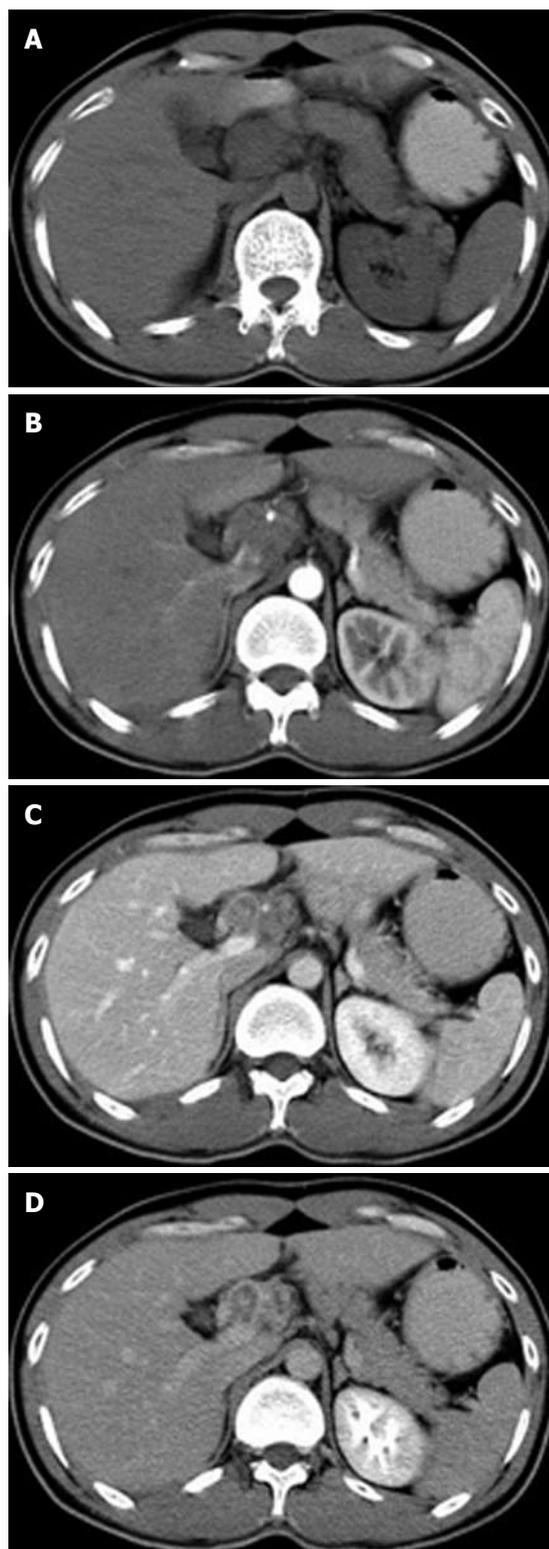


Figure 3 Periportal tuberculous lymphadenopathy. A: Plain computed tomography scan showing hypodense masses in the hepatic portal region; B-D: After injection of contrast material, continuing peripheral enhancement allows individual lymph nodes to be defined, and the common hepatic artery was embedded within the lesion without definite evidence of stenosis.

arterial phase but high in the venous or delay phase in two patients (Figures 2 and 3). The involved lymph nodes were easily distinguished from the pancreas (Figures 1 and 2) in three cases. The common hepatic artery was embed-

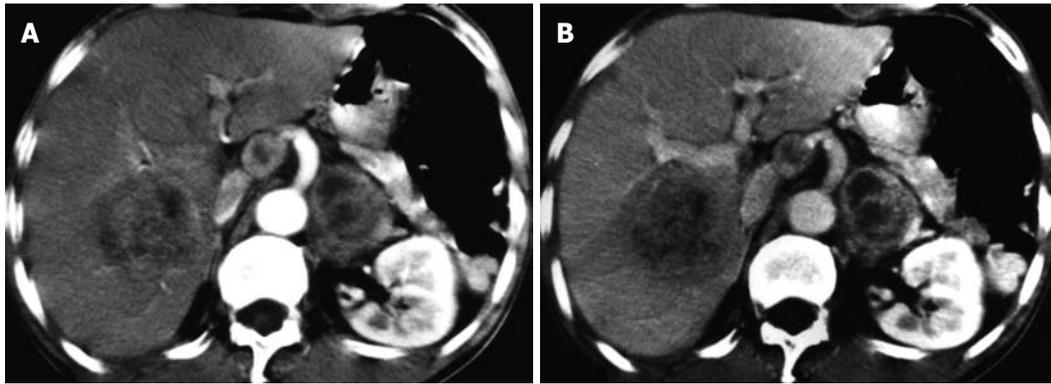


Figure 4 Hepatic tuberculosis and periaortic tuberculous lymphadenopathy. A, B: The lesions within the liver and periaorta show similar irregular rim enhancement on the arterial phase (A) and portal venous phase (B) of contrast-enhanced computed tomography.

ded in the enlarged lymph nodes (Figures 2 and 3) in two patients. None of the patients displayed clinical signs of ascites and/or peritoneum thickening.

One patient had hepatic TB and retroperitoneal lymphadenopathy. The large liver lesion showed irregular rim enhancement with enlarged lymph nodes (Figure 4).

DISCUSSION

The incidence of abdominal TL is low. The disease is thought to result from one of three major routes of transmission of TB. The most common route is through the ingestion of contaminated materials containing the tubercle bacilli. The second source is the hematogenous spread of the bacteria from a distant site of infection (commonly the lungs). The third route is the direct spread from the serosa of the adjacent infected organs or structures^[5]. Our case series identified three patients with X-ray evidence of healed lung TB; none of the patients had an active infection or had been previously treated. Therefore, hematogenous infection was impossible. This finding suggests that the only possible route of these infections was the ingestion of TB bacteria into the gastrointestinal tract. The tubercle bacilli are absorbed into the intestinal submucosal layer and then carried to the draining lymph nodes of the jejunum, ileum and ascending colon. The lymphatic drainage pattern supports our observation that the affected areas included the periportal, peripancreatic, upper periaortic, and mesenteric regions^[6]. Because the tubercle bacilli are hardly absorbed from the left side of the colon, the lower periaortic nodes are rarely involved in cases of nonhematogenous disseminated TL^[7].

The diagnosis of abdominal hematogenous disseminated TL can be made clinically when a patient has active pulmonary miliary TB^[7]. The signs and symptoms of abdominal nonhematogenous disseminated TL in patients include epigastric pain, fever, weight loss, fatigue, and abdominal mass. Other indicative symptoms include the presence of TB at other sites, positive skin TB tests, and night sweats, although these are uncommon in patients with nonhematogenous disseminated TL^[8]. In our review, only two patients had a positive skin TB test and

one patient had night sweats. Obstructive jaundice is a rare complication and may also be caused by periportal lymphadenopathy^[9,10], portal vein thrombosis, or portal hypertension^[11]. Although the clinical presentation of abdominal nonhematogenous disseminated TL has been well characterized, clinical diagnosis is limited because the signs and symptoms are nonspecific.

The CT scan is a useful tool in detecting lesions and making presumptive diagnoses in the abdomen. Lymphadenopathy is the most common manifestation of abdominal TB found on the CT^[12,13]. Plain CT findings are nonspecific in patients with abdominal nonhematogenous disseminated TL. The enlarged lymph nodes display low or soft tissue attenuation values and cannot be used to differentiate TL from neoplasm^[14,15]. On the other hand, contrast-enhanced CT scans can detect the peripheral or rim-like enhancement with a low-attenuation center that was seen in all of our patients. This radiographic feature corresponds histologically to the peripheral inflammatory reaction and neovascularity around central liquefaction or caseous necrosis. Homogeneous enhancement on the CT scan^[6] may reflect an earlier pathologic stage of the disease (i.e., the non caseating epithelioid and giant cell granulomas that precede necrosis) in which the size of the enlarged lymph nodes is often less than 1 cm in diameter. Non-enhancement on the CT scan is presumably due to the diminished inflammatory reaction associated with AIDS or with other immunocompromising diseases in patients. The individuals in this study were selected based upon the following criteria: (1) enlarged lymph nodes greater than 1 cm in diameter and (2) negative HIV infection status and a non-immunocompromised state. These parameters excluded the possibility of the confounding variables discussed above.

Other imaging modalities used for the detection of abdominal nonhematogenous disseminated TL have been described in other studies. Sonography shows TL as a hypoechoic^[16] and homogeneous mass. These features are nonspecific and cannot be used to differentiate TL from a neoplasm^[17]. Limited literature addresses the application of magnetic resonance imaging (MRI) in the diagnosis of abdominal TL. Kim *et al.*^[18] described MRI findings

of abdominal TL in a series of 11 patients who were all diagnosed correctly as having TL. The lesions may show a variety of signal intensities depending on the stage of evolution and are frequently hypointense on the T1-weighted images and hyperintense on the T2-weighted images. The enhancement pattern is similar to that of the CT scans, which show predominant peripheral rim-like enhancement^[18,19]. Furthermore, the MRI seems to be useful in differentiating the enlarged lymph nodes around the pancreas from a cystic neoplasm. Limited evidence suggests that the MRI scan has a valuable role in the diagnosis of abdominal TL. However, MRI scanners are not always available in developing countries. ¹⁸F-fluorodeoxyglucose positron emission tomography (FDG-PET) CT imaging is increasingly used as a tool for detecting malignancies. The accumulation of FDG in tuberculous lymph nodes prevents its use as a diagnostic tool for TL^[20].

Intra-abdominal lymphoma can be confused with abdominal nonhematogenous disseminated TL both clinically and on CT scans. Lymphoma often involves the abdominal lymph nodes in the entire periaortic region, including above and below the third lumbar areas^[6,21]. The enlarged lymph nodes in lymphoma can grow to be as large as 4.0 cm in diameter and appear homogeneous on CT scans in most patients^[6,21,22]. Yu *et al.*^[21] considered embedded vessels to be a unique finding of lymphoma. Nonhematogenous disseminated TL involves the mesentery lymph nodes, the lesser omentum, and the upper periaortic regions in most cases and is rarely found in the lower periaortic region. In general, the size of the lymph nodes is less than 4 cm in diameter. The CT scan shows the peripheral enhancement in TL. On rare occasions, the peripheral enhancement may, however, be seen in untreated lymphoma and lymphoma following radiotherapy. Two of our cases even had embedded vessels that could be seen on a CT scan, and our initial misinterpretation led to a diagnosis of neoplasm. Therefore, the distribution characteristics of the anatomic regions, morphological patterns including size, and enhancement features on the CT scans are all helpful in distinguishing TL from lymphoma, although the results may not be conclusive in some cases.

Another important goal in the development of CT imaging as a screening tool for abdominal nonhematogenous disseminated TL is to differentiate this disease from metastatic lymphadenopathy. Most of the metastases are easily diagnosed because of the presence of a primary tumor, and they generally show homogeneous enhancement on the contrast-enhanced CT scans. Some malignant adenopathies, especially testicular tumors, can result in abdominal metastatic lymphadenopathy with peripheral rim enhancement^[12]. Diagnosis aims to locate the primary tumors first. The anatomic distribution characteristics can sometimes provide clues to the differential diagnosis. For testicular tumors, the metastases may be located in the renal perihilar, paralumbar, and aortic bifurcation regions^[12]. Moreover, other infectious diseases, such as Whipple's disease, can also appear on contrast-enhanced CT scans and may look similar to TL. Although these diseases are

rare, they should be considered in the differential diagnosis.

Three patients received an initial diagnosis of pancreatic tumor following sonography and CT scans. The retrospective review of the CT scans found the clear boundary of lymphadenopathy presented on contrast-enhanced images and could be easily distinguished from the pancreas. This misdiagnosis was likely the result of unfamiliarity with identifying the features of non-malignant lymphadenopathies using CT imaging.

CT or ultrasound-guided needle aspiration cytology has been used to confirm diagnoses^[23,24]. These methods are quicker and less invasive than surgery; moreover, these methods can offer high diagnostic accuracy. In a study by Suri *et al.*^[25], ultrasound-guided needle aspiration cytology yielded a positive diagnosis in 78.6% of 14 cases of abdominal TL and a false negative result in only one patient. However, this technique is heavily operator dependent, particularly when not enough tissues are harvested and when the nodes are situated in close proximity to major blood vessels or important viscera, posing a risk to the patient. In cases where this technique is inconvenient or its result is ambiguous, diagnostic laparoscopy could be scheduled to collect sufficient tissues for histological and microbiological examinations, except in those patients with significant risk of perforation^[26,27].

In conclusion, the characteristics of anatomic distribution, morphological pattern, and enhancement features on contrast-enhanced CT scans will help to differentiate between abdominal nonhematogenous disseminated TL and lymph node neoplasms. Ultrasound-guided needle aspiration cytology performed by an experienced operator should be the first method of diagnosis, although diagnostic laparoscopy is a more reliable method for selected patients.

ACKNOWLEDGMENTS

The authors thank Jian-Sheng Wang for assisting with the manuscript.

COMMENTS

Background

Tuberculous lymphadenopathy (TL) is the most common manifestation of abdominal tuberculosis. The diagnosis of abdominal hematogenous disseminated TL can be made clinically in patients with active pulmonary miliary tuberculosis. Patients with abdominal nonhematogenous disseminated TL typically present with an isolated mass or a mass adhering to the surrounding organs, and these masses may easily be confused with neoplasm. With the widespread use of computed tomography (CT), physicians should be familiar with the features of CT images for these patients and be able to make differential diagnoses based upon such findings.

Research frontiers

CT is a useful method in the diagnosis and differential diagnosis of abdominal lesions. This study reports on CT findings from 12 patients with abdominal non-hematogenous disseminated TL.

Innovations and breakthroughs

Abdominal nonhematogenous disseminated TL still presents a diagnostic dilemma. The authors reviewed 12 patients with this condition and also reviewed the related literature to develop a diagnostic algorithm.

Applications

The recognition of relatively specific CT findings of abdominal nonhematogenous disseminated TL may help avoid misdiagnosis and unnecessary invasive procedures, allowing for the prompt consideration of antituberculosis therapy.

Peer review

The authors describe typical CT features of abdominal nonhematogenous disseminated tuberculous lymphadenopathy in 12 patients. The paper is well organised and informative.

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S- Editor Tian L L- Editor Ma JY E- Editor Xiong L

Collagen-based biological glue after Appleby operation for advanced gastric cancer

Gianluca Baiocchi, Nazario Portolani, Federico Gheza, Stefano M Giulini

Gianluca Baiocchi, Nazario Portolani, Federico Gheza, Stefano M Giulini, Department of Medical and Surgical Sciences, Surgical Clinic, Brescia University, 25127 Brescia, Italy
Author contributions: Baiocchi G and Portolani N clinically managed the patient; Giulini SM and Portolani N performed surgery; Baiocchi G and Gheza F wrote the manuscript. All authors approved the final version of the manuscript.

Correspondence to: Dr. Gian Luca Baiocchi, Department of Medical and Surgical Sciences, Surgical Clinic, Brescia University, 25127 Brescia, Italy. baiocchi@med.unibs.it

Telephone: +39-030-3995600 Fax: +39-030-3397476

Received: January 8, 2010 Revised: February 10, 2011

Accepted: February 17, 2011

Published online: September 21, 2011

Abstract

Pancreatic fistula is a common complication of distal pancreatectomy; although various surgical procedures have been proposed, no clear advantage is evident for a single technique. We herein report the case of a 38-year-old patient affected by an advanced gastric carcinoma infiltrating the pancreas body, with extensive nodal metastases involving the celiac trunk, who underwent total gastrectomy with lymphadenectomy, distal pancreatectomy and resection *en bloc* of the celiac trunk (Appleby operation). At the end of the demolitive phase, the pancreatic stump and the aorta at the level of the celiac ligature were covered with a layer of Tachosil[®], a horse collagen sponge made with human coagulation factors (fibrinogen and thrombin). Presenting this case, we wish to highlight the possible sealing effect of this product and hypothesize a role in preventing pancreatic fistula and postoperative lymphorrhagia from extensive nodal dissection.

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Key words: Gastric cancer; Pancreatic fistula; Vascular

infiltration; Biologic sealant

Peer reviewer: De-Liang Fu, MD, PhD, Professor, Department of Surgery, Pancreatic Disease Institute, Fudan University, 12 Wulumqi Road (M), Shanghai 200040, China

Baiocchi G, Portolani N, Gheza F, Giulini SM. Collagen-based biological glue after Appleby operation for advanced gastric cancer. *World J Gastroenterol* 2011; 17(35): 4044-4047 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/4044.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.4044>

INTRODUCTION

Even though several technical solutions have been proposed, pancreatic fistula remains a frequent complication after distal pancreatectomy, ranging in incidence from 15% to 40%^[1,2]. Almost all serious postoperative sequelae can be ascribed to this complication, and hospitalization times are significantly longer. In the cases in which distal pancreatic resection is only a part of a more complicated, usually oncological, surgical operation, the occurrence of a fistula containing large amounts of proteolytic enzymes can endanger the integrity of visceral sutures and lead to the contamination of prosthetic material, if present. The performance of extensive lymphadenectomy represents an additional risk factor for possible infection of fluids coming from lymphorrhagia. In these cases, achieving a completely dry pancreatic stump and an optimal lymphostasis is a very important goal. No clear evidence is given in the literature with regard to both aspects: there is no indication of a technique or a method guaranteeing a higher level of success.

We would like to report a clinical case during which we successfully experimented with a collagen-based biological glue which had originally been proposed as a hemostatic means. We applied it as a sealer on the pancre-

atic stump after distal resection and on the aorta after a periaortic lymphadenectomy removing the celiac trunk in a young patient affected by advanced gastric carcinoma.

CASE REPORT

A 38-year-old male patient was affected by an averagely differentiated intestinal adenocarcinoma of the esophago-gastric junction recently complicated by a digestive hemorrhage. Multiphase computed tomography (CT) scan showed that the neoplasm had extended to the pancreatic body with an extensive lymph nodal involvement along the left gastric artery which was completely encompassed as far as the celiac trunk. The contact between the celiac trunk and the larger node (3.5 cm) corresponded to 180 degrees (Figure 1). Neoadjuvant chemotherapy with epirubicin, cisplatin, fluorouracil (ECF) cycles was undergone according to the formerly validated protocol^[3]. The restaging CT scan showed a stability of the radiological picture, with no evident regression of the adenopathies along the small curvature and the celiac trunk. We decided therefore to proceed with a demolition surgery. The surgical exploration revealed neither peritoneal carcinosis nor hepatic metastases and confirmed the extensive nodal involvement of the proper hepatic artery and the celiac tripod, the origin of which from the aorta was free (Figure 2). We performed a temporary clamping test of the common hepatic artery and kept the hepatic arterial perfusion under control by inverting the flow of the gastroduodenal artery. We then proceeded to a total gastrectomy and to a distal spleno-pancreatectomy (the pancreas was sectioned by green stitches and a 60 mm GIA stapler), to left adrenalectomy and to *en bloc* removal of perigastric nodes and of the celiac artery (Appleby operation)^[4]. The intervention was then completed by performing a lymphadenectomy of the hepatic pedicle, of the rear-pancreatic and of the interaorto-caval basins. Gastroenteric continuity was restored by mechanical termino-lateral esophago-digiunostomy using a defunctionalized Roux-en-Y loop. The pancreatic stump was then covered with a layer of Tachosil®; a further layer of the same material was then applied to the aorta at the level of the celiac ligature (Figure 3).

The postoperative course was uneventful and the patient left the hospital on the 15th day after surgery. Daily monitoring of the hepatic function did not reveal functional insufficiencies and the increase in cytolysis values was low (AST 92 U/dL, ALT 89 U/dL; normal values < 50 U/dL) and transitory. The outcome of the 3 abdominal drains was always lower than 50 mL/d; the enzymatic dosage in the drained liquid showed a pathological value only on the 1st postoperative day (1500 U/dL; normal value < 286 U/dL), which, according to recent publications^[5], does not indicate the presence of pancreatic fistula. It then dropped to normal values (< 35 U/dL starting from the 4th day). Serum lipases were always normal.

The final pathological report indicated an intestinal type gastric adenocarcinoma of the cardias, infiltrating the whole thickness of the wall and extending to the peri-



Figure 1 Contrast-enhanced computed tomography scan. A cardia tumor is evident (A), associated with multiple enlarged nodes infiltrating the celiac trunk origin (B) and the pancreatic body (C).



Figure 2 Intraoperative findings. Celiac trunk origin from the aorta is free from tumor infiltration.

gastric adipose tissue, directly infiltrating the pancreatic body, the hepatic artery and the left adrenal gland. Lymph node metastases were present in 19 out of 51 removed

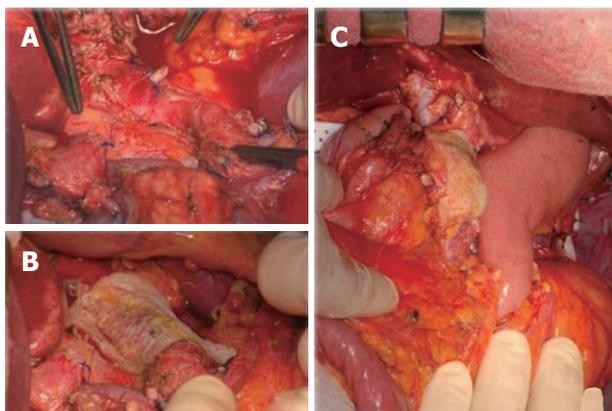


Figure 3 Intraoperative findings after the demolitive phase. A: From the left, the distal hepatic artery stump, the celiac stump and the superior mesenteric artery; B, C: Tachosil® is then applied over the aorta (B) and over the pancreas stump (C).

nodes (pT4N3M0, stage IV). The patient therefore underwent 3 further adjuvant chemotherapy cycles using EFC. After 17 mo the patient developed multiple hepatic metastases treated with a further chemotherapy cycle and died 23 mo after surgery.

DISCUSSION

Distal pancreatectomy involves a higher risk of pancreatic fistulas than duodeno-pancreatectomy. Even though these are “pure” pancreatic fistulas, subsequent complications have a significant effect on the patient postoperative course. It is commonly believed that, along with the clear role played by the structure and consistency of the pancreas, the main risk factors for fistula are the technique used for sectioning the pancreas neck and the treatment of the residual stump. Up to the present, no standardized procedure has been defined^[6]. Several technical proposals have been presented, often employed in an insufficient number of cases; furthermore, patient selection bias and a non-homogeneous definition of the primary end-point (clinical or biochemical pancreatic fistula with different cut-offs in terms of enzymatic values on different postoperative days)^[7] are quite evident. Also, the potentially influential effect of the experience of the surgeon, which, in itself, seems to produce great divergence in results even when the same technique is being used, must be taken into consideration.

The reported clinical case presents some interesting elements; the neoplasm affected the stomach and involved the pancreas as a direct infiltration, with an extensive lymph node involvement mainly along the vascular axes. The clear infiltration of the proper hepatic artery, of the celiac trunk and of the left adrenal gland made an ample periaortic dissection, including the celiac nodes, necessary. The presence of two sutures at risk, the duodenal stump and the origin of the celiac trunk which had been removed *en bloc*, made it particularly important to find a treatment which would reduce the risk

of complications to a minimum. The pancreatic body was sectioned with a mechanical stapler. From a recent review, it seems that this technique presents a lower risk of failure, even though no statistical significance levels have been reached. However, even using this treatment, the fistula rate is higher than 20%^[8]. Among the elements to explain this non-satisfactory result we can consider the compression exerted by the stapler, which can lacerate the parenchyma, especially if it is fragile, and the fact that the Wirsung duct is not identified and individually secured. In the case we are describing, the choice of this method, which we do not routinely use, was justified by the reduced thickness of the pancreas at the point chosen for sectioning. Thus, we deviated from the procedure we generally use for the treatment of distal tumors; specifically, the sectioning of the pancreas with a cutter, the application of 5/0 monofilament fine hemostatic stitches and the meticulous search for the Wirsung duct. This latter phase is particularly important to reduce the risk of fistula; it requires careful preparation of the duct and subsequently the application of a double loop suture or an anchoring stitch before sectioning.

If, as in the described case, a selective treatment of the Wirsung is not possible, we believe that it is useful to perfect the treatment of the residual stump using Tachosil®, a horse collagen sponge on which human coagulation factors (fibrinogen and thrombin) are applied. From a first analysis of the components, it transpires that this product combines the hemostatic characteristics of biological glue with the adhesive/reparatory properties of collagen. On the basis of these characteristics, the product was initially used for hemostasis and, as a logical consequence, it is now being used more and more in the field of hepatic resection surgery, in emergency trauma surgery and in every situation in which one wishes to perfect a hemostasis “which is already sufficiently completed”^[9,10]. However, in the application we are presenting, we wish to highlight the sealing effect of this product resulting from the rapid adhesion of collagen, helped by means of the rapidly activated coagulation process on the pancreatic stump. This consolidates the obliteration of the excretory duct and reduces to a minimum the effect of small, accessory pancreatic ducts left open. The sealing effect is practically immediate; moderate pressure, which we recommend applying for some minutes, mainly aims at letting the product adhere. After wetting it, the product can be modeled onto the section surface, including its margins and also the ventral and dorsal surface, at a depth of at least one centimeter. Although the product does not require a completely dry surface for the best adhesion to the section surface, it is recommended to have a moderately anfractuous plane. The absorption of the product takes a few weeks and therefore the product cannot favor septic complications in the case of fistula.

The present report simply suggests a possible application of Tachosil®; there is no direct evidence of its ability to prevent the pancreatic fistula, so more cases of distal pancreatectomy should be collected, as it is actually ongoing in a multicenter, randomized, prospective Italian trial.

This sealing effect of Tachosil® was also used to reduce the risk of lymphorrhagia after the extensive lymphadenectomy performed in this case because of the metastatic involvement of lymph nodes in the celiac area, as far as the area adjacent to the aorta. In a case such as this, lengthy draining is commonly used to successfully treat lymphatic losses if the quantity exceeds 1 liter a day, if they persist for a few days and if they show a further increase as soon as free feeding is re-introduced. The application of Tachosil® to refreshed areas at higher risk can contribute to sealing open lymphatic channels and to facilitate the healing process, thus making the early removal of drainage possible and reducing the risk of bacterial contamination. This indication has also not been verified in controlled trials and it represents at this time a simple hypothesis for further studies.

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S- Editor Tian L L- Editor Logan S E- Editor Xiong L

Esophageal mucosal lesion with low-dose aspirin and prasugrel mimics malignancy: A case report

Gui-Fen Ma, Hong Gao, Shi-Yao Chen

Gui-Fen Ma, Hong Gao, Shi-Yao Chen, Department of Gastroenterology, Zhongshan Hospital Fudan University, Shanghai 200032, China

Author contributions: Ma GF collected the data and wrote the manuscript; Gao H was responsible for the patient care and revised the manuscript; Chen SY designed and organized the research and performed endoscopy.

Correspondence to: Shi-Yao Chen, MD, Professor, Department of Gastroenterology, Zhongshan Hospital Fudan University, Shanghai 200032, China. syaochen@163.com

Telephone: +86-21-64041990 Fax: +86-21-64432583

Received: January 26, 2011 Revised: May 19, 2011

Accepted: May 26, 2011

Published online: September 21, 2011

Abstract

Dual antiplatelet therapy consisting of low-dose aspirin (LDA) and other antiplatelet medications is recommended in patients with coronary heart disease, but it may increase the risk of esophageal lesion and bleeding. We describe a case of esophageal mucosal lesion that was difficult to distinguish from malignancy in a patient with a history of ingesting LDA and prasugrel after implantation of a drug-eluting stent. Multiple auxiliary examinations were performed to make a definite diagnosis. The patient recovered completely after concomitant acid-suppressive therapy. Based on these findings, we strongly argue for the evaluation of the risk of gastrointestinal mucosal injury and hemorrhage if LDA therapy is required, and we stress the paramount importance of using drug combinations in individual patients.

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Key words: Esophageal injury; Low-dose aspirin; Prasugrel; Gastrointestinal hemorrhage; Drug-eluting stent

Peer reviewers: Bronislaw L Slomiany, PhD, Professor, Re-

search Center, C-875, UMDNJ-NJ Dental School, 110 Bergen Street, PO Box 1709, Newark, NJ 07103-2400, United States; Dr. Ping-Chang Yang, MD, PhD, Department of Pathology and Molecular Medicine, McMaster University, BBI-T3330, 50 Charlton Ave East, Hamilton, L8N 4A6, Canada

Ma GF, Gao H, Chen SY. Esophageal mucosal lesion with low-dose aspirin and prasugrel mimics malignancy: A case report. *World J Gastroenterol* 2011; 17(35): 4048-4051 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/4048.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.4048>

INTRODUCTION

Low-dose aspirin (LDA) is used widely as an antiplatelet therapy for the prevention of cardiovascular and cerebrovascular events^[1]. Dual antiplatelet therapy is more effective than aspirin alone^[2]. However, the risk of severe bleeding, including life-threatening hemorrhaging, increases with the use of aspirin therapy^[3]. The most vulnerable organs are the stomach and duodenum, although recent reports indicate an increased risk of esophageal lesions with LDA use^[4,5]. The US Preventive Services Task Force recommends the use of aspirin for men aged 45 to 79 years when the potential benefit due to a reduction in myocardial infarction outweighs the potential harm due to an increase in gastrointestinal hemorrhage^[6].

Prasugrel, a third-generation thienopyridine, specifically inhibits platelet clotting by binding the adenosine diphosphate receptor. Prasugrel is approved by the Food and Drug Administration (FDA) for acute coronary syndrome (ACS) in patients undergoing percutaneous coronary intervention (PCI). Its recommended loading dose is 60 mg x1, followed by a maintenance dose (10 mg once daily), and combined with concomitant aspirin (81-325 mg, once daily)^[7]. Compared to clopidogrel, prasugrel has been shown to reduce thrombotic cardiovascular events significantly, including stent thrombosis, in patients with ACS who are managed with PCI. However, prasugrel

can cause significant and fatal bleeding, and it has a FDA black-box warning regarding bleeding risk^[7]. Thus, patient selection is important.

Here, we present a case with esophageal lesion mimicking malignancy related to aspirin and prasugrel treatment after drug-eluting stent (DES) implantation.

CASE REPORT

A 58-year-old male was admitted in August 2010 because of melena and hematemesis. About 1.5 mo before admission, the patient underwent PCI and received a DES due to chest pain. Low-dose aspirin (100 mg, once per day) and prasugrel (Effient, 10 mg, once per day) were administered.

One month later, the patient felt epigastric discomfort accompanied by severe chest pain. Coronary arteriography was repeated without positive findings. Gastroendoscopy was then performed, which showed edematous esophageal mucosa with blood clots, a column of violaceous and nontortuous lesions with minimal whitish exudates that occupied more than half of the lumen from the 26 cm to the 42 cm level (Figure 1A). Esophageal candidiasis was suspected. Biopsy indicated that there were necrotic tissues with partly degenerated atypical fusiform to epithelioid cells of undetermined significance.

At 7 d before admission, the patient passed a dark pasty stool of about 250 g after taking some cathartics. Three days later, gastroendoscopy was performed in Shanghai Cancer Center, which revealed a slim ulcer from the 26 cm down to the 40 cm level. Pathology suggested inflammation and granuloma gangraenescens. Chest contrast computed tomography demonstrated thickening of the middle-lower segment of the esophageal wall and a few of the small lymph nodes at the mediastinum (Figure 1B and C) with some pleural effusion. An upper gastrointestinal series (barium X-ray) showed an irregular esophageal wall with stiff movement, and the possibility of malignant tumor could not be excluded (Figure 1D).

One day after the examination, the patient felt nauseous and vomited dark blood clots. He was sent urgently to our emergency room. After treatment with proton pump inhibitors (PPIs), he had no signs of active bleeding. He was transferred to the gastroenterology department for further diagnosis. Another gastroendoscopy was performed after intravenous PPI therapy for 1 wk. Endoscopy by white light imaging (WLI) and narrow band imaging (NBI) showed that the esophageal mucosa was peeled off (Figure 1E and F). Endoscopic ultrasonography suggested that the layers of the esophagus were clear and identifiable; the lesion took on a minimal higher echo (Figure 1G). Tumor markers were normal. Antiplatelet drugs were discontinued after admission, and symptoms improved within 7 d. Clopidogrel and oral PPI were administered afterwards. He was discharged on the 7th day. Two months later, endoscopy showed that the esophageal lesion had completely healed.

DISCUSSION

It was very difficult to identify the esophageal lesion in this patient. After the first gastroendoscopy showed edematous mucosa with blood clots, it was risky to perform a biopsy and difficult to recognize the lesion. It seemed to be a malignant tumor on the basis of the previous auxiliary examinations. Moreover, the accuracy of the biopsy was affected by the focal position and depth; such a situation could easily mislead medical providers.

If discrete erosions had occurred in areas away from the Z line, then drug-induced injury would have been strongly considered^[8]. The occurrence of esophageal mucosal injury by LDA is related closely to the intragastric pH value, especially at night^[9]. A multicenter randomized controlled trial documented that use of the NBI mode could improve the diagnostic accuracy of endoscopy, compared to the WLI mode^[10]. When the auxiliary examination is not consistent with clinical symptoms, further diagnostic modalities are required. If all of the tests are inconclusive, then repeat examinations are needed to confirm or rule out the prior diagnosis after diagnostic treatment. The appropriate use of diverse and repeatable examinations to improve the accuracy of an uncertain disease is of great value.

Esophageal cancer develops rapidly and has a fatal prognosis. It has displayed an increasing incidence in the past decade, with the highest incidence in the age group of 50-70 years. The disease is diagnosed more frequently in males than in females, with an approximate ratio of 3-5^[11]. Whether aspirin has implications for cancer prevention is debatable. Kawai *et al*^[5] investigated 101 consecutive outpatients with cardiovascular diseases who had been on LDA therapy. They found that the incidence of esophageal lesions was 8.9%, whereas the rate of esophageal and gastric cancers was up to 5.9%. Abnet *et al*^[12] found no correlation between the self-reported use of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) and esophageal adenocarcinomas in a prospective cohort followed for 7 years. However, their meta-analysis manifested that aspirin use was inversely associated with esophageal adenocarcinomas, with a summary odds ratios for esophageal adenocarcinomas of 0.64. Considering these previous findings, we speculate that regular endoscopic surveillance might benefit patients on dual antiplatelet therapy.

Upper gastrointestinal hemorrhage is an urgent life-threatening event that is commonly incited by a peptic ulcer or varices. However, evidence indicates that aspirin or other NSAIDs are another potential cause, especially in elderly patients with cardiac or cerebral events. A meta-analysis of 24 randomized controlled trials documented gastrointestinal hemorrhage in 2.47% of patients taking aspirin, compared with 1.42% taking placebo [Odds ratio (OR): 1.68], and no relationship was observed between gastrointestinal hemorrhage and dose^[13]. Among 674 patients with upper gastrointestinal bleeds, the odds ratio for the presence of erosive esophagitis in aspirin users

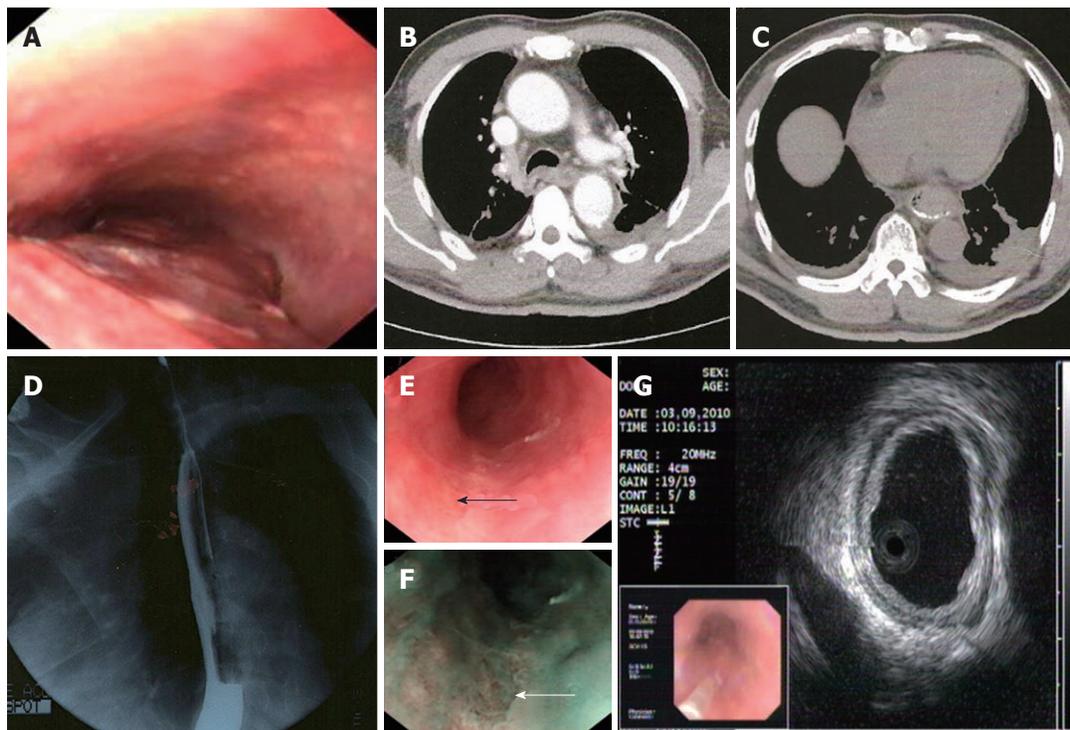


Figure 1 Auxiliary examination. A: Gastroendoscopy showing edematous mucosa with blood clots; B and C: Obstructive esophageal lumen and lymph nodes with some pleural effusion shown by chest contrast computed tomography; D: Barium X-ray displaying irregular esophageal wall; E and F: The mucosal lesion evaluated by white light imaging (black arrow) or narrow band imaging (white arrow) mode of endoscopy; G: Endoscopic ultrasonography showing focal minimal higher echo.

was 2 [95% confidence interval (CI): 1-3], and 3 (95% CI: 2-5) in patients taking other antithrombotic agents, including warfarin, clopidogrel, and dipyridamole. In 41 patients with esophagitis who were taking these drugs, 36 (88%) had cardiovascular disease and only 4 (10%) had peptic symptoms^[14]. These findings suggest that the gastrointestinal risk should be determined, even if LDA is required.

The appropriate use of aspirin is complicated. It is difficult to determine how to balance the prevention of stent thrombosis with the potential injury of the gastrointestinal mucosa. The use of enteric-coated aspirin decreases mucosal damage in the short term, but does not decrease the risk of hemorrhagic gastrointestinal events compared with noncoated aspirin^[15]. Yeomans *et al*^[16] investigated 991 patients who were randomized to take esomeprazole (20 mg, once per day) or placebo for 26 wk. They found that the cumulative proportion of patients with erosive esophagitis was significantly lower for esomeprazole *vs* placebo (4.4% *vs* 18.3%). Esomeprazole-treated patients were more likely to experience resolution of heartburn, acid regurgitation, and epigastric pain.

On the other hand, insufficient antithrombotic drugs might cause stent thrombosis. In November 2009, the FDA warned that the effectiveness of clopidogrel was reduced when clopidogrel and omeprazole were taken together. Warner *et al*^[17] reported that the use of aspirin in the presence of prasugrel could increase the clinical risk of residual ischemic events and bleeding complications. Whether dual antiplatelet therapy should be prescribed routinely to high-risk patients requires a complete un-

derstanding of its long-term consequences. In particular, for an individual patient, this decision must consider the trade-off between the potential benefits of preventing ischemic events weighed against the risk of bleeding complications.

Drug combinations must be prudently selected for individual patients. Compared with the second-generation clopidogrel (Plavix), prasugrel is more potent, faster in onset, and more consistent in inhibiting platelets, but bleeding complications are more frequent^[18]. From the aspect of clinical and cost effectiveness, prasugrel in combination with aspirin could be an option for preventing atherothrombotic events in people having ACS with PCI only when immediate primary PCI for ST-segment-elevation myocardial infarction is necessary, stent thrombosis has occurred during clopidogrel treatment, or in cases of diabetes mellitus. Prasugrel should be used with caution in patients at an increased risk of bleeding, especially in patients who are ≥ 75 years old with a tendency to bleed or with body weight of < 60 kg^[19]. A guideline has recommended that clopidogrel should be continued for at least 12 mo for patients who receive a DES^[7,19]. A randomized controlled trial found no apparent cardiovascular interaction between clopidogrel and omeprazole, and prophylactic use of a PPI reduced the rate of upper gastrointestinal bleeding^[20]. Thus, for such patients with a high risk of bleeding, clopidogrel plus PPI might be a better recommendation to decrease the risk of gastrointestinal bleeding.

In conclusion, this case report of a patient developing an esophageal mucosal lesion mimicking malignancy

while under LDA and prasugrel treatment highlights the need to evaluate the gastrointestinal risk if LDA therapy is required. The findings also stress the importance of the rational selection of drug combinations in individual patients.

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S- Editor Sun H L- Editor O'Neill M E- Editor Zhang DN

Hypergastrinemia and recurrent type 1 gastric carcinoid in a young Indian male: Necessity for antrectomy?

Viplove Senadhi, Niraj Jani

Viplove Senadhi, Division of Gastroenterology and Hepatology, Indiana Institute for Personalized Medicine, Indiana University School of Medicine, Indianapolis, IN 46202, United States
Niraj Jani, Johns Hopkins University/Sinai Hospital and the Greater Baltimore Medical Center, Chief of Division of Gastroenterology, Baltimore, MD 21204, United States

Author Contributions: Senadhi V wrote, revised, and gathered all the data for the manuscript; Senadhi V corresponded with the editors and incorporated the revisions; Jani N reviewed and modified the manuscript; Jani N was the mentor author on the manuscript. Both authors reviewed and approved the final version of the manuscript.

Correspondence to: Dr. Senadhi V, Division of Gastroenterology and Hepatology, Indiana University, 1050 Wishard Blvd, Suite 4100, Indianapolis, IN 46202, United States. vsenadhi@hotmail.com

Telephone: +1-317-9480414 Fax: +1-678-6235999

Received: November 15, 2010 Revised: May 21, 2011

Accepted: May 28, 2011

Published online: September 21, 2011

Abstract

Carcinoid tumors are the most common neuroendocrine tumors. Gastric carcinoids represent 2% of all carcinoids and 1% of all gastric masses. Due to the widespread use of Esophagogastroduodenoscopy for evaluating a variety of upper gastrointestinal symptoms, the detection of early gastric carcinoids has increased. We highlight an alternative management of a young patient with recurrent type 1 gastric carcinoids with greater than 5 lesions, as well as lesions intermittently greater than 1 cm. Gastric carcinoids have a variable presentation and clinical course that is highly dependent on type. Type 1 gastric carcinoids are usually indolent and have a metastasis rate of less than 2%, even with tumors larger than 2 cm. There are a number of experts as well as organizations that recommend endoscopic resection for all type 1 gastric carcinoid lesions less than 1 cm, with a follow-up every 6-12 mo. They also recommend antrectomy for type 1 gastric carcinoids with greater than 5 lesions, lesions 1 cm or greater, or

refractory anemia. However, the American Society of Gastrointestinal Endoscopy guidelines state that type 1 gastric carcinoid surveillance is controversial based on the evidence and could not make an evidence-based position statement on the best treatment modality. Our report illustrates a rare cause of iron deficiency anemia in a young male (without any medical history) due to multiple recurrent gastric carcinoid type 1 lesions in the setting of atrophic gastritis causing hypergastrinemia, and in the absence of a vitamin B12 deficiency. Gastric carcinoid type 1 can present in young males without an autoimmune history, despite the known predilection for women aged 50 to 70 years. Type 1 gastric carcinoids can be managed by endoscopic resection in patients with greater than 5 lesions, even with lesions larger than 1 cm. This course of treatment enabled the avoidance of early antrectomy in our patient, who expressed a preference against more invasive measures at his young age.

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Key words: Gastric carcinoid; Antrectomy; Endoscopic resection; Hypergastrinemia; Iron deficiency anemia

Peer reviewer: Dr. Edward J Ciaccio, Department of Medicine, Columbia University, 180 Fort Washington Avenue, HP804, NY, 10032, United States

Senadhi V, Jani N. Hypergastrinemia and recurrent type 1 gastric carcinoid in a young Indian male: Necessity for antrectomy? *World J Gastroenterol* 2011; 17(35): 4052-4054 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i35/4052.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i35.4052>

TO THE EDITOR

It is with great interest that we read the experiences of Kadikoylu and colleagues in the management of a solitary

Table 1 Differential diagnosis of hypergastrinemia

Elevated antral pH	Gastrinoma
Chronic atrophic gastritis-type A	++++ (> 1000)
Pernicious anemia	++++ (> 1000)
Other immune dz (RA, vitiligo, SS, DM)	+ (150-250)
Chronic atrophic gastritis-type B (<i>H. Pylori</i>), gastric cancer	++ (250-450)
Renal insufficiency/high protein diet	+ (150-250)
Massive small bowel resection	+ or ++
G cell hyperplasia/pyloric outlet obstruction	+ or ++
Calcium, caffeine, insulin, catecholamines	+ (150-250)
H2 blocker/PPI's	+ (H2) ++ (PPI)
Truncal vagotomy/retained antrum s/p surgery	+

Gastrin level in pg/mL: + equals 150-250 pg/mL; ++ equals 250-450 pg/mL; +++ equals 450-1000 pg/mL; ++++ equals > 1000 pg/mL. dz: Diseases; RA: Rheumatoid arthritis; SS: Sjogren's syndrome; DM: Diabetes mellitus type 1; H2: Histamine H2 receptor blockers; PPI's: Proton pump inhibitors.

Table 2 Gastric carcinoid types and differentiating characteristics

	Type 1	Type 2	Type 3
% of gastric carcinoids	70%-80% - most common	Less than 5%	15%-20%
Association	Chronic atrophic gastritis	Gastrinomas (Zollinger-Ellison)	Sporadic carcinoid syndrome
Epidemiology	Typically women 50-70 yrs old	Family hx of MEN type 1 syndrome	Increased in African Americans
Presentation	Asymptomatic or anemia	Peptic ulcer disease	Hepatic mets or carcinoid syndrome
Rate of metastasis over a lifetime	< 2% even if larger than 2 mm	2%-4%	65% metastatic at presentation
Treatment	Observation <i>vs</i> endoscopic resection <i>vs</i> antrectomy	Endoscopic resection <i>vs</i> antrectomy <i>vs</i> octreotide <i>vs</i> gastrectomy	Partial or total gastrectomy with lymph node dissection <i>vs</i> chemotherapy

hx: History; MEN: Multiple endocrine neoplasia.

gastric carcinoid^[1]. Carcinoid tumors are the most common neuroendocrine tumors^[2] and gastric carcinoids represent 2% of all carcinoids and 1% of all gastric masses^[1]. Due to the widespread use of Esophagogastroduodenoscopy (EGD) to evaluate a variety of upper gastrointestinal symptoms, the detection of early gastric carcinoids has increased. We highlight an alternative management of a young patient with recurrent type 1 gastric carcinoids with greater than 5 lesions as well as lesions intermittently greater than 1 cm.

A 28-year-old Indian male with no significant medical history presented with fatigue. He was found to have severe iron deficiency anemia (hemoglobin of 68 gm/L) with a mean corpuscular volume of 77 fL, and an iron level of 370 mcg/L. Endoscopic evaluation for anemia revealed nine sessile polyps in the body and fundus of the stomach ranging from 5 mm to 9 mm, which were all resected. An Endoscopic Ultrasound showed the lesions to be within the mucosa and there was no evidence of gastrinoma or metastatic disease to the liver or pancreas. The serum gastrin level was 1534 ng/L and other causes of hypergastrinemia were considered (Table 1)^[3-6]. Histopathological examination of the polyps confirmed carcinoid tumors with positive synaptophysin and chromogranin. The body of the stomach revealed autoimmune atrophic gastritis without oxyntic mucosa, helicobacter pylori, or evidence of parietal cell hyperplasia. Capsule endoscopy and colonoscopy did not reveal any other sources of blood loss or further carcinoid tumors. Octreotide scans, vitamin B12 levels, as well as Computed Tomography

scans of the thorax, abdomen, and pelvis were normal. Surveillance EGD 6 mo later showed recurrence with 5 polyps, with the largest measuring 1.1 cm, which was resected. Since resection, the patient has experienced a resolution of his anemia along with normal gastrin levels. The patient has not had more than 5 lesions or a lesion greater than 1 cm for over two years.

Gastric carcinoids have a variable presentation and clinical course that is highly dependent on type (Table 2)^[7]. Type 1 gastric carcinoids are usually indolent and have a metastasis rate of less than 2%, even with tumors larger than 2 cm^[8]. Kadikoylu *et al*^[1] recommend endoscopic resection for all type 1 gastric carcinoid lesions less than 1 cm with follow-up every 6-12 mo and antrectomy for type 1 gastric carcinoids with greater than 5 lesions, lesions 1 cm or greater, or refractory anemia. However, the American Society of Gastrointestinal Endoscopy guidelines state that type 1 gastric carcinoid surveillance is controversial based on the evidence and could not make an evidence-based position statement on the best treatment modality^[9].

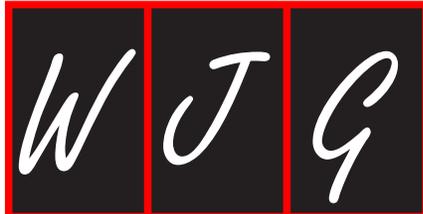
This report illustrates a rare cause of iron deficiency anemia in a young male (without any medical history) due to multiple recurrent gastric carcinoid type 1 lesions in the setting of atrophic gastritis causing hypergastrinemia and in the absence of a vitamin B12 deficiency. Gastric carcinoid type 1 can present in young males without an autoimmune history, despite the known predilection for women aged 50 to 70 years. Type 1 gastric carcinoids can be managed by endoscopic resection in patients with

greater than 5 lesions, even with lesions larger than 1 cm. This course of treatment enabled the avoidance of early antrectomy in our patient, who expressed a preference against more invasive measures at his young age.

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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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Vito Annese, MD, Associate Professor, Department of Surgical and Medical Sciences, Unit of Gastroenterology, University Hospital Careggi, Pad. S. Luca Nuovo 16c, Largo Brambilla, 3, 50134 Florence, Italy

Jan Bures, MD, PhD, Professor, University Department of Gastroenterology, Charles University, Faculty of Medicine, University Teaching Hospital, Sokolska 581, 50005 Hradec Kralove, Czech Republic

Mark J Czaja, MD, Liver Research Center, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx, NY 10461, United States

Dr. Devinder Kumar Dhawan, Professor, Department of Biophysics & Coordinator, Nuclear Medicine, Panjab University, Chandigarh 160014, India

Piers Gatenby, MA, MD, MRCS, Department of Surgery, Royal Free and University College Medical School, London NW3 2PF, United Kingdom

Dr. Vui Heng Chong, Gastroenterology and Hepatology Unit, Department of Medicine, Raja Isteri Pengiran Anak Saleha Hospital, Bandar Seri Begawan BA 1710, Brunei Darussalam

Jae J Kim, MD, PhD, Associate Professor, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

Dr. Sang Geon Kim, PhD, MS, BS, Professor, Chairman, College of Pharmacy, Seoul National University, Sillim-dong, Kwanak-gu, Seoul 151-742, South Korea

Takumi Kawaguchi, MD, PhD, Department of Digestive Disease Information & Research, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan

Dr. Kemal Kismet, MD, 4th General Surgery Department, Ankara Training and Research Hospital, Ankara 06430, Turkey

John K Marshall, MD, Associate Professor of Medicine, Division of Gastroenterology (4W8), McMaster University Medical Centre, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada

Giuseppe Montalto, Professor, Clinical Medicine and Emerging Diseases, University of Palermo, *via* del Vespro, 141, Palermo 90100, Italy

Paola De Nardi, MD, Department of Surgery, Scientific Institute San Raffaele Hospital, *via* Olgettina 60, Milan 20132, Italy

Dr. Matthias Ocker, MD, Professor, Department of Medicine 1, University Hospital Erlangen, Ulmenweg 18, 91054 Erlangen, Germany

Dr. Rene Schmidt, PhD, Department of Anesthesiology, Freiburg University Medical Center, Hugstetter Strasse 55, Freiburg 79106, Germany

Bruno Stieger, Professor, Department of Medicine, Division of Clinical Pharmacology and Toxicology, University Hospital, Zurich 8091, Switzerland

Gabor Veres, MD, Assistant Professor, First Department of Pediatrics, Semmelweis Medical University, Bókay street. 53, Budapest 1083, Hungary

Frank Zerbib, MD, PhD, Professor, Department of Gastroenterology, Hopital Saint Andre, CHU de Bordeaux, 1 rue Jean Burguet, 33075 Bordeaux Cedex, France

Events Calendar 2011

January 14-15, 2011

AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011

Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011

Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011

9. Gastro Forum München, Munich, Germany

February 4-5, 2011

13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011

Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011

APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver Bangkok, Thailand

February 22, 2011-March 04, 2011

Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011

Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011

2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011

International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011

Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011

Childhood & Adolescent Obesity:

A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011

42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011

Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011

British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011

41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011

Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011

UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011

MedicRes IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011

26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011

IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011

International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011

Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011

Pediatric Emergency Medicine: Detection, Diagnosis and Developing

Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011

9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011

The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011

Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011

4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011

Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011

2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011

1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011

22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venice, Italy

May 25-28, 2011

4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011

The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011

Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011

International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011

ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011

XI Congreso Interamericano de 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

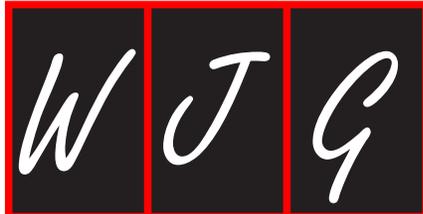
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

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



GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

World Journal of Gastroenterology

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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 Identifier, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 Gastroenterology and Hepatology).

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There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

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Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

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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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World Journal of Gastroenterology®

Volume 17 Number 36
September 28, 2011



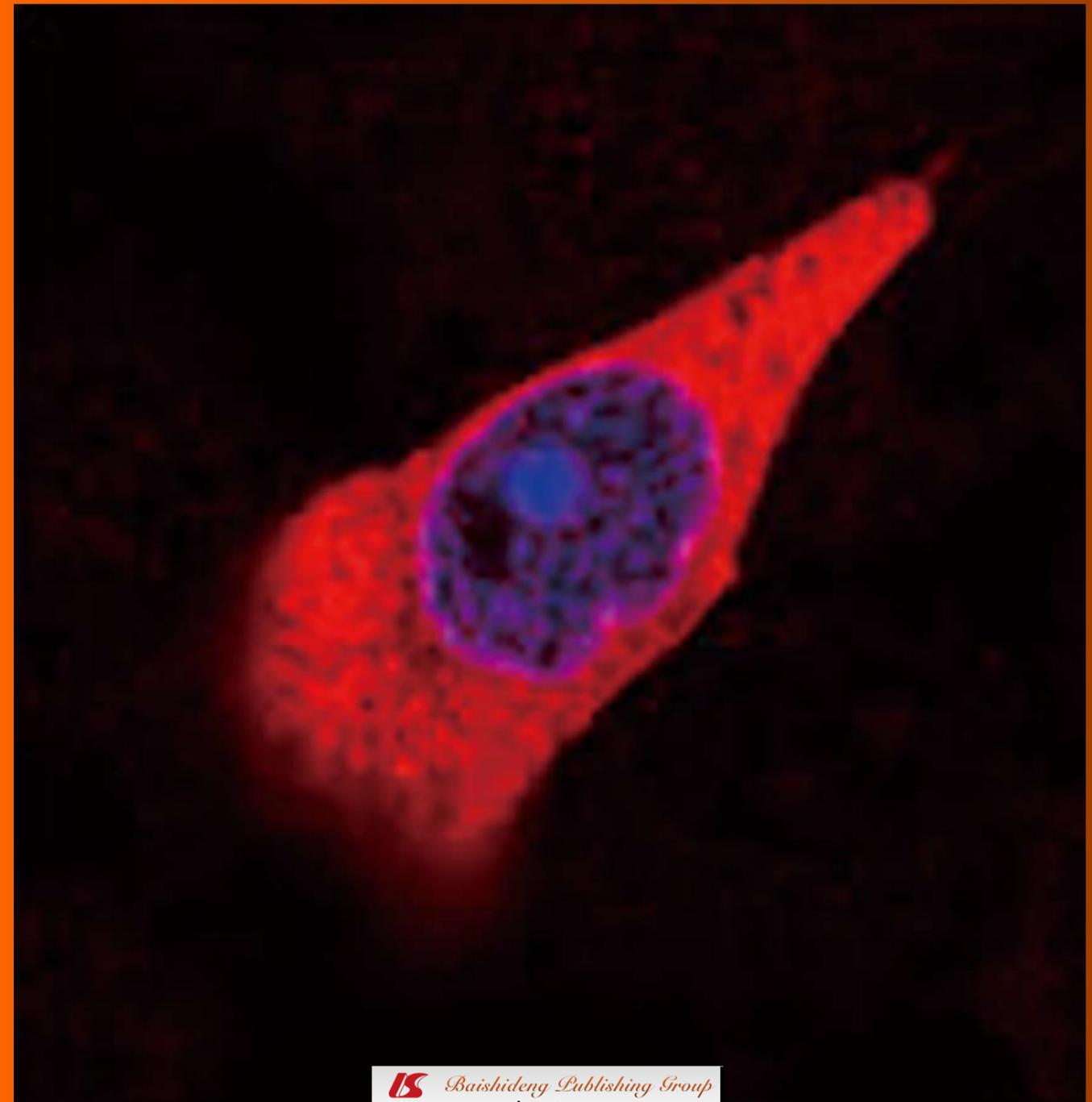
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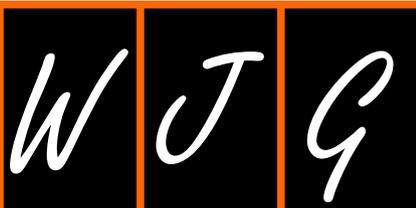
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ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

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<http://www.wjgnet.com/1007-9327/full/v17/i36/4090.htm>

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

PUBLICATION DATE
September 28, 2011

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

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Pathophysiology of insulin resistance and steatosis in patients with chronic viral hepatitis

Metin Basaranoglu, Gökçen Basaranoglu

Metin Basaranoglu, Division of Gastroenterology and Hepatology, Teaching and Consulting, Ankara Yüksek İhtisas Hospital Gastroenterology Clinic, Sıhhiye, Ankara 06100, Turkey
Gökçen Basaranoglu, Department of Anaesthesiology, Bezmialem Vakıf University, Fatih, İstanbul 34590, Turkey

Author contributions: Basaranoglu M and Basaranoglu G performed the literature search and designed and wrote the study.

Correspondence to: Metin Basaranoglu, MD, Division of Gastroenterology and Hepatology, Teaching and Consulting, Ankara Yüksek İhtisas Hospital Gastroenterology Clinic, Sıhhiye, Ankara 06100, Turkey. metin_basaranoglu@yahoo.com

Telephone: +90-212-6217580 Fax: +90-212-6217580

Received: March 17, 2011 Revised: May 16, 2011

Accepted: May 23, 2011

Published online: September 28, 2011

Abstract

Chronic hepatitis due to any cause leads to cirrhosis and end-stage liver disease. A growing body of literature has also shown that fatty liver due to overweight or obesity is a leading cause of cirrhosis. Due to the obesity epidemic, fatty liver is now a significant problem in clinical practice. Steatosis has an impact on the acceleration of liver damage in patients with chronic hepatitis due to other causes. An association between hepatitis C virus (HCV) infection, steatosis and the onset of insulin resistance has been reported. Insulin resistance is one of the leading factors for severe fibrosis in chronic HCV infections. Moreover, hyperinsulinemia has a deleterious effect on the management of chronic HCV. Response to therapy is increased by decreasing insulin resistance by weight loss or the use of thiazolidinediones or metformin. The underlying mechanisms of this complex interaction are not fully understood. A direct cytopathic effect of HCV has been suggested. The genomic structure of HCV (suggesting that some viral sequences are involved in the intracellular accumulation of triglycerides), lipid metabolism, the molecular links between the HCV core protein and lipid droplets (the core protein of HCV and its transcriptional

regulatory function which induce a triglyceride accumulation in hepatocytes) and increased neolipogenesis and inhibited fatty acid degradation in mitochondria have been investigated.

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Key words: Adipocytokines; Fatty acids; Hepatitis B virus; Hepatitis C virus; Inducible nitric oxide synthase; Insulin resistance; Signal transduction and activator of transcription-3; Steatosis; Sterol regulatory element-binding protein-1c; Suppressors of cytokine signaling; Tumor necrosis factor- α

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Basaranoglu M, Basaranoglu G. Pathophysiology of insulin resistance and steatosis in patients with chronic viral hepatitis. *World J Gastroenterol* 2011; 17(36): 4055-4062 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4055.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4055>

INTRODUCTION

Chronic hepatitis due to any cause leads to cirrhosis and end-stage liver disease. A growing body of literature has also shown that fatty liver due to overweight or obesity is a leading cause of cirrhosis^[1-3]. Due to the obesity epidemic, fatty liver is now a significant problem in clinical practice. An association between hepatitis C virus (HCV) infection, steatosis and the onset of insulin resistance has been reported^[4-6]. Moreover, steatosis has an impact on the acceleration of liver damage in patients with chronic hepatitis due to other causes. The underlying mechanisms of this complex interaction are not fully understood. A direct cytopathic effect of HCV has been suggested. The genomic structure of HCV (suggesting

that some viral sequences are involved in the intracellular accumulation of triglycerides, lipid metabolism, and the molecular links between the HCV core protein and lipid droplets (the core protein of HCV and its transcriptional regulatory function which induce a triglyceride accumulation in hepatocytes) and increased neolipogenesis and inhibited fatty acid degradation in mitochondria have been investigated (Figure 1).

BACKGROUND OF FATTY LIVER

Excessive accumulation of triglycerides in hepatocytes in the absence of significant alcohol consumption, defined as > 5% fat by weight, occurs in about 20%-30% of adults^[1-3]. Excessive fat in the liver predisposes to the development of steatohepatitis which is a significant risk factor for developing cirrhosis and its complications, including hepatocellular carcinoma.

Background of insulin resistance in patients with HCV

The frequency of type 2 diabetes is more common in patients with chronic HCV infection than in hepatitis B infection (21% *vs* 12%, respectively) which is evidence of a link between HCV infection and diabetes mellitus (DM)^[4-6]. This relationship is independent of the existence of cirrhosis. A large cross-sectional United States study which included over 9000 individuals showed that the frequency of type 2 DM is 3-fold more common in hepatitis C patients. Both older age and higher body mass index (BMI) are more common among patients with both hepatitis C and type 2 diabetes.

Insulin resistance (IR) is a specific feature of chronic HCV, associated with genotypes 1 and 4 and high serum HCV RNA level^[7]. Chronically HCV infected subjects present a 3-fold increased risk of IR and glucose metabolism impairment, with IR occurring in the very early stages of hepatic lesions (fibrosis stage 0 or 1), with a worsening tendency as hepatic fibrosis progresses^[8-10]. There is also an association between IR severity and DM, with higher viral load and an improvement in IR after a sustained viral response.

A recently published study which investigated 600 patients [chronic hepatitis C (CHC) in 500 and chronic hepatitis B (CHB) in 100] reported that IR was present in 32.4% of the 462 nondiabetic CHC patients and was associated with the metabolic syndrome, genotypes 1 and 4, significant fibrosis, and severe steatosis^[7]. IR was diagnosed in 15% of 145 CHC patients without metabolic syndrome or significant fibrosis, and was associated with genotypes 1 and 4, high serum HCV RNA level, and moderate-to-severe necroinflammation. IR was less frequent in CHB patients than in matched CHC patients (5% *vs* 35%, respectively, $P < 0.001$).

In our clinic, we investigated 76 patients. Of these 76, 12 had hepatitis B, 19 had hepatitis C, 34 had simple steatosis and 11 were control subjects. We found that IR was only significant and associated with severe fibrosis in patients with HCV^[11].

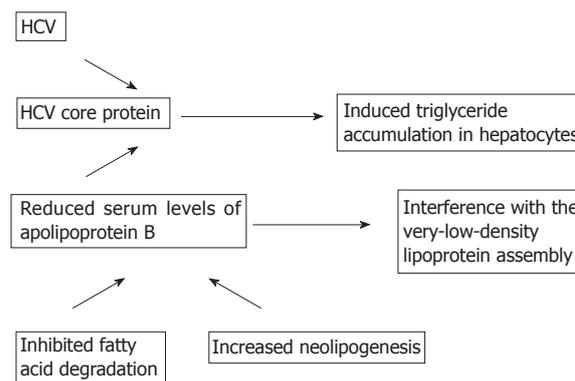


Figure 1 Underlying mechanisms of the complex interaction resulting in steatosis in patients with hepatitis C virus. HCV: Hepatitis C virus.

Whether fat in the liver is an important determinant of IR is debatable. The study showed that insulin secretion assessed by intravenous glucose injection was not impaired in CHC patients compared to the controls^[12]. When they studied the IR of 29 people with hepatitis C (14 with genotype 1 and 15 with genotype 3) and confirmed they had high IR, nearly all the IR was found to be in the muscle and hardly any in the liver. Of the 29 patients, 15 had very high levels of fat in the liver and had the same degree of IR as the 14 patients who did not have fatty livers.

IR is one of the leading factors for severe fibrosis in CHC infections independent of steatosis, as compensatory hyperinsulinemia is fibrogenic^[12]. Moreover, a relationship between exogenous hyperinsulinemia and hepatocellular carcinoma has been reported. Hyperinsulinemia decreases therapy response and has a deleterious effect on the management of chronic HCV infection. Response to therapy is increased by decreasing insulin resistance by weight loss or the use of thiazolidinediones and metformin. Metformin improved virologic response when added to hepatitis C interferon-ribavirin therapy in those with IR^[13].

A relationship between type 1 diabetes and hepatitis C, and type 2 diabetes and hepatitis B has not so far been reported^[14].

Background of steatosis in patients with HCV infection

The prevalence of steatosis is 20%-30% in the general population and is 50%-80% in patients with HCV infection. HCV itself has the ability to directly promote steatosis and IR^[15-18]. If all steatogenic co-factors are excluded, the prevalence of steatosis remains at 50% resulting in a 2.5-fold increased prevalence as compared with the general population and other forms of chronic liver disease. The prevalence of steatosis is 18% in hepatitis B virus infection^[19].

There are 2 types of liver steatosis: metabolic steatosis which is related to high BMI in patients with genotype 1, and viral steatosis which is related to hepatitis C genotype 3. Steatosis is more frequent in association with genotype 3a as compared to other genotypes such

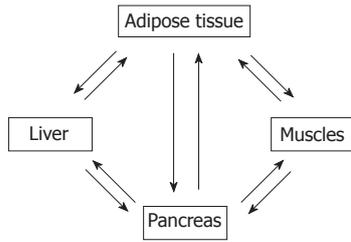


Figure 2 Cross-talk among the insulin sensitive organs.

as genotype 1, 2 and 4 (74% vs 50%), which suggests that some sequences of the viral genome may be involved in intracellular lipid accumulation^[16]. Additionally, steatosis correlates with viral load and can revert after effective treatment and reoccurs with re-infection in genotype 3 infection.

The localization of steatosis, particularly in genotype 3 infected patients, is predominantly in the periportal zone (acinar 1) and not in the centrilobular zone (acinar 3) and is more typical of metabolic associated steatohepatitis^[20-23]. All genotypes are steatogenic, however, genotype 3 is three times more potent. Transgenic mouse models showed that the core protein can induce the appearance of lipid droplets. One possible molecular explanation for the greater steatogenic property of genotype 3, could be a phenylalanine residue at position 164 in core protein domain II, instead of tyrosine as in other genotypes, which results in a higher affinity to lipids.

Hyperinsulinism, IR related, directly activates stellate cells and, in association with hyperglycemia, increases connective tissue growth factor, a key cytokine in hepatic fibrogenesis^[24,25]. Steatosis also relates to more advanced fibrosis and to accelerated fibrosis progression. Thus, treating HCV infected patients with evidence of hepatic steatosis, even if they only present mild inflammatory activity, has been suggested. How? Steatosis may sensitize the liver to inflammation and apoptosis, and subsequently enhance fibrosis. A recent study showed that hepatic steatosis is associated with higher programmed cell death by apoptosis with stellate cell activation^[26].

Background of insulin resistance

A balance exists between energy demand and intake in the human body. Obesity and its consequences such as IR and the metabolic syndrome, is a growing threat to the health of people in developed nations. While insulin receptor defects cause severe IR, most patients with IR have impaired post-receptor intracellular insulin signaling^[27].

INSULIN SIGNALING PATHWAY AND GLUCOSE HOMEOSTASIS

There is cross-talk among insulin sensitive tissues such as skeletal muscle, adipose tissue, and liver (Figure 2). Insulin binds α -subunits of its receptor which is a cell surface receptor on insulin sensitive cells such as skeletal

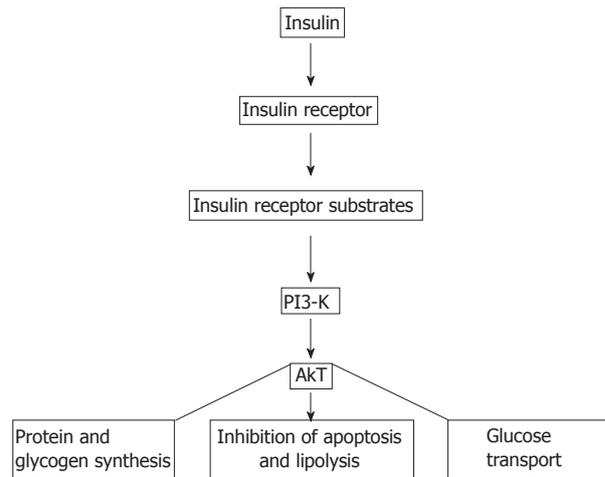


Figure 3 Insulin signaling pathways. PI3-K: Phosphatidylinositol 3-kinase; Akt: A serine/threonine protein kinase.

muscle, adipocytes, and hepatocytes leading to autophosphorylation of the cytoplasmic domains (β -subunits) of the receptor^[25-40]. The insulin receptor has intrinsic tyrosine kinase activity activated by insulin binding and the autophosphorylated receptor activates its substrates that include insulin receptor substrate (IRS)-1, IRS-2, Src homology collagen (Shc), and an adaptor protein with a pleckstrin homology (PH) and Src homology (SH) 2 domain by tyrosine phosphorylation (Figure 3). These phosphorylated docking proteins bind and activate several downstream components of the insulin signaling pathways. Activated IRS-1 associates with phosphatidylinositol 3-kinase (PI3-K), which then activates Akt. Akt substrate of 160 kDa (AS160), a serine/threonine kinase, was identified in 3T3-L1 adipocytes. In both skeletal muscle and adipose tissue, these insulin-mediated phosphorylation-dephosphorylation signaling cascades induce the translocation of glucose transporters (GLUT), predominantly GLUT4-containing vesicles, from intracellular storage sites to the plasma membrane, increasing glucose uptake to prevent abnormal glucose and insulin elevations in the plasma (insulin-stimulated glucose transport). These events and insulin-dependent inhibition of hepatic glucose output maintain glucose homeostasis. Insulin also affects glucose homeostasis indirectly by its regulatory effect on lipid metabolism. Any interference in this insulin signaling pathway causes glucotoxicity, insulin resistance and, when islet β cells are capable of responding, compensatory hyperinsulinemia. Hepatitis C virus Genotype 1b diminishes IRS-1 levels and causes IR.

Hepatic expression of insulin receptor protein was decreased in chronic hyperinsulinemic states. IRS-1 was more closely linked to glucose homeostasis with the regulation of glucokinase expression, while IRS-2 was more closely linked to lipogenesis with the regulation of lipogenic enzymes sterol regulatory element-binding protein-1c (SREBP-1c) and fatty acid synthase. Moreover, insulin activates synthesis and inhibits catabolism

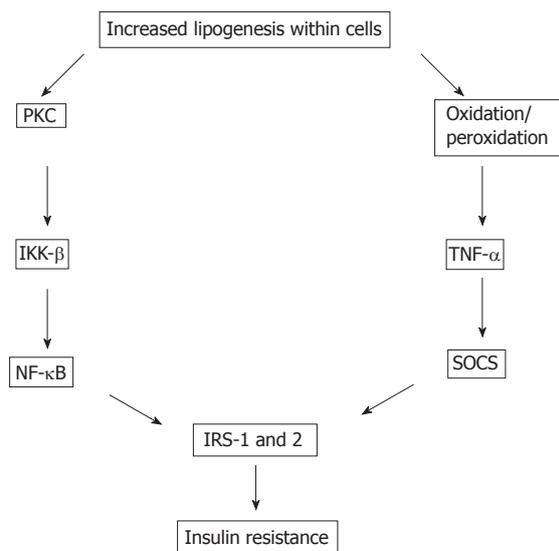


Figure 4 Pathways to insulin resistance. PKC: Protein kinase C; IKK: Inhibitor κ B kinase; TNF: Tumor necrosis factor; NF: Nuclear factor; SOCS: Suppressors of cytokine signaling; IRS: Insulin receptor substrate.

of lipids, while shutting off the synthesis of glucose in the liver.

Adipose tissue is one of the major insulin sensitive organs in the human body and the process of differentiation of preadipocytes to adipocytes, induced by insulin, is called adipogenesis. Within the adipose tissue, insulin stimulates triglyceride synthesis (lipogenesis) and inhibits lipolysis by upregulating lipoprotein lipase activity which is the most sensitive pathway in insulin action, facilitating free fatty acid uptake and glucose transport, inhibiting hormone sensitive lipase, and increasing gene expression of lipogenic enzymes. Insulin also induces the degradation of apolipoprotein B100 (apo B100), a key component of very-low-density lipoprotein, in the liver^[41].

Insulin resistance

Insulin resistance can be defined as the failure of insulin sensitive cells to respond to insulin normally. It is characterized by elevated plasma glucose and, before attrition of pancreatic β -cells develops, elevated insulin levels. Chronic hyperinsulinemia is a major contributor to glucose and lipid metabolism abnormalities. Insulin resistance also inappropriately activates peripheral lipolysis and stimulates free fatty acid mobilization from adipocytes in the fed state. Increased circulating free fatty acids contribute to fat accumulation in the liver and muscle, further causing these tissues to be insulin resistant by disturbing their downstream insulin signaling cascades.

Mechanisms of insulin resistance (role of tumor necrosis factor- α and plasma free fatty acids)

The most common mechanism of IR is disturbed post-receptor insulin signaling (Figure 4)^[42-48]. Whereas most

insulin signaling is propagated by tyrosine phosphorylation, serine (Ser) phosphorylation is often inhibitory. Ser phosphorylation of IRS-1 decreases both insulin stimulated tyrosine phosphorylation of IRS-1 (phosphorylated Ser residues of IRS-1 become poor substrates for insulin receptor) and PI3-K activation. This diminishes the downstream insulin signaling and insulin sensitivity of insulin target tissues. IRS-1 has several Ser residues including Ser 307, Ser 612 and Ser 632 which can be phosphorylated. Insulin and tumor necrosis factor- α (TNF- α) can phosphorylate the same Ser residues of IRS-1. IR occurs very early in HCV infection, in parallel with an elevation in TNF- α levels. HCV also directly promotes IR through the proteasomal degradation of IRS-1.

TNF- α and plasma free fatty acids have been shown to be major stimuli of Ser 307 phosphorylation of IRS-1. Inhibition of IRS-1 due to the phosphorylation of its Ser 307 residues also requires the activation of both c-Jun N-terminal kinase (JNK) and inhibitor κ B kinase (IKK) β . Both TNF- α and free fatty acids induce JNK and IKK- β activation.

TNF- α stimulates phosphorylation of Ser residues of both IRS-1 and IRS-2 in hepatocytes^[48-50]. It was recently reported that monocyte-derived macrophages increasingly accumulated within the adipose tissue of obese patients. In addition to the dysregulated production of adipocytokines by adipocytes, adipose tissue macrophages also produce proinflammatory cytokines such as TNF- α , interleukin-6, and C-reactive protein. Both adipose tissue and its macrophages contribute to the TNF- α burden. Indeed, its circulating concentrations are very low, commonly undetectable even in obese mice or humans.

Elevated free fatty acids in the circulation are also major contributors to IR in both humans and mice by stimulating Ser 307 phosphorylation of IRS-1. Adipose tissue triglycerides are the main source of circulating free fatty acids in obesity. One mechanism of elevated free fatty acid-induced IR in muscle is the impaired activation of protein kinase C lamda (PKC λ) and protein kinase C XI (PKC ξ)^[50-52]. PKC θ can also activate IKK- β which phosphorylates Ser 307 residues of IRS-1. Additionally, increased acyl CoAs or ceramide which is a derivative of acyl CoAs, promote IR by diminishing Akt1 activation. Increased ceramide activates a phosphatase (protein phosphatase 2A) that reverses tyrosine phosphorylation of Akt/protein kinase B (PKB). Inactivated PKB inhibits the insulin downstream signaling cascade leading to IR in muscles [Le]. Several oxidative stress mediators might also induce IR by affecting insulin downstream signaling. Phosphatases such as phosphatase and tensin homolog, small heterodimer partner 2, and protein tyrosine phosphatase 1B are now recognized to be major mediators involved in IR. Another possible mechanism for IR is defective glucose transport such as down-regulation of GLUT4.

JNK is one of the stress-related kinases and plays an important role in the development of IR^[48,52]. The

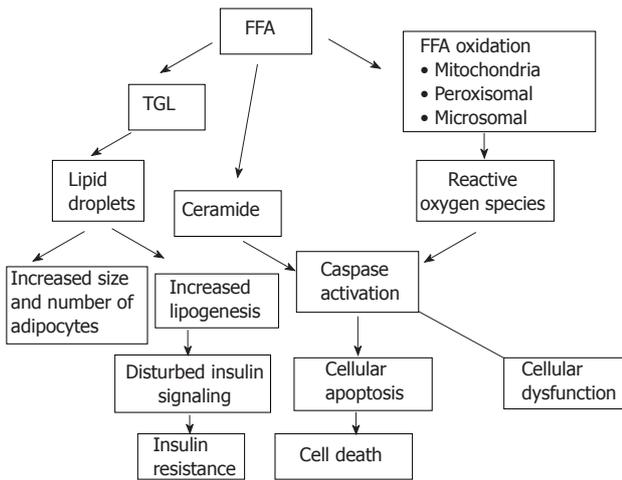


Figure 5 Insulin resistance and cell death. FFA: Free fatty acids; TGL: Tryg-lycerides.

three members of the JNK group of serine/threonine kinases, namely JNK-1, -2, and -3 are activated by proinflammatory cytokines such as TNF- α as well as free fatty acids and endoplasmic reticulum stress due to metabolic overload, which is an intracellular abnormality found in obesity. Activated JNK induces Ser 307 phosphorylation of IRS-1, disturbs insulin downstream signaling, and subsequently causes insulin resistance. JNK activity has been found to be elevated in liver, muscle, and adipose tissue of experimental obese models. Additionally, the loss of JNK-1 activity such as in JNK-1 knockout mice has been shown to prevent the development of IR in leptin-deficient *ob/ob* mice or mice with high-fat induced dietary obesity.

PKC θ and IKK- β are two proinflammatory kinases involved in insulin downstream signaling. They are activated by lipid metabolites such as high plasma free fatty acid concentrations and there is a positive relationship between the activation of PKC θ and the concentration of intermediate fatty acid products. PKC θ activates both IKK- β and JNK, leading to increased Ser 307 phosphorylation of IRS-1 and IR. IKK- β is a mediator of IR and one of the other stress-related kinases^[53,54]. Activation or overexpression of IKK- β diminishes insulin signaling and causes IR, whereas inhibition of IKK- β improves insulin sensitivity. IKK- β phosphorylates the inhibitor of nuclear factor (NF)- κ B leading to the activation of NF- κ B by the translocation of NF- κ B to the nucleus. NF- κ B is an inducible transcription factor and promotes specific gene expression in the nucleus. NF- κ B has both apoptotic and anti-apoptotic effects. The finding that NF- κ B deficient mice were protected from high-fat diet-induced IR suggests that NF- κ B directly participates in processes that impair insulin signaling.

Suppressors of cytokine signaling (SOCS) and inducible nitric oxide synthase (iNOS) are two inflammatory mediators recently recognized to play a role in insulin signaling^[54-61]. Induction of SOCS proteins [SOCS 1-7 and cytokine-inducible src homology 2 domain-contain-

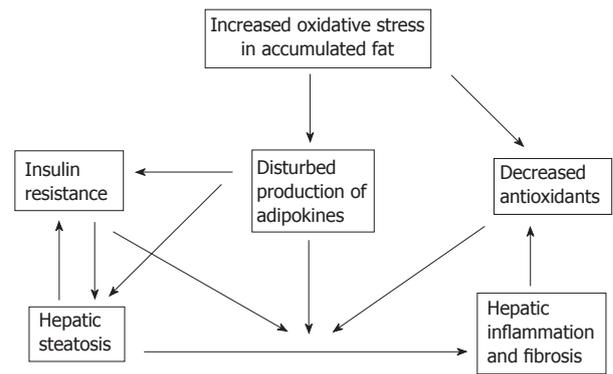


Figure 6 Fate of accumulated fat within hepatocytes.

ing protein [CIS]) by proinflammatory cytokines might contribute to the cytokine-mediated IR in obese subjects. SOCS-3 might also regulate central leptin action and play a role in the leptin resistance of obese human subjects. SOCS-1 knockout mice showed low glucose concentrations and increased insulin sensitivity. In animal studies, inactivation of SOCS-3 or SOCS-1 or both in the livers of *db/db* mice partially improved insulin sensitivity and decreased hyperinsulinemia, whereas overexpression of SOCS-1 and SOCS-3 in obese animals caused IR and also increased activation of SREBP-1c^[62]. SREBP-1c is one of the key mediators of lipid synthesis from glucose and other precursors (*de novo* lipogenesis) in the liver. Indeed, SOCS proteins markedly induce *de novo* fatty acid synthesis in the liver by both the up-regulation of SREBP-1c and persistent IR with hyperinsulinemia which stimulates SREBP-1c-mediated gene expression.

The molecular mechanism that leads to IRS-1 degradation varies according to genotype in patients with hepatitis C virus infection. Genotype 1 promotes the expression of SOCS-3 as genotype 3 promotes SOCS 7 expression, with a mechanism of IRS-1 degradation similar to that induced by SOCS 3; it also inhibits PPAR- γ , further worsening IR. One of the steatogenic mechanisms is the promotion of *de novo* fatty acids synthesis by induced expression of SREBP-1c by HCV infection.

Nitric oxide synthase-2 (NOS2) or iNOS production are also induced by proinflammatory cytokines^[63,64]. High-fat diet in rats causes up-regulation of iNOS mRNA expression and increases iNOS protein activity. Increased production of NOS2 might reduce insulin action in both muscle and pancreas and decreased iNOS activity protects muscles from high-fat diet-induced IR.

HCV induces protein phosphatase 2A expression, through an endoplasmic reticulum stress response pathway, which dephosphorylates protein kinase B (Pkb)/Akt (a main enzyme in the insulin signaling pathway), and thereby lowers its kinase activity^[65].

THE PATHOGENESIS OF HEPATOCELLULAR INJURY IN STEATOSIS

The accumulation of fat within the hepatocytes sensitizes

the liver to injury from a variety of causes and the regenerative capacity of a fatty liver is impaired (Figures 5 and 6)^[66-68]. An interacting network of cytokines and adipokines that regulate inflammation is disrupted. **p53 is involved in the mechanisms of hepatocellular injury accompanied by steatosis**^[69].

CONCLUSION

Steatosis is one of the characteristic histopathologic features of HCV caused by chronic liver disease, and is also closely related to IR. Insulin resistance is one of the leading factors for severe fibrosis in chronic HCV infections. Moreover, hyperinsulinemia has a deleterious effect on the management of chronic HCV. The underlying mechanisms of this complex interaction are not fully understood. A direct cytopathic effect of HCV has been suggested. The genomic structure of HCV, lipid metabolism, the molecular links between the HCV core protein and lipid droplets and increased neolipogenesis and inhibited fatty acid degradation in mitochondria have been investigated.

ACKNOWLEDGMENTS

This article is dedicated to my mentors Professor Stephen H Caldwell from the University of Virginia Health System and Professor Brent A Neuschwander-Tetri from the St. Louis University Hospital in gratitude for having guided me into Basic and Clinical Hepatology.

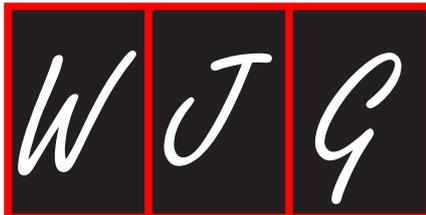
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S- Editor Tian L L- Editor Webster JR E- Editor Zhang DN



Digestive manifestations of parathyroid disorders

Bassam Abboud, Ronald Daher, Joe Boujaoude

Bassam Abboud, Ronald Daher, Department of General Surgery, Hotel Dieu de France Hospital, Faculty of Medicine, Saint-Joseph University, Beirut 166830, Lebanon

Joe Boujaoude, Department of Gastroenterology, Hotel Dieu de France Hospital, Faculty of Medicine, Saint-Joseph University, Beirut 166830, Lebanon

Author contributions: Abboud B designed the research; Daher R, Abboud B and Boujaoude J performed the research; Daher R, Abboud B and Boujaoude J analyzed the data; Daher R and Abboud B wrote the paper.

Correspondence to: Bassam Abboud, MD, Department of General Surgery, Hotel Dieu de France Hospital, Alfred Naccache Street, Beirut 166830, Lebanon. dbabboud@yahoo.fr
Telephone: +961-1-615300 Fax: +961-1-615295

Received: December 29, 2010 Revised: March 25, 2011

Accepted: April 2, 2011

Published online: September 28, 2011

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Key words: Dysparathyroidism; Hypoparathyroidism; Hyperparathyroidism; Digestive manifestations; Steatorrhea; Pancreatitis; Peptic ulcer

Peer reviewer: Dan L Dumitrascu, Professor, President, Romanian Society of Neurogastroenterology 2nd Medical Department University of Medicine and Pharmacy Iuliu Hatieganu Cluj, Romania

Abboud B, Daher R, Boujaoude J. Digestive manifestations of parathyroid disorders. *World J Gastroenterol* 2011; 17(36): 4063-4066 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4063.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4063>

Abstract

The parathyroid glands are the main regulator of plasma calcium and have a direct influence on the digestive tract. Parathyroid disturbances often result in unknown long-standing symptoms. The main manifestation of hypoparathyroidism is steatorrhea due to a deficit in exocrine pancreas secretion. The association with celiac sprue may contribute to malabsorption. Hyperparathyroidism causes smooth-muscle atony, with upper and lower gastrointestinal symptoms such as nausea, heartburn and constipation. Hyperparathyroidism and peptic ulcer were strongly linked before the advent of proton pump inhibitors. Nowadays, this association remains likely only in the particular context of multiple endocrine neoplasia type 1/Zollinger-Ellison syndrome. In contrast to chronic pancreatitis, acute pancreatitis due to primary hyperparathyroidism is one of the most studied topics. The causative effect of high calcium level is confirmed and the distinction from secondary hyperparathyroidism is mandatory. The digestive manifestations of parathyroid malfunction are often overlooked and serum calcium level must be included in the routine workup for abdominal symptoms.

INTRODUCTION

The parathyroid glands play a major role in calcium homeostasis, and ultimately have an effect on all organs because of the complexity of intracellular calcium physiology. The gut and accessory organs are not spared. However, digestive manifestations of dysparathyroidism are not well known and typically rely on old articles and theories. This paper summarizes the digestive consequences of parathyroid disorders and highlights recent theories based on older studies.

DIGESTIVE MANIFESTATIONS OF HYPOPARATHYROIDISM

Hypoparathyroidism may be transient, genetically inherited, or acquired due to an autoimmune process. It may also be secondary to surgery or neck irradiation^[1]. Digestive manifestations of hypoparathyroidism are few and consist mainly of steatorrhea.

Steatorrhea related to hypoparathyroidism is a consequence of bilio-pancreatic exocrine deficit due to insufficient meal-stimulated cholecystokinin secretion by the

duodenal mucosa^[2]. The treatment of fat malabsorption in idiopathic hypoparathyroidism comprises: medium-chain triglycerides diet^[3], correction of hypoparathyroidism, administration of vitamin D^[4], and normalization of hypocalcemia^[5]. In contrast, secondary hyperparathyroidism, as a consequence of malabsorption and steatorrhea, is accompanied by normal or sub-normal serum calcium level.

Idiopathic hypoparathyroidism can be associated with other digestive autoimmune diseases that may cause diarrhea. Few reports have been published on the coexistence of primary hypoparathyroidism and celiac disease^[6-8]. Kumar *et al*^[9] have explored this association in a cross-reactive immunological pathway. If suspected by resistance to vitamin D supplementation^[10], the coexistence of celiac sprue must be ruled out by duodenal biopsy. In such cases, gluten-free diet should be included in the treatment regimen^[11,12]. Moreover, in the specific context of celiac sprue, Parathyroid hormone (PTH) level might not be elevated because of parathyroid atrophy, and secondary hyperparathyroidism might not appear^[13]. Finally, since its description by Reisner *et al*^[14] more than 50 years ago, the coexistence of idiopathic hypoparathyroidism and pernicious anemia has not been further reported.

DIGESTIVE MANIFESTATIONS OF HYPERPARATHYROIDISM

The gastrointestinal manifestations of primary hyperparathyroidism (PHPT) have been described many decades ago^[15]. Truly asymptomatic hyperparathyroidism is rare when thorough anamnesis looks for subtle symptoms. Most frequent digestive manifestations are constipation, heartburn, nausea and appetite loss that occur in 33%, 30%, 24% and 15% of cases, respectively^[16]. Significant reduction in symptom rates is found after parathyroidectomy. Vague abdominal pain can be as frequent as 29%^[17]. The exact pathophysiological mechanism is not fully understood. Alterations in gene expression secondary to sustained stimulation of PTH receptors may help explain the symptoms^[18]. As a result, gut atony occurs and leads to constipation in the colon and dyspepsia in the stomach^[17]. Finally, PHPT has been associated with increased incidence of malignancies, especially of the colon^[19].

The association between PHPT and peptic ulcer disease is a yet-to-be-resolved issue. Most studies about this subject date were performed several decades ago^[18,20,23], did not include prospective large-scale studies, and led to controversial results. Compared to 30% in adults with hyperparathyroidism^[18], peptic ulcer was found in 5% of autopsies in the general population before the advent of the proton pump inhibitors^[20]. Other studies have reported results between these two extremes^[21]. On the other hand, among patients with duodenal ulcer, Frame *et al*^[22] have shown a 10-fold increase in the incidence of PHPT. As reported in old studies, complete correlation between hyperparathyroidism and increased gastric acid secretion could not be found, and normalization of the latter was

not systematic after parathyroidectomy^[21,23-28]. Again, the correlation between hypergastrinemia and hyperparathyroidism was not constant throughout previous studies^[28,29], although Reeder *et al*^[30] have found a direct calcium-to-gastric hypersecretion relationship in hypergastrinemia. The only prospective study conducted by Corleto *et al*^[31] failed to confirm these findings. Zollinger-Ellison syndrome (ZES) may coexist with PHPT in the context of multiple endocrine neoplasia type 1. In a prospective study, Norton *et al*^[32] reported a significant biochemical improvement of ZES in 20% of patients who underwent resection of more than three parathyroid glands. Finally, pancreatic polypeptide was once correlated with hyperparathyroidism^[33].

Acute pancreatitis caused by PHPT was first described by Cope *et al*^[34] in 1957. Since that date, the exact relationship between these two entities has been questioned, until PHPT was accepted as an etiology for pancreatitis^[35]. Incidence of acute pancreatitis in patients with PHPT has varied from 1%^[36] to 12%^[37] in retrospective series, with intermediate values^[38,39]. Jacob *et al*^[40] have shown a 28-fold increased risk of pancreatitis in hyperparathyroid patients compared to the general population. After eliminating all other causes, mean plasma calcium level seems to be the only predictive factor for pancreatitis development^[37,40,41]. Its dosage must be included in the etiological work-up, although hyperparathyroidism is found in < 1% of patients who present with acute pancreatitis^[42]. Carnaille *et al*^[37] have shown that most patients had single adenoma, which suggested that pancreatitis was a consequence (and not the cause) of hyperparathyroidism. Additionally, acute pancreatitis may be the presenting form of PHPT^[38,43,44], even in its ectopic localization^[45,46]. In contrast, Felderbauer *et al*^[39] have stressed that genetic mutations constitute a greater risk factor for pancreatitis than serum calcium.

The pathophysiological mechanism that leads to pancreatitis seems more related to hypercalcemia than to PHPT. It has been shown that hypercalcemia from any cause can lead to pancreatitis^[47-49]. As confirmed by experimental studies, calcium ions cause calculus deposition within the pancreatic ductules, with consequent obstruction and inflammation^[50]. Moreover, calcium can trigger the pancreatitis cascade by promoting conversion of trypsinogen to trypsin^[51,52].

Interrelation between acute pancreatitis and parathyroid function can be summarized as follows: (1) acute pancreatitis results in a tendency to hypocalcemia and secondary hyperparathyroidism^[53,54]. **Compensation need** is correlated to pancreatitis severity as shown by PTH level^[55]; (2) **severe and/or complicated pancreatitis** can lead to overt hypocalcemia through relative deficiency in PTH secretion^[54], because exogenous administration of PTH normalizes calcium level^[56]; (3) **in severe pancreatitis**, resistance to PTH action in bones and kidneys may occur because of fluid sequestration and reduction in efficient arterial blood volume^[53]; (4) **once the diagnosis** of PHPT-induced acute pancreatitis is established, parathyroidectomy is mandatory because it prevents recurrence^[37,42].

Bhadada *et al*^[57] have studied PHPT-induced chronic pancreatitis and compared it to pancreatitis of other causes. PTH and calcium levels are significantly more elevated in PHPT, while in others, elevated PTH level is secondary to maintain normocalcemia. With regard to complications, it seems that chronic pancreatitis secondary to PHPT does not differ from chronic pancreatitis of other causes. This entity needs to be studied by larger studies for further understanding.

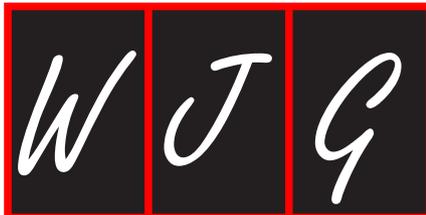
In conclusion, serum calcium level must be considered among the usual tests in patients with rare and/or non-specific abdominal symptoms. Hypoparathyroidism mainly manifests in the gut as malabsorptive diarrhea. Laboratory tests are essential for the diagnosis of secondary hypocalcemia when treatment is medical. PHPT causes non-specific digestive symptoms that are consequent to smooth-muscle atony. Association of peptic ulcer with PHPT is not as clear as described by old literature except for ZES in MEN 1. In contrast, PHPT is a confirmed risk factor for acute pancreatitis that can be its presenting form. Finally, PHPT-induced chronic pancreatitis needs further study for confirmation.

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S- Editor Sun H L- Editor Kerr C E- Editor Xiong L



Current treatment for colorectal liver metastases

Evangelos P Misiakos, Nikolaos P Karidis, Gregory Kouraklis

Evangelos P Misiakos, Third Department of Surgery, Medical School, University of Athens, Attikon University Hospital, Athens 12462, Greece

Nikolaos P Karidis, Gregory Kouraklis, Second Propedeutic Department of Surgery, Medical School, University of Athens, General Hospital Laiko, Athens 11527, Greece

Author contributions: Misiakos EP organized and prepared the draft of the present review; Karidis NP contributed to reference collection and final preparation of the manuscript; Kouraklis G coordinated and reviewed the manuscript.

Correspondence to: Nikolaos P Karidis, MD, General Surgeon, Second Propedeutic Department of Surgery, University of Athens, General Hospital Laiko, Athens 11527, Greece. npkaridis@gmail.com

Telephone: +30-69-74779016 Fax: +30-21-07791456

Received: October 3, 2010 Revised: November 30, 2010

Accepted: December 7, 2010

Published online: September 28, 2011

Key words: Colorectal liver metastases; Multidisciplinary treatment

Peer reviewer: Ferenc Sipos, MD, PhD, Cell Analysis Laboratory, 2nd Department of Internal Medicine, Semmelweis University, Szentkirályi u. 46, Budapest 1088, Hungary

Misiakos EP, Karidis NP, Kouraklis G. Current treatment for colorectal liver metastases. *World J Gastroenterol* 2011; 17(36): 4067-4075 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4067.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4067>

Abstract

Surgical resection offers the best opportunity for survival in patients with colorectal cancer metastatic to the liver, with five-year survival rates up to 58% in selected cases. However, only a minority are resectable at the time of diagnosis. Continuous research in this field aims at increasing the percentage of patients eligible for resection, refining the indications and contraindications for surgery, and improving overall survival. The use of surgical innovations, such as staged resection, portal vein embolization, and repeat resection has allowed higher resection rates in patients with bilobar disease. The use of neoadjuvant chemotherapy allows up to 38% of patients previously considered unresectable to be significantly downstaged and eligible for hepatic resection. Ablative techniques have gained wide acceptance as an adjunct to surgical resection and in the management of patients who are not surgical candidates. Current management of colorectal liver metastases requires a multidisciplinary approach, which should be individualized in each case.

INTRODUCTION

Colorectal cancer (CRC) is the fourth most common cancer in the West and the second most common cause of cancer related mortality after lung cancer in Europe and North America^[1,2]. More than 50% of patients with CRC will develop liver metastases during their lifespan^[2,3]. A quarter of patients with primary CRC are found to have synchronous hepatic secondaries^[4]. Almost half of patients undergoing resection for primary CRC eventually develop metachronous liver secondaries^[5]. Despite improvements in chemotherapies and biological agents, survival is rarely longer than three years^[6,7].

Evidence based on numerous retrospective and comparative studies indicates that hepatic resection is the only available treatment that allows long-term survival^[8]. Experiences with liver resection is associated with a 25% to 51% 5-year survival^[9,10]. By contrast, five-year survivors with chemotherapy alone are anecdotal. Historically, only 5%-10% of patients with colorectal liver metastases were resectable; currently, with the advances in diagnostic methods and new therapies, resectability rates have increased to 20%-25%^[11].

Emerging strategies designed to increase the proportion of patients who are candidates for complete surgical resection have been introduced in clinical practice. Neoadjuvant chemotherapy^[11], preoperative portal vein embolization^[12], and the two-stage resection approach^[13]

contribute to this aim. However, even with these new strategies, the majority of patients with colorectal liver metastases are not candidates for a curative resection.

In this review, the current data supporting the use of liver resection in the management of colorectal liver metastases are analyzed. For this purpose, the role of new imaging techniques for the preoperative evaluation and new staging systems to stratify the patients are extensively reported. Moreover, the most recently introduced chemotherapies and biological therapies to prevent recurrence after surgery or to downstage unresectable tumors are analyzed.

NATURAL HISTORY

Liver metastases from colorectal cancer carry a median survival of 5 to 20 mo if left untreated; two-year survival is unusual, and five-year survival is extremely rare^[4,14]. Factors associated with a significant disadvantage in the unresected group include extent of liver disease, presence of extrahepatic disease, age of the patient, and carcinoembryonic antigen (CEA) level^[14]. Prognosis is closely related to the extent of liver replacement by the tumor^[4,15]. Indeed, Wood *et al*^[15] in a retrospective study of 113 patients undertaken in the Glasgow Royal Infirmary, reported a one-year survival rate of 5.7% for patients with widespread liver disease, 27% for patients with metastases localized to one hepatic lobe, and 60% for patients with solitary metastases.

Even when hepatic resection is performed with curative intent^[16], 60% to 70% of patients will develop local or distant recurrence^[17]. Recurrence occurs equally at intrahepatic and extrahepatic sites; 80% of all recurrences occur within two years. The median survival of patients with recurrent disease is 8 to 10 mo without any treatment^[14]. Repeat resection is feasible in 10% to 15% of these cases and may achieve a five-year overall survival rate of 15% to 40% in selected patients. Cure is considered after the achievement of 10-year disease-free survival^[18].

Chemotherapy alone, whether administered systemically or regionally, has a palliative role and rarely results in prolonged survival. Several retrospective studies have reviewed the clinical outcome of patients with potentially resectable liver metastases treated with chemotherapy alone. An obvious survival advantage for patients undergoing curative resection compared to those treated with chemotherapy was noted^[19]. Scheele *et al*^[19] compared 183 patients with resected hepatic metastases with 62 patients with resectable lesions who did not undergo surgery and 920 patients with unresectable disease. The median survival for the three groups was 30 mo, 14.2 mo and 6.9 mo, respectively. Although the patients of the second group lived longer than those of the third group, no patient in either group survived more than five years.

These poor results in untreated hepatic metastases from colorectal cancer and the continuous improvements in hepatic surgery provided the rationale for increasingly aggressive hepatic resections for the treatment of this condition^[20].

CURRENT CRITERIA OF HEPATIC RESECTION

During the past two decades the five-year survival rates for hepatic colorectal metastases patients have almost doubled, from 30% to 60%^[14]. The introduction of new chemotherapeutic agents and the shift in the criteria of surgical resection were the main factors in this progress^[21]. Previous absolute or relative contraindications to resection included the presence of extrahepatic disease^[8], involvement of hepatic pedicle lymph nodes^[22], and an inadequate resection margin of < 1 cm^[23]. All above contraindications for hepatic resection have been challenged and have already lost their importance in patient selection for hepatectomy^[24,25].

The current criteria focus on what should be left after hepatic resection. Previous criteria for resection, such as the size, location, number of intrahepatic metastases, and the presence of bilobar or extrahepatic disease have been largely abandoned^[14,26,27]. Nowadays, the definition of resectability includes a complete resection with tumor-free surgical margins (R0 resection), sparing at least two liver segments having an independent inflow, outflow, and biliary drainage. The amount of the liver remnant after resection should not be less than 20% and 30% of the total liver volume in normal and cirrhotic patients, respectively. This can be accurately predicted by computed tomography (CT) or magnetic resonance imaging (MRI) during preoperative evaluation.

PREOPERATIVE EVALUATION

Preoperative investigations before resection of colorectal liver metastases are focused on: (1) determining the diagnosis; (2) anatomically defining the lesion in the liver parenchyma for surgical planning; and (3) meticulous staging to rule out extrahepatic disease^[28].

Preoperative biopsy

Fine needle aspiration (FNA) cytology is a well established approach for diagnosis. The potential benefit of FNA in suspect cases is the cytological confirmation of diagnosis, although this can be effectively obtained by other examinations, together with the patient's history. However, there is a potential for false negative results. Nevertheless, the benefit of this examination may be outweighed by the serious risk of needle tract seeding^[29,30]. For these reasons, FNA cytology has been virtually abandoned in the preoperative evaluation of colorectal liver metastases.

Preoperative investigation

Metastatic liver tumors can usually be differentiated by imaging modalities, including ultrasound, CT, MRI and positron emission tomography (PET). CT plays a pivotal role in selecting patients for hepatic resection. The use of multidetector helical CT scans has improved resolution and increased the previously low sensitivity (53%) of detecting colorectal liver metastases to 70%-90%^[14,31,32]. Liv-

er metastases can be distinguished as hypodense lesions in the portal phase. A CT scan may provide information regarding the anatomical characteristics of the metastatic lesions and their relation to lobar architecture and major vascular structures. However, a CT scan cannot detect subcentimeter lesions^[14]. Colorectal liver metastases usually respect the liver capsule and the intersegmental planes and push these structures away. Even large lesions that appear to involve the inferior vena cava or the diaphragm on a CT scan, often do not do so and such appearances should not preclude surgical exploration^[28].

MRI is more useful than CT in detecting small metastatic lesions in a fatty liver, and in defining the relationship of the lesions to the hepatic vasculature and the biliary tree with MR cholangiopancreatography^[28]. However, it has a sensitivity of 70% to 80% and it does not offer any significant advantage over a CT scan^[14]. Furthermore, MRI angiography and CT angiography have gradually replaced the more invasive direct hepatic angiography.

Ultrasonography is an inexpensive test that may identify small metastatic lesions within the hepatic parenchyma. It can give information regarding the size of the metastatic tumor and the extent of liver involvement. Moreover, Duplex ultrasound can define the relation of the tumor to the hilar structures, the hepatic veins, and the inferior vena cava. Ultrasound may be used as a first-line modality in the diagnostic evaluation of hepatic metastases^[28].

A new modality in the diagnosis of colorectal liver metastases is whole body PET. The most common tracer in PET scanning is fluoro-18-deoxyglucose (FDG)-PET, a glucose analog, which can proceed down the glycolytic pathway, and accumulate within the glucose-avid cancer cells. A recent meta-analysis reported a sensitivity and specificity for FDG-PET of 88% and 96%, respectively, for the detection of hepatic metastases, and 90% to 95% for the detection of extrahepatic disease^[33]. The combination of CT and FDG-PET increases sensitivity and allows the selection of surgical therapy for patients likely to gain the most benefit^[34]. The main limitation of a PET scan is the reduced sensitivity in detecting subcentimeter lesions, mucinous lesions, and lesions that have been treated with neoadjuvant chemotherapy^[35].

During the last two decades, laparoscopy has emerged as a new diagnostic modality for patients with liver malignancies. When laparoscopy is employed, unnecessary laparotomy can be avoided in 78% of patients with unresectable disease^[35]. In these cases, laparoscopy can decrease the morbidity of surgery, and shorten the delay to systemic therapy^[36]. Laparoscopy is indicated in cases in which the results of imaging studies are suspicious, but not diagnostic for extrahepatic tumor, such as enlarged lymph nodes or possible peritoneal dissemination.

PREOPERATIVE TREATMENT

Chemotherapy

Current chemotherapy regimens including oxaliplatin

and irinotecan in addition to 5-fluorouracil (5-FU), and leucovorin (LV) have achieved improved response rates in colorectal liver metastases, with significant reduction in disease bulk in almost 50% of patients and a median survival approaching two years^[37]. New biological agents, such as those targeting epithelial and vascular endothelial growth factor pathways (bevacizumab, cetuximab) have added a significant survival benefit in these patients^[38,39].

The successful use of combination chemotherapy in colorectal liver metastases has led to the concept that these agents could also be used before hepatic resection. In fact, the use of neoadjuvant chemotherapy has the benefit of downstaging the tumor, rendering a previously unresectable tumor resectable. This approach may assess the responsiveness of the tumor to chemotherapy, as the initial response to chemotherapy is strongly predictive of a favorable long-term outcome^[40,41]. The development of steatohepatitis is a complication of preoperative chemotherapy, which results in a significantly increased 90-d postoperative mortality^[42].

Neoadjuvant chemotherapy

The use of preoperative chemotherapy may exert a downsizing effect on the metastatic tumors, so one may perform surgery as soon as resectability is technically feasible. According to the Paul Brousse experience^[43], modern chemotherapeutic regimens allow 12.5% of patients with unresectable colorectal liver metastases to be rescued by hepatic resection. This strategy may offer a possibility of long-term survival (33% at five years and 22% at 10 years) with a low operative risk. It is noteworthy that this strategy involves the wide use of repeat hepatectomies and extrahepatic resections in an effort to eradicate all tumors. Currently most reports suggest that infusional FU/LV with oxaliplatin and/or irinotecan are the most effective protocols for this purpose^[31,44]. However, although the response rates are very high when used as first-line therapy, the response rates for second-line therapy are very low^[31,45]. Therefore, tumors that progress while on chemotherapy usually have a low likelihood of becoming resectable with second-line chemotherapy.

Neo-adjuvant chemotherapy can also be used *via* hepatic arterial infusion (HAI) with high response rates, as first or second-line therapies^[46]. Patients with metastatic lesions confined to the liver, without severe ascites or jaundice, are ideal candidates^[47]. Preliminary data from several clinical trials with oxaliplatin or irinotecan *via* HAI have been promising^[48]. However, HAI is rarely used outside specialized treatment centers, because of limited expertise, high cost of infusion pumps, and ongoing concerns regarding the considerable morbidity due to catheter-related complications, particularly sclerosing cholangitis^[49].

Portal vein embolization

Portal vein embolization (PVE) is another modality used preoperatively for patients where the extent of liver

resection is expected to result in less than the optimal functional liver volume of 25% to 40%, necessary to prevent postoperative liver failure^[21,50]. This technique, which induces ipsilateral atrophy and contralateral hypertrophy, is used to expand the number of patients undergoing curative hepatectomy for colorectal liver metastases. The most commonly used agents for embolization include gelatin sponge particles (Gelfoam) with iodized oil (Lipiodol), cyanoacrylate, alcohol, fibrin glue, or gelatin sponge, and they are usually administered percutaneously^[14,51]. The amount of liver tissue gained is about 15% of the total liver volume, and the time for maximum regeneration ranges from three to nine weeks^[52].

Azoulay *et al*^[51] have reported on a group of 30 patients who were deemed ineligible for liver resection because the estimated remnant liver was considered too small. These patients underwent PVE with minimal morbidity and no mortality. PVE substantially increased the remnant liver volume, rendering liver resection feasible in 19 patients (63%), with low morbidity and mortality rates and survival rates similar to the patients who did not undergo PVE. In conclusion, PVE followed by hepatic resection represents a two-stage hepatectomy: progressive atrophy of the embolized area, which triggers compensatory hypertrophy of the future remaining parenchyma, followed by liver resection. Therefore, PVE increases the resectability of colorectal liver metastases with a survival benefit comparable to that obtained with primary liver resection.

Several disadvantages of PVE have emerged as more experience is collected. Thrombosis, and/or migration of the emboli to the contralateral hepatic lobe, hemobilia, hemoperitoneum, and transient liver insufficiency, are complications occurring in 10% of cases and can be easily managed^[50]. Another adverse side effect is the possibility that PVE may stimulate the growth of tumors in the contralateral liver lobe, although this has yet to be clarified^[53]. A way of counteracting this effect is the administration of concurrent chemotherapy soon after PVE, the so-called “interterm chemotherapy”^[14].

LIVER RESECTION

Over the last two to three decades, an aggressive surgical approach has been followed for the treatment of colorectal hepatic metastases, based on the fact that the liver is the first isolated site of metastases for colorectal cancer. This direct treatment of hepatic metastases prevents dissemination of the disease from the liver to other sites^[54].

The role of hepatic resection as an effective treatment for colorectal liver metastases was established in 1988 from the registry of hepatic metastases^[9]. In a retrospective review on 859 patients with colorectal liver metastases who were surgically treated between 1948 and 1985, the five-year actuarial survival rate and the disease-free survival rate were 33% and 21%, respectively. Along with the gradual improvement in imaging techniques,

better understanding of liver anatomy, recent refinements of surgical techniques, and the continuous progress in pre- and postoperative care, the postoperative mortality rate after hepatectomy has been reduced to < 3% and the five-year survival rate after resection of colorectal liver metastases has reached 26%-58%^[10,25].

Initially, liver resection was based on the anatomic system described in the early 1950s by Couinaud^[55], who defined the intrahepatic divisions of blood vessels and bile ducts. However, there was significant confusion regarding the description of liver anatomy and hepatic resections until the first universally accepted terminology system was introduced. The “Brisbane 2000 terminology of liver anatomy and resections”^[56] was based on the internal anatomy and described the several levels of division of the liver segments; today, it has gained wide acceptance among liver specialists.

The main purpose of liver resection is to resect the tumor with a sufficient tumor-free margin, while preserving as much normal parenchyma as possible. Hepatic resections have regularly been along the liver segmental anatomy planes^[51]. An alternative approach is a non-anatomical or wedge resection, removing a smaller volume of liver with reduced postoperative morbidity and mortality. However, this carries a higher risk of positive resection margins^[41]. However, in a recent series where wedge resections were performed for single rather than multiple lesions, the incidence of positive resection margins was equivalent for both wedge resection and segmental resection (8.3%), and the five-year survival was equivalent in both groups^[57].

Intraoperative ultrasound can delineate the interior anatomy of the liver, including intrahepatic vessels, and allows hepatic resection to be performed more safely and anatomically. Moreover, intraoperative ultrasound may identify extrahepatic sites of the disease, such as infiltrated lymph nodes in the celiac axis and the liver hilum, or deposits in the peritoneal cavity^[58]. Extrahepatic disease sites in the peritoneal cavity impart a significant disadvantage in prognosis, whereas an excellent five-year survival (20% to 48%) can be achieved with pulmonary metastases with an R0 resection^[59].

There is a variety of techniques and devices used for hepatic resection, including the clamp crushing technique, Cavitron Ultrasonic Surgical Aspirator (CUSA, Covidien, Mansfield, MA, United States), Hydrojet (Hydro-Jet, Erbe, Tübingen, Germany), and bipolar sealing devices. Among these, the clamp crushing technique remains the most efficient in terms of reduced operation time, blood loss and total costs^[60].

Synchronous disease

Synchronous hepatic metastases occur in about 20%-30% of newly diagnosed colorectal cancers, and they present a challenging problem in the management of these patients^[9]. Consensus has not been reached as to the timing of surgical resection of the hepatic secondaries and the primary colorectal tumor. Traditionally, these patients

were managed by a second laparotomy 12-16 wk after the resection of the primary tumors^[61]. The advantage of this approach is that it provides less surgical insult to the patient as the incision used in the two operations is different^[14]. However, with advances in perioperative care and the continuous improvements regarding the postoperative morbidity and mortality rates after liver resection, most researchers today support simultaneous resection^[62,63]. In fact, very few reports in the last decade still strongly oppose the simultaneous procedure.

Today, a simultaneous resection is preferred when there is a right colon primary, or when a single hepatic lesion is contemplated, whereas a staged resection is often done in case of rectal primaries, or multiple liver secondaries^[31]. However, no real indications or contraindications exist for simultaneous resection of hepatic metastases, and it seems that the final decision depends on the surgeon's experience and the patient's physical status. In general, the results of simultaneous resection are comparable to staged resection in terms of morbidity, and mortality rates; additionally simultaneous resection offers the advantage of completing the local control of the disease in a single procedure, allowing the use of adjuvant chemotherapy for systemic micrometastases^[64].

Locally ablative modalities in combination with liver resection

Locally ablative modalities, such as radiofrequency ablation (RFA)^[65], cryotherapy^[66], or high intensity focused ultrasound^[67], can be used in combination to hepatic resection, to offer curative treatment in patients with unresectable tumors. RFA is the most widely used modality. The goal of the combined approach is to resect the bulk of the metastatic load and to ablate the residual smaller lesions, to achieve a R0 status, preserving at the same time adequate liver parenchyma to avoid postoperative hepatic failure^[68]. According to the MD Anderson Cancer Center's experience^[65] in the combined approach for advanced hepatic malignancies (72% were hepatic colorectal metastases), the perioperative mortality and morbidity rates were 2.3% and 19.8%, respectively. In addition, patients with colorectal secondaries had a median actuarial survival of 37.3 mo. The authors point out that the functional residual hepatic volume has to be accurately estimated to avoid fatal hepatic failure postoperatively, which is quite common in this combined approach.

The use of RFA in combination with surgical resection allows the hepatic surgeon to ablate small lesions while removing the large ones. RFA combined with hepatectomy is well tolerated by the patients and adds minimal complexity and morbidity to the operation. However, RFA is inferior for local control of metastatic lesions, systemic spread, and long-term survival. Indeed, there is a higher local recurrence rate associated with RFA than with resection, resulting in inferior disease-free survival rate^[21]. Therefore, for the treatment of solitary hepatic metastases, the application of RFA cannot be primarily recommended^[69]. On the other hand, RFA can be used

as palliative treatment for unresectable metastases, as it achieves better survival than chemotherapy^[21]. The only limitations in the use of RFA and other locally ablative modalities are the size of the lesion and its location close to major biliary or vascular structures^[31].

Bilobar metastases

The management of bilobar liver metastases demonstrates the advantages of a multidisciplinary approach with a step-by-step strategy and restaging at regular intervals, to achieve a complete resection in most of these patients. The prognostic significance of bilobar distribution of multiple metastases is controversial. Some researchers report bilobar distribution as a poor prognostic factor^[9], whereas others support the view that bilobar distribution does not affect overall patient survival^[8,10]. In fact, the total tumor volume of liver metastases seems to have a stronger influence on survival than the number or location of metastatic lesions^[70].

Surgical resection should be performed only if all the metastatic load of the liver can be removed (R0 resection). In case of involvement of lymph nodes in the hepatic pedicle, with frozen section confirmation, an extensive lymphadenectomy should be performed from the liver hilum to the celiac axis. Moreover, in patients who have more than three poorly differentiated metastatic lesions in segments IV and V, a routine extended lymphadenectomy of the hepatic pedicle seems justified^[71,72].

In general, hepatic lymph node involvement is a poor prognostic factor affecting survival of these patients^[9], but according to a multi-center study by the Association Francaise de Chirurgie, the five-year survival rate of patients with hepatic pedicle lymph node involvement who underwent lymphadenectomy was 12%, compared to the expected 0% to 2% without resection^[10].

The presence of extrahepatic disease is no longer a contraindication to hepatic resection. Recently, encouraging results have been reported in patients treated for liver metastases and peritoneal carcinomatosis^[73]. However, this approach is suitable only for expert teams with experience in liver surgery and intraperitoneal chemotherapy^[72].

Two-stage hepatectomy

The aim of this approach is to achieve in two steps a complete resection of the metastases in cases initially considered unresectable. In these cases, a single hepatectomy would have left too small a remnant liver after surgery, with a high risk of liver insufficiency after surgery^[72]. In two-stage hepatectomy the highest possible number of tumors are resected first, and the remaining tumors are resected in a second procedure after a period of liver regeneration^[13].

The aim of the first hepatectomy is to make the second hepatectomy potentially curative. Mapping permits the surgeon to achieve this by resecting the highest possible number of liver tumors or by clearing the metastatic load from the less invaded hepatic lobe, leaving

the other to be resected after regeneration. Neoadjuvant chemotherapy is given after the first operation, beginning three weeks postoperatively, so it does not interfere with initial liver regeneration. The usual interval between the two stages should be usually around 4 mo, (from 2 to 14 mo), depending on the progress of liver regeneration^[13]. Patients with multiple bilobar liver metastases and too small a future remnant liver could be treated with a two-stage procedure with the use of portal vein embolization^[72].

This approach can also be used at the time of colectomy when multiple synchronous hepatic lesions preclude a single curative hepatectomy. In such cases, a limited resection of the metastatic load of one hemiliver could be done at the same time as the colectomy, leaving the second major hepatectomy to be done in a second stage^[72].

FOLLOW UP AFTER RESECTION

Patients who have undergone hepatic resection of colorectal metastases are monitored to identify early recurrence that may be amenable to repeat resection for cure. Most patients undergo serial physical examination, serum CEA level, chest X-ray, and CT of the upper and lower abdomen every 3 to 4 mo for the first two years and then every 6 mo for the following five years^[28]. Most patients surviving after liver resection present with recurrent disease at the liver or lung. The liver is the site of recurrence in 45% to 75% of cases after liver resection^[5], and this explains the fact that most chemotherapeutic regimens address mainly the liver.

ADJUVANT CHEMOTHERAPY

Postoperative chemotherapy following complete resection of metastatic disease may lead to improvement in long-term prognosis. The past decade has been marked with significant changes in the options available for this group of patients. In addition to 5-FU, which has been used since 1996, several new drugs have been introduced on the market for the treatment of metastatic colorectal cancer (2006): irinotecan, oxaliplatin, capecitabine, bevacizumab, and cetuximab. Therefore the efficacy of treatment regimens has substantially increased^[28].

Adjuvant chemotherapy is used to increase survival and decrease the rate of recurrence. Recently, the first randomized clinical trial by Portier *et al*^[74], which compared surgery alone to surgery plus adjuvant chemotherapy, provided clear evidence that adjuvant chemotherapy is beneficial for patients with colorectal liver metastases. In this study, 173 patients were randomly assigned to surgery and observation or surgery plus 6 mo of systemic adjuvant chemotherapy. The results showed a significantly improved five-year disease-free survival in the surgery plus chemotherapy group compared to surgery alone (33.5% *vs* 26.7%), with a trend towards improved overall five-year survival.

Adjuvant chemotherapy does not decrease the meta-

static recurrence rate in the remnant liver after resection^[75]. Indeed, according to another study^[76], in patients with complete clinical response to chemotherapy according to CT imaging, in situ recurrence was observed in 78% one year after surgery, because of non-visible but viable tumor cells or microscopic disease.

REPEAT RESECTION

As mentioned in the natural history section, the majority of patients with colorectal liver metastases (55%-60%) will develop recurrent disease in the liver within the first two years after surgery, despite any mode of treatment that they have received^[17]. For these patients, the only chance to prolong life would be a repeat resection, usually combined with a locally ablative therapy (RFA). The results of repeat curative resection are comparable to the first one^[14].

The only problem with a second or third hepatectomy on the same patient is increased technical difficulty. Repeat resection carries perioperative morbidity and mortality rates of 5%-7% and 20%-39%, respectively^[27,77]. Therefore, repeat hepatectomy provides similar long-term survival to primary hepatectomy, without increasing perioperative morbidity and mortality^[78]. Indeed, Pessaux *et al*^[79] showed that overall five-year survival rates after the first, second and third hepatectomy are similar: 33%, 21% and 36%, respectively.

There are a number of prognostic factors determining patient eligibility and probable success after a third hepatectomy. These factors are: the curative nature of the first two hepatectomies, an interval of more than one year between the two procedures, the number of recurrent tumors, serum carcinoembryonic antigen levels, and the presence of extrahepatic disease^[80,81]. The best candidates for repeat resection are patients with a low tumor load, no extrahepatic disease, and removal of all visible metastatic load during the second hepatectomy^[69]. However, the role of repeat liver resection in patients with intrahepatic recurrence still remains controversial, because of the disputable survival benefit and the additional risks of repeat surgery.

CONCLUSION

There is an ongoing progress in the diagnostic imaging, chemotherapeutic regimens, and surgical techniques in the management of hepatic colorectal metastases. Hepatic resection has been recognized as the only treatment that could offer long-term survival. Traditional risk factors, indications, and contraindications have been abandoned. The present principle as to resectability is that resection should be performed if all metastases could be removed, while leaving a sufficient remaining liver parenchyma, regardless of their size, number, location and distribution.

Proper use of modern chemotherapy, PVE and/or two-stage hepatectomy and locally ablative modalities

might improve the resectability and prognosis in these patients. This review emphasizes the importance of a multidisciplinary approach for the optimal management of this disease. Moreover, decision making and patient care requires careful assessment of the risks and benefits for each individual, as well as balancing the technical feasibility and oncological options for each case.

ACKNOWLEDGMENTS

We would like to thank Professor Gregory Kouraklis for his constant support throughout the design and preparation of this review.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Li JY

Nitric oxide-releasing aspirin but not conventional aspirin improves healing of experimental colitis

Malgorzata Zwolinska-Wcislo, Tomasz Brzozowski, Agata Ptak-Belowska, Aneta Targosz, Katarzyna Urbanczyk, Slawomir Kwiecien, Zbigniew Sliwowski

Malgorzata Zwolinska-Wcislo, Gastroenterology and Hepatology Clinic, Jagiellonian University Medical College, 31-531 Cracow, Poland

Tomasz Brzozowski, Agata Ptak-Belowska, Aneta Targosz, Slawomir Kwiecien, Zbigniew Sliwowski, Department of Physiology, Jagiellonian University Medical College, 31-531 Cracow, Poland

Katarzyna Urbanczyk, Department of Pathomorphology, Jagiellonian University Medical College, 31-531 Cracow, Poland

Author contributions: Zwolinska-Wcislo M designed the study, performed the majority of the experiments and helped with writing of the manuscript; Ptak-Belowska A and Targosz A measured the plasma levels of cytokines, the generation of mucosal prostaglandins and assessed the expression of cytokines in the colonic tissue using molecular techniques; Urbanczyk K performed the histological evaluation of the colonic mucosa; Kwiecien S performed experiments in rats and measured the colonic blood flow; Sliwowski Z evaluated the lesion score, colonic tissue weight and MPO activity; Brzozowski T designed the study, wrote the manuscript and worked on the revised version of this paper.

Supported by The financial grant K/PBW/000067 of the Polish Ministry of Science and Higher Education

Correspondence to: **Dr. Tomasz Brzozowski, Professor, Chairman, Department of Physiology, Jagiellonian University Medical College, 16 Grzegorzeczka Street, 31-531 Cracow, Poland.** mpbrzozo@cyf-kr.edu.pl

Telephone: +48-12-4211006 Fax: +48-12-4222014

Received: February 21, 2011 Revised: June 15, 2011

Accepted: June 22, 2011

Published online: September 28, 2011

Abstract

AIM: To determine the effect of non-selective cyclooxygenase (COX) inhibitors, selective COX-2 inhibitors and nitric oxide (NO)-releasing aspirin in the healing of ulcerative colitis.

METHODS: Rats with 2,4,6 trinitrobenzenesulfonic acid (TNBS)-induced colitis received intragastric

(ig) treatment with vehicle, aspirin (ASA) (a non-selective COX inhibitor), celecoxib (a selective COX-2 inhibitor) or NO-releasing ASA for a period of ten days. The area of colonic lesions, colonic blood flow (CBF), myeloperoxidase (MPO) activity and expression of proinflammatory markers COX-2, inducible form of nitric oxide synthase (iNOS), IL-1 β and tumor necrosis factor (TNF)- α were assessed. The effects of glyceryl trinitrate (GTN), a NO donor, and 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide, onopotassium salt (carboxy-PTIO), a NO scavenger, administered without and with ASA or NO-ASA, and the involvement of capsaicin-sensitive afferent nerves in the mechanism of healing the experimental colitis was also determined.

RESULTS: Rats with colitis developed macroscopic and microscopic colonic lesions accompanied by a significant decrease in the CBF, a significant rise in colonic weight, MPO activity and plasma IL-1 β and TNF- α levels. These effects were aggravated by ASA and 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC-560), but not celecoxib and counteracted by concurrent treatment with a synthetic prostaglandin E₂ (PGE₂) analog. Treatment with NO-ASA dose-dependently accelerated colonic healing followed by a rise in plasma NO_x content and CBF, suppression of MPO and downregulation of COX-2, iNOS, IL-1 β and TNF- α mRNAs. Treatment with GTN, the NO donor, significantly inhibited the ASA-induced colonic lesions and increased CBF, while carboxy-PTIO or capsaicin-denervation counteracted the NO-ASA-induced improvement of colonic healing and the accompanying increase in the CBF. These effects were restored by co-treatment with calcitonin gene related peptide (CGRP) and NO-ASA in capsaicin-denervated animals.

CONCLUSION: NO-releasing ASA, in contrast to ASA,

COX-1 inhibitors, and SC-560, accelerated the healing of colitis *via* a mechanism involving NO mediated improvement of microcirculation and activation of sensory nerves releasing CGRP.

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Key words: Nitric oxide-releasing aspirin; Colitis; Cyclooxygenase-2; Aspirin; Celecoxib; Colonic blood flow; Interleukin-1 β ; Tumor necrosis factor- α

Peer reviewers: Dr. Paulino Martínez Hernández Magro, Department of Colon and Rectal Surgery, Hospital San José de Celaya, Eje Vial Norponiente No 200-509, Colonia Villas de la Hacienda, 38010 Celaya, México; Benjamin Perakath, Professor, Dr., Department of Surgery Unit 5, Christian Medical College, Vellore 632004, Tamil Nadu, India

Zwolinska-Wcislo M, Brzozowski T, Ptak-Belowska A, Targosz A, Urbanczyk K, Kwiecien S, Sliwowski Z. Nitric oxide-releasing aspirin but not conventional aspirin improves healing of experimental colitis. *World J Gastroenterol* 2011; 17(36): 4076-4089 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4076.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4076>

INTRODUCTION

In humans inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease, considered as chronically relapsing disorders. The pathogenesis of IBD is complex, with individuals displaying a genetic predisposition, specific immunological properties of the gastrointestinal (GI) mucosa and the type of GI microflora all being involved^[1-4]. The features of animal models of colitis do not necessary mimic the human scenario of UC with the use of chemical stimuli and the magnitude of inflammatory changes. However, it is believed that an increase in mucosal prostaglandin (PG) synthesis, mainly cyclooxygenase (COX)-2 derived, correlates with the disease activity of human IBD and experimental colitis in rats^[5]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used in the treatment of pain and inflammation, but are also recommended prophylactically and used as therapeutic strategies against both neurological and cardiologic disorders. The therapeutic effects and side effects of NSAIDs in the gut are the consequence of the inhibition of COX activity, which is the key enzyme in the biosynthesis of prostanoids from arachidonate^[6]. It was reported that COX-2 is undetectable or expressed at very low levels in the healthy GI mucosa of humans and animals but is upregulated in the GI tract of individuals with inflammatory conditions^[7]. Thus, it has been suggested that the anti-inflammatory action of NSAIDs depend on the inhibition of COX-2 activity, whereas side effects, such as gastrointestinal damage and renal toxicity, is a consequence of COX-1 inhibition^[8,9]. It is believed that COX-2 derived PG play an important role in the healing

process of colitis, which is similar to that observed for the mechanisms of gastric ulcers^[10,11]. Results of studies on the influence of COX-2 inhibitors in experimental colitis and human IBD thus far have provided conflicting evidence for the exacerbation of colitis, and the attenuation of inflammation by COX-2 inhibitors^[12,13].

The mechanism of ulcerogenic activity of NSAIDs on the healing of the intestinal and colonic lesions has not been fully explained. Reuter *et al*^[12] reported that NSAIDs can exacerbate colitis by a mechanism attributable to the suppression of COX-2 derived PG synthesis. Tanaka *et al*^[14] showed that the non-selective COX inhibitor indomethacin, suppressed the mucosal prostaglandin E₂ (PGE₂) level, which in turn caused intestinal damage that was accompanied by the upregulation of COX-2 expression. It should be emphasized that neither a selective COX-1 inhibitor [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC-560)], nor a COX-2 selective inhibitor (rofecoxib) when applied alone caused intestinal damage^[8,15,16]. Recent evidence indicates that incorporation of a nitric oxide (NO) generating moiety into the basic structure of NSAIDs, such as aspirin, attenuates the ulcerogenic activity of native NSAID^[17]. Under basal conditions, NO derived from the activity of constitutive NO synthase (cNOS) contributes to the maintenance of intestinal integrity and the control of intestinal motility^[2,18].

In human IBD and experimental colitis, the inducible form of nitric oxide synthase (iNOS), excessive production of NO derived from iNOS and abundant amounts of toxic peroxynitrite are observed^[2]. On the other hand, NO was shown to possess anti-inflammatory properties and contributes to the resolution of intestinal inflammation^[18]. Moreover, iNOS deficient mice with colitis were more susceptible to inflammation as compared to animals with normal iNOS expression^[3]. The role of NO released from NO-NSAIDs in the mechanism of gastroprotection has been well documented^[19-21], but the efficacy of NO-aspirin (ASA) in the healing of experimental colitis has yet to be determined.

In this study, we focused mainly on the mechanism of healing of colitis by NO-ASA and therefore we compared the effect of vehicle, conventional NSAIDs such as ASA and indomethacin, SC-560 (a selective COX-1 inhibitor), celecoxib (a selective COX-2 inhibitor) with the new derivative of ASA, NO-releasing ASA, on the intensity of inflammation and the accompanying alterations in the colonic blood flow (CBF), myeloperoxidase (MPO) activity and expression of mRNA for COX-2, IL-1 β , tumor necrosis factor (TNF)- α and iNOS and their activities in rats with trinitrobenzenesulfonic acid (TNBS)-induced colitis. An attempt was made to determine the involvement of NO in the mechanism of healing of colitis by using NO-ASA and ASA-treated rats with 2-(4-carboxyphenyl)-4, 5-dihydro-4, 4, 5, 5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide, onopotasium salt (carboxy-PTIO), which is a NO scavenger and glyceryl trinitrate (GTN), an NO donor, respectively.

Also, the importance of sensory nerve neurotransmitters in the healing of colitis were determined in rats by the functional ablation of sensory nerves by capsaicin, in the absence and the presence of NO-derivative of ASA.

MATERIALS AND METHODS

Animal studies were carried out on male Wistar rats weighing 180-220 g. The animals had free access to water and food and were adapted to laboratory conditions and 12 h day/night cycles for 10 d after TNBS administration. The study was approved by the local Ethical Committee at the Jagiellonian University Medical College in Cracow, Poland and run in accordance with the Helsinki declaration.

Colitis in rats was induced by rectal administration of TNBS (Sigma, Slough, United Kingdom) at a dose of 10 mg/kg, dissolved in 50% solution of ethanol as reported in our previous study^[22]. Briefly, the animals were anaesthetized with phenobarbital (60 mg/kg ip) and TNBS was administered into the colon in a volume of 0.25 mL per rat at a depth of 8 cm from the rectum with the use of a soft polyethylene catheter. Until the moment of awakening the rats were positioned in the Trendelenburg position so as to avoid loss of the TNBS solution *via* the rectum. Animals in the control group were given 0.9% saline or in some cases 50% ethanol in the same volume, corresponding to the rats that were administered TNBS.

Animals with TNBS-induced colitis were randomized into 8 experimental groups (A-H), consisting of 6-10 rats per group. Rats received ig the following daily treatments: A: vehicle (saline); B: ASA (80 mg/kg) or indomethacin (5 mg/kg) suspended in 0.25% carboxy methylcellulose (CMC); C: SC-560 (5 mg/kg); D: celecoxib (10 mg/kg); E: NO-ASA (80 mg/kg; NicOx SA, Sophia Antipolis, France); F: PGE₂ (5 µg/kg) combined with non-selective COX-1 (ASA) and selective COX-2 (celecoxib) inhibitors; G: GTN (10 mg/kg ig), a NO donor combined with ASA and carboxy-PTIO (5 mg/kg ig) combined with NO-ASA, both NSAID administered in a dose of 80 mg/kg. In series H consisting of 30 rats, capsaicin was administered in a large neurotoxic dose of 125 mg/kg applied in three doses of 25, 50 and 50 mg/kg 14 d prior to TNBS administration to induce the functional ablation of sensory nerves as described previously^[23]. On day 15, capsaicin-denervated rats received TNBS and were subsequently given NO-ASA (80 mg/kg ig) with or without calcitonin gene related peptide (CGRP) (10 µg/kg sc), and administration was similar to those treated with vehicle (saline). The doses of NO-ASA and selective COX-1 and COX-2 inhibitors were selected on the basis of our group and others published evidence^[14,15,19-21]. In the doses used in this study, the COX-1 and COX-2 selective inhibitors failed to produce gastrointestinal lesions after single or prolonged administration. The dose of 80 mg/kg of ASA was selected based on our preliminary determination of the dose-dependency of this

conventional ASA applied in graded doses starting from 10 mg/kg up to 160 mg/kg on healing of TNBS colitis. ASA given in a dose of 10 mg/kg failed to significantly affect the healing of colitis but when this NSAID was administered ig in graded doses of 40 mg/kg, 80 mg/kg and 160 mg/kg, the area of colonic damage was significantly increased by 23%, 42% and 74%, respectively, at day 10 upon TNBS administration (data not shown). We have previously published that 80 mg/kg of ASA is equimolar to 128 mg/kg of NO-ASA and this dose of NO-ASA by itself does not influence the gastrointestinal integrity but markedly increases organ blood flow^[21].

After 1, 3, 10 and 14 d from induction of colonic lesions with TNBS, the animals were weighed and anaesthetized to determine CBF using the H₂-gas clearance technique^[22,23]. The abdominal cavity was opened and after separation of the colon, the CBF in the areas of the mucosa not affected by inflammatory lesions was measured. CBF was expressed as a percentage of the CBF in the vehicle-control rats without TNBS administration.

At the termination of the experiment, the entire colon was removed, isolated from surrounding tissues, opened along the antimesenteric border, rinsed, weighed, and processed for gross and histology determinations. The areas of colonic damage were evaluated planimetrically (Morphomat, Carl Zeiss, Berlin, Germany) by two independent researchers. Subsequently, fragments of the colon (2 mm × 10 mm) with colonic lesions were sampled, fixed with formaldehyde, embedded in paraffin and routinely stained with haematoxylin and eosin for histological assessment.

The presence and intensity of histological changes was evaluated for the following criteria: presence, area and depth of ulceration, presence and intensity of inflammatory infiltrations, ulcerations and fibrosis^[24].

Determination of plasma IL-1 β and TNF- α levels, NO concentration the mucosal generation of prostaglandin E₂ and gastric mucosal MPO activity

Immediately after CBF measurements, a venous blood sample was drawn from the vena cava and placed into EDTA-containing vials and used for the determination of plasma IL-1 β and TNF- α . Blood was collected and placed into sterile, plastic syringes, kept in ice till centrifugation. The blood samples were centrifuged at a speed of 1000 g for 10 min at a temperature of 15 °C temperature and the sera were stored at -80 °C. The serum levels of proinflammatory cytokines IL-1 β and TNF- α were evaluated with high sensitive enzyme-linked immunosorbent assay (ELISA) (Quantikine HS, R and D Systems, Minneapolis, Minn., United States) according to manufacturer's instructions. Intensity of the color reaction was estimated in the spectrophotometer Stat Fax 2100 (Awareness Technology Inc., Pal City, FL, United States) at 490 nm. The intra- and inter-assay coefficients of variation were 8.5% and 10.6%, respectively, for TNF- α , and 10.2% and 10.4%, respectively, for IL-1 β . The plasma NO concentration was quantified indi-

Table 1 Primer sequence used in reverse transcription polymerase chain reaction determination of gene expression of proinflammatory factors

Gene	Primer
<i>β-actin</i>	Upstream 5'-TTG TAA CCA ACT GGG ACG ATA TGG-3'
	Downstream 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3'
<i>IL-1β</i>	Upstream 5'-GCT ACC TAT GTC TTG CCC GT-3'
	Downstream 5'-GAC CAT TGC TGT TTC CTA GG-3'
<i>TNF-α</i>	Upstream 5'-TAC TGA ACT TCG GGG TGA TTG GTC C-3'
	Downstream 5'-CAG CCT TGT CCC TTG AAG AGA ACC-3'
<i>COX-2</i>	Upstream 5'-ACA ACA TTC CCT TCC TTC-3'
	Downstream 5'-CCT TAT TTC CTT TCA CAC C-3'
<i>iNOS</i>	Upstream 5'-CCA CAA TAG TAC AAT ACT ACT TGG-3'
	Downstream 5'-ACG AGG TGT TCA GCG TGC TCC ACG-3'

TNF: Tumor necrosis factor; COX: Cyclooxygenase; iNOS: Inducible form of nitric oxide synthase.

rectly as nitrate (NO₃⁻) and nitrite (NO₂⁻) levels using the nitrate/nitrite kit purchased from Cayman Lab, Michigan, United States as described before^{125,26}. This method is based on the Griess reaction and the generation of a chromophore absorbing at 595 nm, according to the original procedure reported previously¹²⁶. Since NO in the colonic mucosa is quickly transformed into NO₃⁻ and NO₂⁻¹²⁵, the total nitrate and nitrite concentration (NO_x) is routinely used as an index of NO production. In order to determine NO_x, the blood was withdrawn and centrifuged for 10 min at 3000 r/min, the samples were mixed with Griess reagent from the commercially available kit.

In rats with colitis treated with or without concurrent COX-1 and COX-2 inhibitors, the mucosal samples were taken by biopsy (about 200 mg) from unchanged colon mucosa without mucosal lesions to determine PGE₂ generation by radioimmunoassay (RIA) as described previously^{119,211}. Briefly, the mucosal samples were placed in preweighed Eppendorf vial with 1 mL of Tris buffer (50 mmol/L, at pH 9.5) added to each vial. The samples were finally minced (15 s) with scissors, washed and centrifuged for 10 s, with the pellet being resuspended again in 1 mL of Tris. Then, each sample was incubated on a vortex mixer for 1 min and centrifuged for 15 s. The pellets were weighed and the supernatant was transferred to a second Eppendorf vial containing indomethacin (10 mmol/L) and kept at -20 °C until the time for RIA. The capability of the mucosa to generate PGE₂ was expressed in nanograms of wet tissue weight.

Fragments of colonic tissue weighing about 200 mg were collected and frozen in -70 °C for the determination of MPO activity by ELISA as reported before¹²².

Expression of COX-2, IL-1β, TNF-α and iNOS transcripts in the rat colonic mucosa determined by reverse transcriptase-polymerase chain reaction

The mRNA expression for COX-2, IL-1β, TNF-α and iNOS were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) in the unchanged colon mucosa of intact rats or those with TNBS colitis given vehicle, ASA and NO-ASA. Samples of the colon

mucosa (about 200 mg) were scrapped off into ice using glass slides and then immediately snapped frozen in liquid nitrogen and stored at -80 °C. The total RNA was isolated from the colon mucosa according to the technique using Trizol Reagent (Invitrogen, Carlsbad, United States) and the manufacturer's protocol¹²⁷. The first strand of cDNA was synthesized from total cellular RNA (2 μg) using a Reverse Transcription System (Promega, Madison, United States). The PCR was carried out in an automatic DNA thermal cycler, using 1 μg of cDNA and Promega PCR reagents. For amplification of β-actin, COX-2, TNF-α, IL-1β and iNOS cDNA, and gene-specific primers (SIGMA-Aldrich St. Louis, United States) were used. The sequences for primers used in this study are presented in Table 1. Primer annealing was carried out as follows: at 56 °C, 60 °C, 60 °C and 58 °C for COX-2, IL-1β, TNF-α and iNOS, respectively. Amplification of the control rat β-actin was performed on the same samples to verify the RNA integrity. PCR products were separated by electrophoresis in 2% agarose gel containing 0.5 μg/mL ethidium bromide and then visualized under UV light. Location of the predicted PCR product was confirmed by using O'Gene Ruler 50 bp DNA ladder (Fermentas, Life Sciences, San Francisco, United States) as a standard marker. Comparison between different treatment groups was made by determination of COX-2, IL-1β, TNF-α and the iNOS/β-actin ratio of the immunoreactive area by densitometry (Gel-Pro Analyzer, Fotodyne Incorporated, Hartland, WI, United States).

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was done using Student *t* test or analysis of variance and two-way ANOVA test with Tukey *post hoc* test where appropriate. Differences of *P* < 0.05 were considered significant.

RESULTS

Effect of vehicle, non-selective and selective COX-1 and COX-2 inhibitors on TNBS-induced colitis and accompanying changes in CBF and MPO activity

Intrarectal administration of TNBS caused severe damage to the colonic mucosa manifested by inflammatory changes in the colon with extensive ulcerations of the mucosa. The area of these lesions was at a maximum at 24 h, it was not significantly decreased on day 3 but then it significantly declined at day 10 and day 14 (Figure 1). The CBF was decreased by about 46% and 43% on day 1 and 3, respectively, but it was significantly increased on day 10 and 14 (Figure 1). The 10 d administration of NO-ASA applied ig in gradual concentrations ranging from 20 mg/kg up to 120 mg/kg produced a dose-dependent decrease in the area of colonic lesions and this effect was accompanied by a significant rise in plasma NO_x concentrations and CBF (Figure 2). In contrast, after 10 d of ASA, indomethacin and SC-560 administration, a significant aggravation of the area of colonic lesions and a significant fall in CBF when

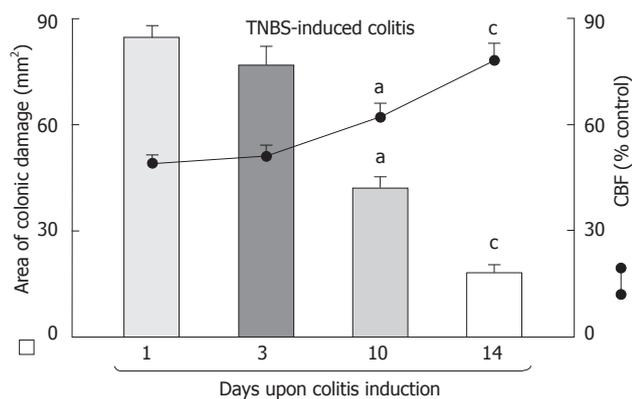


Figure 1 Time-sequence of the healing of trinitrobenzenesulfonic acid-induced colonic lesions, and accompanying changes in the colonic blood flow following day 1, 3, 10 and 14 upon induction of colitis. The area of trinitrobenzenesulfonic acid-induced lesions was maximal on day 1 and then it significantly declined on day 10 and day 14, respectively. Mean \pm SE of 6-8 rats. ^a*P* < 0.05 vs values obtained on day 3; ^c*P* < 0.05 vs values on day 3 and day 10. TNBS: Trinitrobenzenesulfonic acid; CBF: Colonic blood flow.

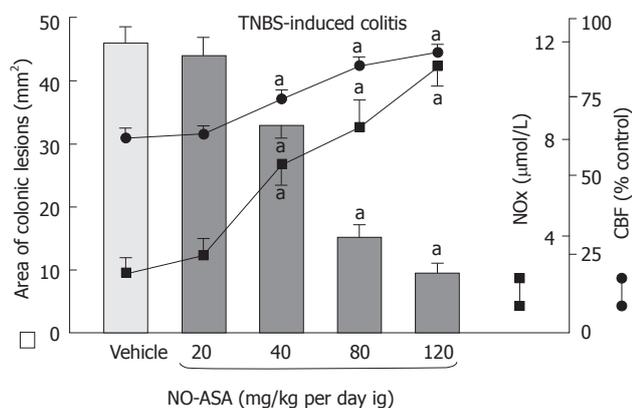


Figure 2 The effect of 10 d administration of nitric oxide-aspirin, applied ig in gradual concentrations ranging from 20 mg/kg up to 120 mg/kg on the area of colonic lesions, alterations in colonic blood flow and plasma NOx concentrations in rats with trinitrobenzenesulfonic acid-induced colitis on day 10. Mean \pm SE of 6-8 rats. ^a*P* < 0.05 vs vehicle (control) and animals treated with a dose of 20 mg/kg nitric oxide-aspirin (NO-ASA). TNBS: Trinitrobenzenesulfonic acid; CBF: Colonic blood flow.

compared with vehicle was observed (Figure 3). Similarly as shown in Figure 2, the treatment with NO-ASA applied in a dose of 80 mg/kg ig produced a significant decrease in the area of colonic damage and significantly increased the CBF comparing to vehicle-control (Figure 3). Treatment with celecoxib also significantly decreased the area of colonic damage and produced a significant increase in CBF; however, these changes were significantly less pronounced as compared to those achieved with NO-releasing ASA (Figure 3). Ten days after colitis induced by TNBS treatment all animals had a significant reduction in body weight compared with the control rats without TNBS administration and this loss of body weight was counteracted by treatment with NO-ASA. In contrast, treatment with ASA and indomethacin failed to attenuate the loss of weight induced by TNBS administration (data not shown). As shown in Figure 4A, the intact colonic mucosa

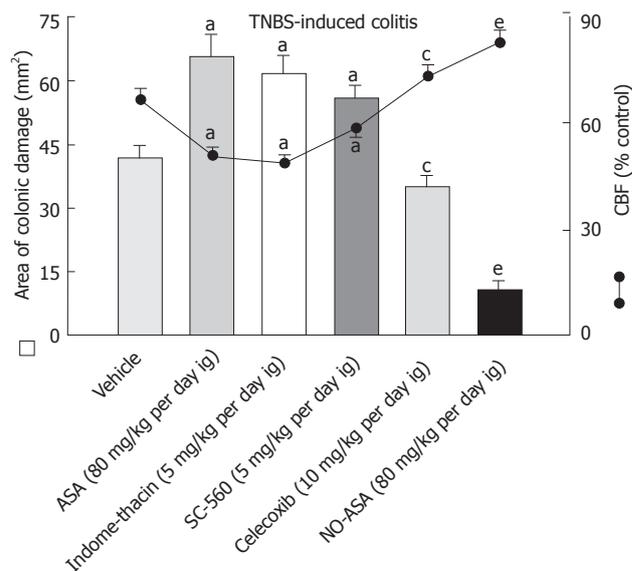


Figure 3 Effect of administration of vehicle, aspirin or indomethacin, non-selective cyclooxygenase inhibitors, a selective cyclooxygenase-1 inhibitor 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole, a selective cyclooxygenase-2 inhibitor (celecoxib) and nitric oxide releasing aspirin, on the area of colonic lesions and alterations in colonic blood flow on day 10 after colitis induction. Mean \pm SE of 6-8 rats. ^a*P* < 0.02 vs vehicle (control); ^c*P* < 0.05 vs aspirin (ASA), indomethacin- and 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC-560) groups; ^e*P* < 0.02 vs vehicle, ASA, indomethacin, SC-560 and celecoxib groups. CBF: Colonic blood flow; NO-ASA: Nitric oxide-ASA; TNBS: Trinitrobenzenesulfonic acid.

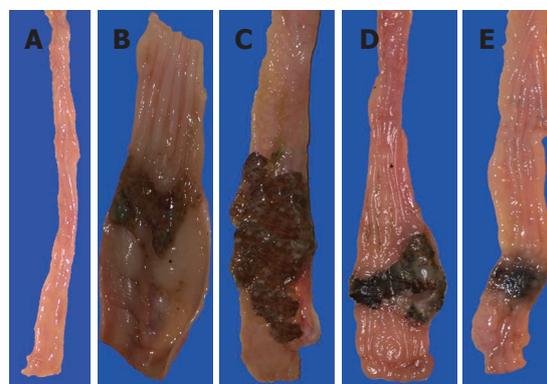


Figure 4 Gross appearance of the intact colon (A), and that of trinitrobenzenesulfonic acid-induced colitis rats treated with vehicle (B), aspirin (C), celecoxib (D) and nitric oxide-aspirin (E) at day 10 of colitis induction. In aspirin-treated rats (C) the area of colonic damage was larger than in the control trinitrobenzenesulfonic acid rats, which were treated with vehicle (B). In the celecoxib group (D), the area of colonic damage was significantly smaller when compared to the aspirin (ASA) and vehicle groups. The healing of colonic lesions was significantly improved in nitric oxide-ASA treated rats as documented by the small ulceration area and scar formation.

showed a normal macroscopic appearance. At day 10 after TNBS administration, colonic damage was still observed in vehicle-control animals (Figure 4B). This gross damage, as reflected by the area of ulceration, was exacerbated by treatment with ASA but significantly reduced by treatment with celecoxib. The area of TNBS-induced damage was significantly smaller in NO-ASA-treated animals as compared to that in rats treated with vehicle or celecoxib

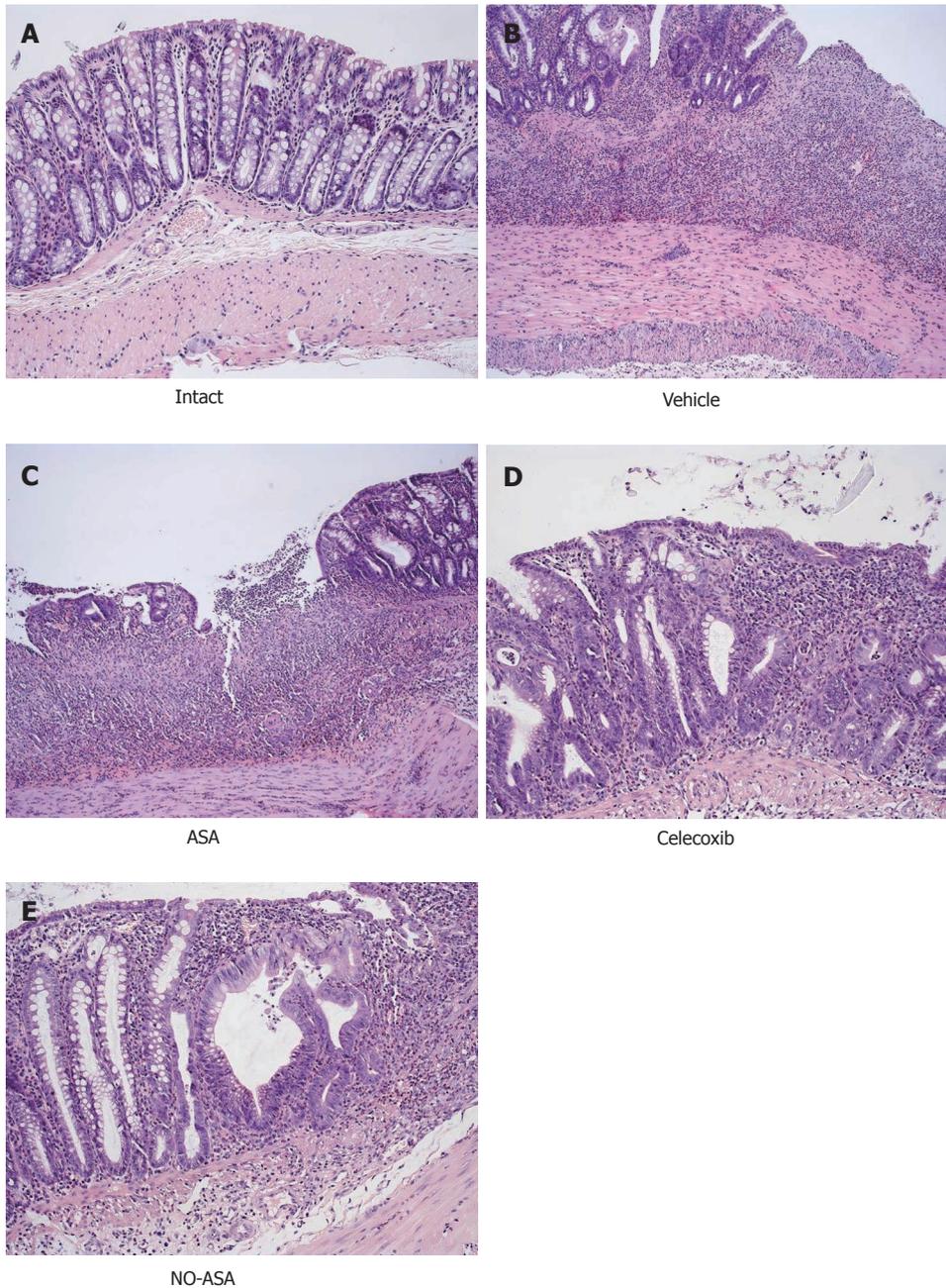


Figure 5 Histological appearance of the intact colonic mucosa (A) and that treated with vehicle (B), aspirin (C), celecoxib (D) and nitric oxide releasing aspirin on day 10 in rats with trinitrobenzenesulfonic acid-induced colitis. Intact rat colon shows regular colonic architecture and normal colonic mucosa continuity with no signs of inflammation (A). In trinitrobenzenesulfonic acid rats treated with vehicle, deep ulceration and an intense neutrophil infiltration with the presence of numerous neutrophils penetrating the muscularis mucosa and submucosa were observed. The features of regeneration adjacent to the ulcer margin are clearly visible. In aspirin (ASA)-treated rats with colitis (C), there is deep ulceration with necrosis and an intense inflammation followed by severe neutrophil infiltration and the formation of granulation tissue penetrating the muscle layer of the *muscularis propria*. Less regeneration is observed with ASA (C) than vehicle (control) animals (B). The partially healed epithelium and abnormal crypt architecture with much more pronounced regeneration was observed in the colonic mucosa of rats with colitis treated with celecoxib (D) as compared with other cyclooxygenase inhibitors. In nitric oxide (NO)-ASA treated rats with colitis (E), the most advanced healing process of colonic ulcers as reflected by scar formation, epithelial regeneration and significantly smaller neutrophil infiltration was observed. Part of the colonic crypts distant to the scar already shows a normal appearance.

(Figure 4E). By histology, intact colonic mucosa showed regular colonic architecture and continuity with no signs of inflammation (Figure 5A). In vehicle-treated TNBS rats on day 10 deep ulcerations and an intense infiltrate with the presence of numerous neutrophils penetrating the *muscularis mucosa* and submucosa were observed. In addition, features of regeneration, adjacent to the ulcer margin were

clearly visible (Figure 5B). In ASA-treated rats with colitis (Figure 5C) there was a deep ulceration with necrosis and an intense inflammation followed by severe neutrophil infiltration and formation of granulation tissue penetrating the muscle layer of the *muscularis propria*. Less regeneration was observed with ASA (Figure 5C) than in vehicle (control) colonic mucosa (Figure 5B). The area of colonic damage

Table 2 Evaluation of related markers in trinitrobenzenesulfonic acid rats treated ig with non-selective and selective cyclooxygenase-1 and cyclooxygenase-2 inhibitors or nitric oxide-aspirin throughout the period of 10 d in 6-10 rats per group (mean \pm SE)

Treatment	Weight (mg)	MPO (ng/mL)	IL-1 β (pg/mL)	TNF- α (pg/mL)
Vehicle	1150 \pm 23.1	51 \pm 8.1	42 \pm 3.4	4.8 \pm 0.6
ASA (80 mg/kg)	1580 \pm 41.4 ^a	78 \pm 9.3 ^a	65 \pm 5.1 ^a	8.5 \pm 0.95 ^a
Indomethacin (5 mg/kg)	1450 \pm 45.8 ^a	65 \pm 5.2 ^a	58 \pm 4.1 ^a	7.8 \pm 0.62 ^a
SC-560 (5 mg/kg)	1310 \pm 32.4 ^a	52 \pm 6.8 ^a	53 \pm 3.9 ^a	7.5 \pm 0.46 ^a
Celecoxib (10 mg/kg)	1082 \pm 14.9 ^c	44 \pm 4.3 ^c	36 \pm 2.2 ^c	3.8 \pm 0.33 ^c
NO-ASA (80 mg/kg)	710 \pm 12.3 ^c	35 \pm 3.2 ^c	18 \pm 1.5 ^c	2.4 \pm 0.21 ^c

MPO: Myeloperoxidase; TNF: Tumor necrosis factor; ASA: Aspirin; SC-560: 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole; NO: Nitric oxide. ^a*P* < 0.05 vs vehicle; ^c*P* < 0.05 vs vehicle, ASA, indomethacin or SC-560 groups; ^e*P* < 0.05 vs groups treated with non-selective and selective COX-1 or COX-2 inhibitors.

was significantly smaller in celecoxib-treated animals and in those treated with ASA and vehicle (Figure 5B and D). Partially healed epithelium and abnormal crypt architecture possessing a more pronounced regeneration were observed in colonic mucosa of the rat with colitis treated with celecoxib (Figure 5D) when compared to those receiving ASA. By histology, the area of colonic damage was significantly decreased in NO-ASA treated colitis rats as compared to all other COX inhibitors and vehicle-treated rats (Figure 5E). NO-ASA treated rats showed the most advanced healing of colitis, as reflected by the degree of epithelial regeneration and the significantly smaller infiltration of neutrophils. Part of the crypts, which were distant to the scar, displayed a more normal appearance (Figure 5E). In the indomethacin-group, similar to the ASA-group, the massive hemorrhagic lesions and transmural necrosis coexisting with acute inflammatory infiltrate were observed (data not shown).

As shown in Table 2, treatments with ASA, indomethacin and SC-560 were accompanied by a greater increase in the weight of colonic tissue and a significant rise in MPO activity in TNBS-treated animals as compared to vehicle control. In contrast, treatment with celecoxib decreased the weight of colonic tissue and MPO activity below those in the vehicle group; however, these changes were significantly smaller than those caused by NO-ASA (Table 2). In contrast, treatment with NO-ASA resulted in a significant decrease in the area of colonic lesions and colonic tissue weight, accompanied by a significant rise in CBF and a fall in MPO activity compared to the respective values in rats treated with vehicle or those treated with non-selective and selective COX-1 and COX-2 inhibitors (Table 2, Figure 3).

Effect of non-selective and selective COX-1 and COX-2 inhibitors on plasma proinflammatory cytokine levels and PGE₂ generation

Plasma levels of proinflammatory cytokines IL-1 β and TNF- α , which were negligible in intact rats (data not shown), were markedly elevated in animals administered

Table 3 The generation of prostaglandin E₂ in intact rats and those treated rats (mean \pm SE)

Group	PGE ₂ generation in the colonic mucosa (pg/mg tissue weight)
Intact	182 \pm 15.4
Vehicle	409 \pm 23.2 ^a
ASA (80 mg/kg per day)	130 \pm 11.7 ^c
Indomethacin (5 mg/kg per day)	124 \pm 17.8 ^c
SC-560 (5 mg/kg per day)	145 \pm 15.6 ^c
Celecoxib (10 mg/kg per day)	191 \pm 18.3 ^c
NO-ASA (80 mg/kg per day)	175 \pm 17.3 ^c

PGE₂: Prostaglandin E₂; ASA: Aspirin; SC-560: 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole; NO: Nitric oxide. ^a*P* < 0.05 vs intact colonic mucosa; ^c*P* < 0.05 vs vehicle (control); ^e*P* < 0.05 vs ASA, indomethacin and SC-560 groups.

with ASA, indomethacin and SC-560, when compared to those treated with vehicle (Table 2). Plasma IL-1 β and TNF- α levels were significantly attenuated by treatment with the COX-2 inhibitor (celecoxib) when compared to the levels of those cytokines achieved in animals treated with non-selective COX inhibitors (ASA and indomethacin). NO-ASA was administered at a similar dose to that of native ASA, which accelerated the healing of colonic damage, and significantly attenuated plasma IL-1 β and TNF- α levels as compared to those achieved with non-selective COX inhibitors (Table 2).

As shown in Table 3, the intestinal level of PGE₂ increased in the colonic mucosa of animals with TNBS colitis as compared to that measured in vehicle-controls that did not receive TNBS. Administration of ASA and indomethacin significantly reduced the colonic PGE₂ content when compared to the respective value in vehicle-treated control animals. Treatment with ASA and SC-560, the selective COX-1 inhibitor, also significantly reduced PGE₂ generation; however this inhibition was less potent than in the case of ASA and indomethacin (Table 3). Administration of the selective COX-2 inhibitor celecoxib, and NO-ASA significantly inhibited the colonic PGE₂ generation when compared to the value measured in vehicle-control rats (Table 3).

Effect of NO donor, GTN and NO scavenger carboxy PTIO on area of colonic damage and alteration in CBF in rats with TNBS colitis

As shown in Figure 6, the area of colonic damage was significantly increased in animals treated for 10 d with ASA and this effect was accompanied by a significant fall in CBF as compared with the respective values observed in vehicle-control animals. Concurrent treatment with GTN, which by itself significantly attenuated the area of colonic damage markedly decreased these lesions and significantly improved the CBF.

Figure 7 shows the effect of 10 d administration of NO-ASA applied ig at a dose of 80 mg/kg on the area of colonic damage and accompanying alterations in CBF. Treatment with NO-ASA caused a similar decrease in

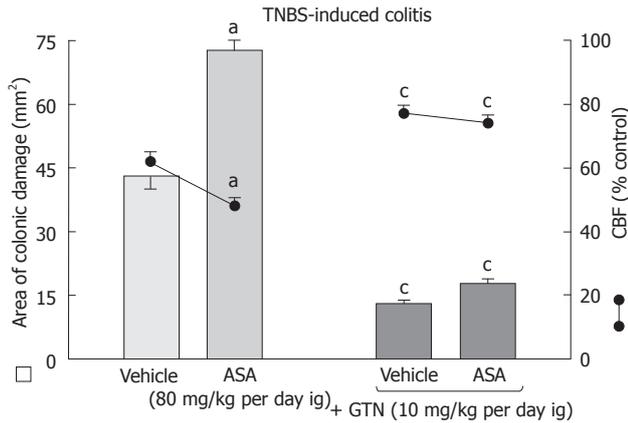


Figure 6 The effect of concurrent administration of nitric oxide donor glyceryl trinitrate on the area of colonic damage and alterations in colonic blood flow in rats with trinitrobenzenesulfonic acid-induced colitis, treated with vehicle or aspirin, on day 10. Mean ± SE of 6-8 rats. ^a*P* < 0.02 vs vehicle (control); ^c*P* < 0.02 vs vehicle or aspirin alone. TNBS: Trinitrobenzenesulfonic acid; ASA: Aspirin; GTN: Glyceryl trinitrate; CBF: Colonic blood flow.

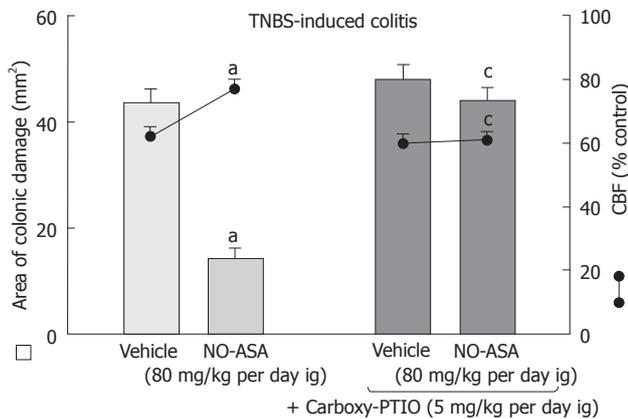


Figure 7 The effect of concurrent administration of nitric oxide scavenger 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxo-3-oxide, monopotassium salt on the area of colonic damage and alterations in colonic blood flow in rats with trinitrobenzenesulfonic acid-induced colitis, treated with vehicle or nitric oxide-aspirin (80 mg/kg per day ig) on day 10. Mean ± SE of 6-8 rats. ^a*P* < 0.05 vs vehicle group (control); ^c*P* < 0.05 vs vehicle or nitric oxide-aspirin (NO-ASA) without 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxo-3-oxide, monopotassium salt. TNBS: Trinitrobenzenesulfonic acid; CBF: Colonic blood flow.

area of colonic damage and a significant rise in CBF as presented in Figure 2. The concomitant treatment of carboxy-PTIO, the NO scavenger, which by itself had no influence on the CBF, completely abolished the beneficial effect of NO-ASA on healing of TNBS-induced colonic lesions and reversed an increase in CBF induced by this agent (Figure 7).

Effect of replacement therapy with exogenous PGE₂ on TNBS-induced colonic damage and changes in the weight of colonic tissue, CBF, plasma IL-1 β and TNF- α levels in rats treated with COX inhibitors

The role of PGE₂ in the process of healing of colonic lesions in TNBS-induced colitis animals given ASA and celecoxib was determined using rats exogenously admin-

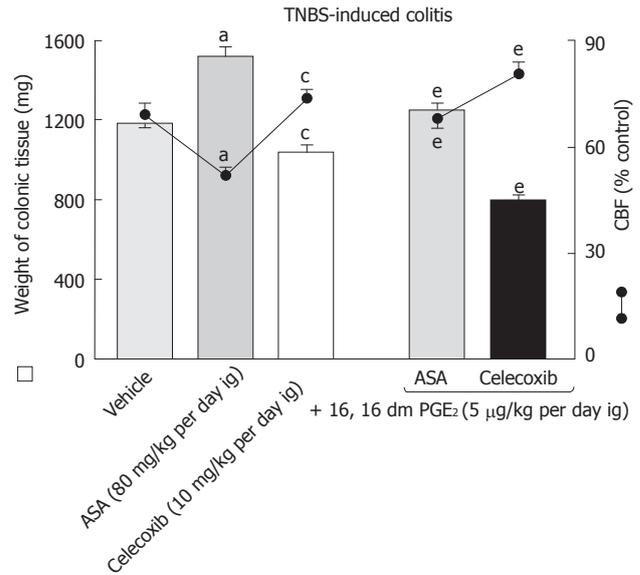


Figure 8 The weight of colonic tissue and alterations in colonic blood flow on day 10 after induction of trinitrobenzenesulfonic acid colitis in rats treated with aspirin or celecoxib with or without 16, 16 dm prostaglandin E₂ (5 µg/kg per day ig). Mean ± SE of 6-8 rats. ^a*P* < 0.02 vs vehicle (control); ^c*P* < 0.05 vs vehicle (control) and aspirin (ASA) groups; ^e*P* < 0.05 vs ASA and celecoxib groups without concurrent prostaglandin E₂ (PGE₂) administration. TNBS: Trinitrobenzenesulfonic acid; CBF: Colonic blood flow.

istered with PGE₂ in doses of 5 µg/kg added to both COX-1 and COX-2 inhibitors. An increase in both the area of colonic lesions and colonic weight as well as a significant fall in CBF induced by ASA and celecoxib were counteracted by concomitant treatment with exogenous PGE₂ (Figures 8 and 9). Moreover, the administration of this synthetic analogue of PGE₂ not only reduced the area of colonic damage but also significantly suppressed the rise in plasma IL-1 β and TNF- α compared to those in ASA- or celecoxib-treated rats without PGE₂ administration (Figure 8).

Effect of NO-ASA on TNBS-induced colonic damage and alterations in CBF in rats with capsaicin induced sensory denervation

Capsaicin-deactivation of sensory nerves, which by itself increased the area of colonic lesions and produced a significant fall in CBF when compared to those in vehicle-controlled rats, significantly attenuated the NO-ASA induced acceleration of healing of these colonic lesions and the accompanying increase in CBF (Figure 10). Concurrent administration of CGRP (10 µg/kg sc) with NO-ASA restored the healing of colonic damage as reflected by the significant decrease in colonic damage and the increase in CBF induced by this NO-derivative of ASA in rats with capsaicin denervation.

Effect of vehicle, ASA and NO-ASA treatments on the mucosal expression of COX-2, IL-1 β , TNF- α and iNOS in rats with colitis

As shown in Figure 11 (left panel), the signal for the expression of COX-2, IL-1 β , TNF- α and iNOS was significantly increased in vehicle-treated colonic mucosa (lane 2) in rats

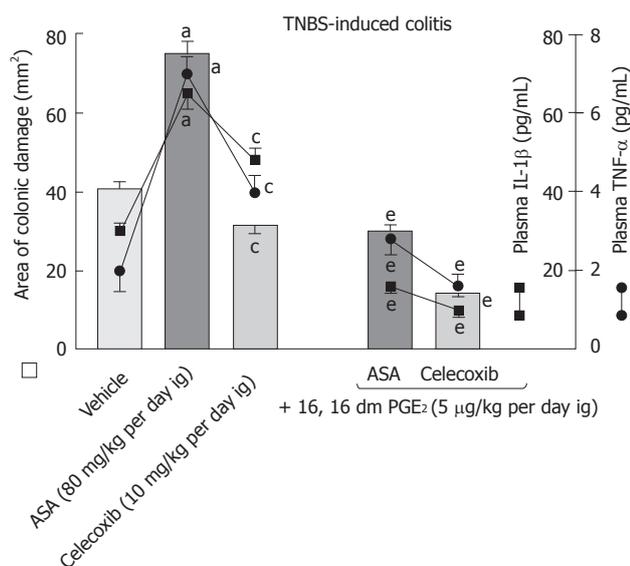


Figure 9 The area of colonic lesions and plasma levels of proinflammatory cytokines IL-1 β and tumor necrosis factor- α on day 10 after induction of colitis in rats treated with aspirin or celecoxib with or without 16, 16 dm prostaglandin E $_2$ (5 μ g/kg per day ig). Mean \pm SE of 6-8 rats. ^a*P* < 0.02 vs vehicle (control); ^b*P* < 0.02 vs vehicle-treated and aspirin (ASA)-treated groups; ^c*P* < 0.02 vs ASA and celecoxib groups without concurrent prostaglandin E $_2$ (PGE $_2$) administration. TNBS: Trinitrobenzenesulfonic acid; TNF: Tumor necrosis factor.

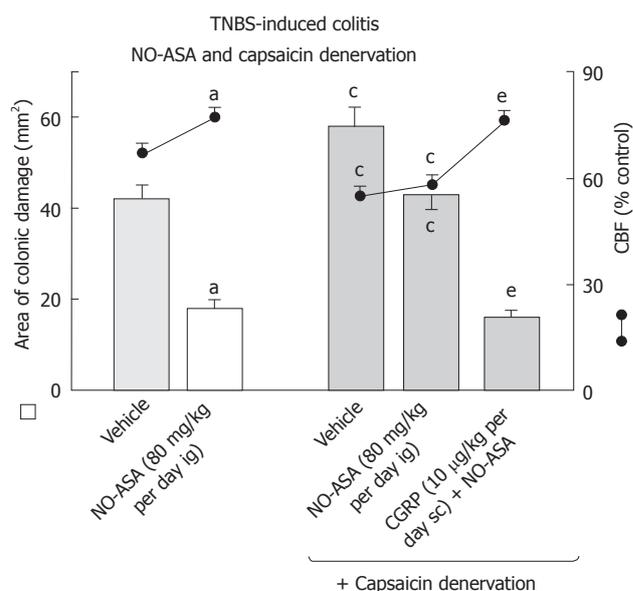


Figure 10 The area of colonic damage and changes in colonic blood flow on day 10 after colitis induction in rats with intact sensory nerves and in those with capsaicin-sensory denervation treated with vehicle (saline) or nitric oxide-aspirin (80 mg/kg per day ig) with or without administration of calcitonin gene related peptide (10 μ g/kg per day sc). Mean \pm SE of 6-8 rats. ^a*P* < 0.05 vs vehicle (control); ^b*P* < 0.05 vs trinitrobenzenesulfonic acid (TNBS) rats without capsaicin denervation; ^c*P* < 0.02 vs rats with TNBS colitis treated with nitric oxide-aspirin (NO-ASA). CGRP: Calcitonin gene related peptide; CBF: Colonic blood flow.

with colitis when compared to that in the intact mucosa (lane 1). The ratio of COX-2, IL-1 β , TNF- α and iNOS mRNA over β -actin mRNA, confirmed that expression of COX-2, IL-1 β , TNF- α and iNOS mRNAs were significantly elevated in TNBS-treated animals (Figure 11, right panel). Treatment with ASA resulted in a strong signal of mRNAs for COX-2, IL-1 β , TNF- α and iNOS. The semi-quantitative ratio of COX-2, IL-1 β , TNF- α and iNOS (lane 3) confirmed that the expression of these inflammatory markers was significantly increased in the colonic mucosa of rats treated with ASA (Figure 11, right panel). In NO-ASA-treated animals the signal for COX-2, IL-1 β , TNF- α and iNOS mRNAs was less pronounced (lane 4) and the determination of the ratio of COX-2, IL-1 β , TNF- α and iNOS confirmed that expression of mRNAs for these inflammatory factors was significantly inhibited as compared to those recorded in ASA-treated animals (Figure 11, right panel).

DISCUSSION

Worldwide, NSAIDs are among the most widely prescribed medications, and are often the drugs of choice for the treatment of various inflammatory conditions. Currently, NSAIDs, including ASA, are recommended as a prophylactic therapy against neurological and cardiologic disorders including strokes and heart infarcts^[28]. In humans, UC is a chronic relapsing disorder, characterized by colon mucosa inflammation, ulcerations, diarrhea, bloody stools and abdominal pain^[1,3]. The inflamed mucosa of the lower GI tract produces a high amount of PG derived from COX-2 expression and activity in response to stimulation by proinflammatory cytokines and growth

factors, also co-expressed at a site of inflammation^[14]. Although the NSAIDs effect on the upper GI tract is well documented, the mechanisms by which NSAIDs and their new NO-releasing derivatives affect the course and the healing of colitis in humans and experimental animals has not been fully explored. In the present study, using a rodent model of colitis we determined the effect of the new ASA derivative NO-ASA, and selective and non-selective COX-1 and COX-2 inhibitors on the healing process of this colonic damage, and the effects on weight of colonic tissue, the CBF and MPO activity. Moreover, we assessed the colonic expression of COX-2 which in contrast to COX-1 expression is negligible in normal GI mucosa, but has been shown to be significantly upregulated in most GI-related disorders such as gastritis, gastric mucosal damage, ulcers and ulcerative colitis^[3,11,14,29].

Interestingly, the inhibition of COX-2 activity by selective COX-2 inhibitors enhances gastric damage induced by stress and ischemia-reperfusion and delays the healing process of gastric ulcers in the GI tract^[29,30]. Moreover, a single application of either COX-1 or COX-2 inhibitors does not cause GI damage, but concurrent treatment with inhibitors of COX-1 and COX-2 activity, resulted in both gastric and intestinal damage. For instance, administration of celecoxib alone or SC-560 alone failed to cause gastric damage, but administration of both selective COX-1 together with COX-2 inhibitors resulted in the formation of gastric mucosal injury. It was concluded that both COX-1 and COX-2 are essential for the maintenance of the integrity of the

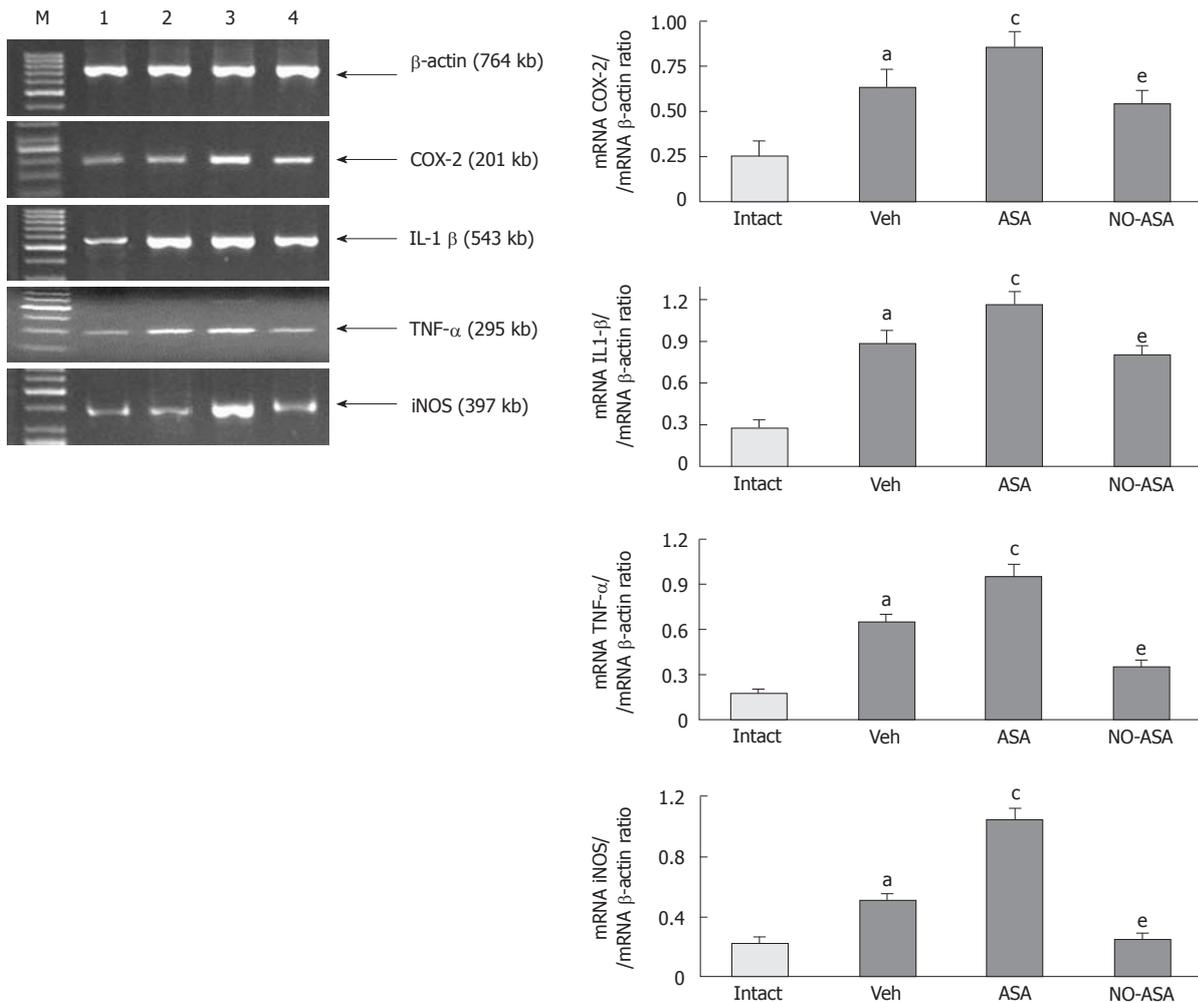


Figure 11 The expression of mRNA for cyclooxygenase-2, IL-1 β , tumor necrosis factor- α and inducible form of nitric oxide synthase in colonic mucosa of intact rats (lane 1) those treated with vehicle (lane 2), aspirin (lane 3) and nitric oxide-aspirin (lane 4) at day 10 after induction of colitis. Mean \pm SE of 4 determinations in 4 rats. ^a $P < 0.05$ vs intact colonic mucosa; ^c $P < 0.05$ vs rats with trinitrobenzenesulfonic acid colitis administered with vehicle (Veh); ^e $P < 0.05$ vs vehicle and aspirin (ASA) groups. COX: Cyclooxygenase; TNF: Tumor necrosis factor; iNOS: Inducible form of nitric oxide synthase; M: Marker.

upper GI-tract^[7,31]. Tanaka *et al.*^[31] have shown that only TNF- α was influenced by the administration of SC-560 or celecoxib but other cytokines were not affected in mice models of dextran sulphate (DSS)-induced colitis. In their model of colitis produced by adding 3% DSS to drinking water, the inhibition of both COX-1 and COX-2 resulted in exacerbation by these NSAIDs of a widespread intestinal inflammation in mice. This model has, however, different histological appearance characteristics and time course of pathology^[31] from that used in our present study^[31]. We have utilized a colitis model with TNBS which rather mimics some features of human UC and Crohn's disease (CD) which do not readily apply to the DSS model.

Expression of TNF- α is considered an important pathogenic feature of human IBD, especially CD^[13]. Another cytokine, IL-1 β , plays an immunoregulatory role in amplifying the inflammatory response by inducing the cascade activation of immune cells. In high doses, IL-1 β is responsible for the formation of epithelial cell necrosis, edema and neutrophil infiltration^[24]. In our

study TNBS-induced colonic damage was accompanied by a prominent increase in colon tissue weight as well as the rise in the MPO, the gene expression and the plasma levels of both IL-1 β and TNF- α . This data is in keeping with previous observations^[12] that pathogenesis of colitis is associated with an increase in expression and activity of TNF- α and IL-1 β . Furthermore, treatment with the non-selective COX-1 and COX-2 inhibitors such as ASA or indomethacin in our study produced a further rise in plasma IL-1 β and TNF- α levels. In contrast, the administration of celecoxib moderately improved the healing of colitis followed by a minor increase in plasma IL-1 β and TNF- α levels, while a significant improvement of this healing, accompanied by the suppression of these proinflammatory cytokines, was observed in NO-ASA-treated animals. We have also found significant differences not only in macroscopic and microscopic appearance of the colonic mucosa treated with the selective COX-2 inhibitor and NO-releasing ASA *vs* the conventional NSAID (aspirin) on healing of colonic lesions but also in functional alterations such as CBF and MPO activity.

We documented that an increase in plasma levels of IL-1 β and TNF- α in colitis was reduced to a greater extent by treatment with NO-ASA and to a lesser extent by a COX-2 inhibitor, which was not the case with the non-selective COX inhibitor, ASA. In ASA-treated rats, the expression and activity of these cytokines were both enhanced. The involvement of PG in healing of colitis is supported by our finding that an exogenously administered synthetic analog of PGE₂ reversed the delay in this healing, the increase in colonic weight and the rise in plasma levels of both IL-1 β and TNF- α induced by ASA and celecoxib. It is concluded that the lack of intestinal PGE₂ plays a crucial role in the pathomechanism of exacerbation of inflammatory colonic lesions and the perturbations in CBF in animals administered non-selective COX inhibitors but not to the same extent as in the case of selective COX-2 inhibitors. This is in keeping with the observation that PGE₂ is essential to the process of regeneration of epithelial crypts during the time course of DSS-induced colitis^[32]. In another report, PGE₂ was shown to inhibit production of proinflammatory cytokines, particularly that of TNF- α ^[33]. We found an apparent increase in MPO activity, which is considered to be an indicator of neutrophil-induced inflammatory infiltration, which was significantly elevated in colitis. The colonic MPO activity was further enhanced by non-selective COX inhibitors and selective COX-1 inhibitors such as ASA, indomethacin and SC-560, respectively, while both celecoxib and especially NO-ASA attenuated MPO activity in colitis rats treated with vehicle. This deleterious effect of non-selective COX-inhibitors, ASA and indomethacin, observed in our study could not be attributed to GI toxicity of these NSAIDs, since e.g. indomethacin was used in our study in a dose which by itself failed to induce gastric mucosal lesions but as shown before, prolonged the healing of acute and chronic gastric ulcers, mostly due to inhibition of protective PG^[19,21]. Indomethacin was previously reported to induce experimental colitis at doses up to 10 mg/kg^[34] that was higher than that in our present study.

We were particularly interested in exploring the mechanism of accelerated colonic healing of the new class of so-called “safer NSAID” such as NO-ASA. In our study the TNBS-induced colitis was associated with a fall in CBF, but this impairment in the colonic microcirculation was worsened by native ASA and indomethacin. Celecoxib moderately enhanced colonic healing and slightly increased CBF, suggesting that this COX-2 inhibitor exerts the opposite effect on colonic microcirculation than non-selective COX inhibitors. In clear contrast, NO-ASA greatly improved CBF and reduced both MPO activity and the expression of mRNAs and reduced plasma IL-1 β and TNF- α levels thus contributing to the process of healing of inflammatory lesions. We conclude that these healing and anti-inflammatory effects could be attributed to NO being released from NO-ASA, which ultimately was responsible for the improvement of CBF observed in our study. This is supported by the fact that

GTN which is an NO donor, when co-administered with ASA, significantly reduced colonic damage and counteracted the fall in CBF induced by this NSAID. Moreover, the mechanism of the acceleration of colonic healing involves the release of NO from NO-ASA. This notion is supported by the observation that NO-ASA dose-dependently accelerated healing of these lesions followed by an increase in plasma NO_x levels. Second, carboxy-PTIO, an NO-scavenger, abolished the healing efficacy of this NO-derivative of ASA and the accompanying increase in CBF. In addition, NO released from NO-ASA could contribute to the intestinal elimination of bacteria, protozoa and fungi as reported previously^[35]. In other studies, the addition of a NO-releasing moiety to mesalamine exerted an immunomodulatory effect and suppressed intestinal inflammation by inhibiting T-helper cells while stimulating the activity of the anti-inflammatory cytokine IL-10, TGF β and mucosal Treg pathway whereas standard mesalamine was less effective^[36].

It is known that the maintenance of GI mucosal integrity depends on different protective mechanisms against injury, which involves the stimulation of two populations of afferent nerves, vagal and spinal. As shown previously, these sensory neurons are involved in GI protection *via* activation of vasodilatory and anti-inflammatory reflexes^[37]. It was reported that the afferent neurons from the dorsal root ganglia play a role in the local regulation of GI circulation, secretion, motility and the process of mucosal repair and healing after injury^[37]. CGRP, a neurotransmitter, is released from the peripheral fibers of sensory neurons and plays an important role in GI mucosal defense^[37]. Capsaicin, an active ingredient of red pepper has been found to act on the capsaicin sensitive afferent nerves releasing CGRP^[38]. When applied in small doses, capsaicin induces a protective response, but a large dose of this neurotoxin renders the gastric mucosa more susceptible to damage induced by indomethacin, ischemia and reperfusion and cold stress^[23,26]. This has also been shown to result in a delay in the process of gastric ulcer healing, an event associated with decreased tissue levels of gastric CGRP^[37].

In our study, capsaicin-induced functional ablation of sensory nerves with capsaicin, which by itself markedly prolonged the healing process of colonic lesions, attenuated the increase in the healing of these lesions and the accompanying rise in CBF induced by NO-ASA. The impaired healing and evident fall in CBF were restored by concurrent administration of CGRP with NO-ASA in capsaicin-denervated rats. These findings suggest that sensory nerve neuropeptides such as CGRP may contribute to the healing effect and hyperemia induced by NO-ASA. Both CGRP and NO contribute to hyperemia in the GI tract mucosa and facilitate other mechanisms of defense, such as bicarbonate secretion^[21,23]. Existing evidence in the lower GI tract revealed an increased inflammatory reaction in experimental colitis of transient receptor potential vanilloid-1 (TRPV-1) knockout mice^[37,38]. TRPV-1 is expressed by many afferent nerves and its activation results

in the release of vasodilatory peptides such as CGRP^[38,39].

In summary, conventional NSAIDs, such as ASA and indomethacin or selective COX-1 inhibitors such as SC-560^[40], delay the healing of experimental colitis and this effect is accompanied by a fall in CBF and an enhancement in gene expression and release of proinflammatory cytokines IL-1 β and TNF- α . These deleterious effects are less pronounced with the use of the selective COX-2 inhibitor, celecoxib, which differs with respect to colonic healing with that of conventional ASA. NO-ASA exerts the opposite effects to those of native ASA and selective COX-1 inhibitors on the delay in healing of TNBS colitis and accompanying fall in colonic microcirculation induced by these agents. The beneficial healing action of NO-ASA, involves the NO mediated suppression of proinflammatory cytokines and the activation of sensory nerves resulting in a local release of sensory vasodilatory neuropeptides such as CGRP.

ACKNOWLEDGMENTS

This article contains data from the paper presented in preliminary form by Zwolinska-Wcislo M, Kwiecien S, Sliwowski Z, Brzozowski T, Drozdowicz D, Mitis-Musiol M, Konturek SJ, Pawlik WW. (2008) "Classic aspirin, COX-2 selective inhibitor and NO derivative of aspirin in the mechanism of healing of ulcerative colitis" GUT 32: Abstract A124 awarded first prize at Gastroenterology XXV UEGW Meeting, Vienna 2008. Authors would like to acknowledge the careful reading of the manuscript by Mrs. Nily Osman.

COMMENTS

Background

Nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin, are among the most prescribed drugs because of their anti-inflammatory, antipyretic, analgesic effects. Inhibition of prostaglandin synthesis seems to be the major mechanism implicated in both the beneficial and adverse effects of NSAIDs. Aside from the systemic activity of NSAIDs, resulting from the inhibition of mainly cyclooxygenase (COX)-1 activity, the NSAIDs exert a deleterious influence on the upper and lower gastrointestinal (GI) mucosal integrity by affecting the local inflammatory mechanisms, such as neutrophil recruitment, the activation of proinflammatory cytokines, impairment of microcirculation and the release of free oxygen metabolites. A new class of nitric oxide (NO)-releasing NSAIDs was developed to limit the serious adverse effects associated with NSAIDs ingestion, but their effect in lower GI healing with respect to colitis in animal models has not been carefully investigated. Sensory neurons releasing neuropeptides such as calcitonin gene related peptide (CGRP) are involved in the mechanism of GI protection and ulcer healing due to activation of vasodilatory mediators such as CGRP. Currently, it is unknown whether these sensory neuropeptides could influence the healing of ulcerative colitis (UC) in animals treated with NO-releasing aspirin (ASA).

Research frontiers

Both UC and Crohn's disease belong to the category of inflammatory bowel diseases (IBD). It is believed that three major factors influence the pathogenesis of these two diseases: an individual's susceptibility, microflora of the gastrointestinal tract and immunological properties of the gastrointestinal mucosa. UC is a disease of the colon which is characterized in humans by chronic inflammation in both the mucosal and submucosal layer with a cellular and humoral immunological response. Patients suffering from UC frequently require anti-inflammatory analgesics such as NSAIDs because of inflammatory conditions

such as arthritis, sacroiliitis or osteoporosis-related fractures. On the other hand conventional NSAIDs may either induce development of colitis in the healthy colon or exacerbate preexisting colitis. Previous studies revealed that COX exists in the following two isoforms: the inducible COX-2, which is detected at the site of inflammation, and COX-1, which functions in a housekeeping fashion, is present in the majority of human tissues and organs, and is responsible for homeostasis and GI tract integrity. PGs induced by COX-2 are involved in maintaining the intestinal mucosa integrity, in the healing of gastrointestinal ulcers and the modulation of IBD. But the inhibition of both COX-1 and COX-2 isoforms seems to be necessary to induce significant intestinal damage. The new class of NSAIDs containing the NO moiety, such as NO-releasing ASA, could be an alternative strategy in the attenuation of GI side effects of conventional NSAIDs such as ASA. This is due to the fact that NO released from ASA possesses anti-inflammatory properties and contributes to the resolution of intestinal inflammation.

Innovations and breakthroughs

The authors attempted to determine the involvement of NO released from NO-ASA and its role in the mechanism of the healing of colitis. This was accomplished by using NO-ASA and glyceryl trinitrate (GTN), an NO donor, added to classic ASA to mimic the protective action of NO-ASA. Treatment with NO-ASA improved the healing of colitis, with these effects as well as the anti-inflammatory properties of NO-ASA being attributed to the NO released from NO-ASA, which was ultimately responsible for the improvement of colonic blood flow (CBF) observed in this study. Furthermore, the authors found that both NO and the activation of sensory nerve neuropeptides such as CGRP may contribute to the healing effects and the hyperemia induced by NO-ASA. This could be responsible for the attenuation of the expression and the release of pro-inflammatory cytokines such as IL-1 β and tumor necrosis factor (TNF)- α . This is supported by the fact that GTN, which is an NO donor, when co-administered with ASA, significantly decreased colonic damage and counteracted the decrease in CBF induced by this NSAID. The study demonstrates that NO-ASA is beneficial when compared to its parent drug ASA in the healing of experimental colitis. Furthermore, celecoxib, the selective COX-2 inhibitor, showed greater efficacy in healing colitis than was observed with non-selective COX-1 and COX-2 inhibitors (conventional ASA, indomethacin). However, the prolonged use of coxibs in clinical practice may be associated with prothrombotic action and increased risk of acute myocardial infarction. This significant finding of the authors with respect to selective COX-2 inhibitors and healing of ulcerative colitis requires further investigation and confirmation in clinical trials.

Applications

(1) Inhibition of both COX-1 and COX-2 isoforms by non-selective and selective COX-1 inhibitors exacerbates colonic damage and leads to functional impairment of the colonic mucosa blood flow during the process of healing of colitis; (2) The importance of PG inhibition by NSAIDs in the pathogenesis of colitis is confirmed by the finding that supplementation with exogenous PGs of animals concurrently treated with COX-1 or COX-2 inhibitors attenuated the colonic damage and the decrease of CBF induced by these agents; and (3) The NO released from ASA may be an alternative option to native ASA in patients with lower GI tract disorders such as UC.

Terminology

Incorporation of NO generating moiety into the basic structure of NSAIDs, such as aspirin (NO-ASA) attenuates the ulcerogenic activity of native NSAID. Under basal conditions, NO derived from the activity of constitutive NO synthase (cNOS) contributes to the maintenance of intestinal integrity and the control of intestinal motility. NO and CGRP exhibit a protective action against NSAIDs induced impairment of colonic healing. GTN, an NO donor, and 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide, monopotassium salt, an NO scavenger were used for the evaluation of the involvement of NO in the process of colonic healing in rats with colitis. CGRP is a sensory neurotransmitter released from the peripheral endings of afferent sensory neurons implicated in the defense mechanism of the GI mucosa. Capsaicin, an active ingredient of red pepper, has been found to induce a functional ablation of the capsaicin-sensitive afferent nerves releasing CGRP. Previous studies revealed that when applied in small doses capsaicin exerts a protective action, but large doses of this neurotoxin render the GI mucosa more susceptible to damage induced by various ulcerogens and stressors.

Peer review

This work is good, with proper design of research and interesting results.

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S- Editor Sun H L- Editor O'Neill M E- Editor Zhang DN

Inhibition of high-mobility group box 1 expression by siRNA in rat hepatic stellate cells

Wen-Song Ge, Jian-Xin Wu, Jian-Gao Fan, Yao-Jun Wang, Ying-Wei Chen

Wen-Song Ge, Jian-Xin Wu, Jian-Gao Fan, Ying-Wei Chen, Department of Gastroenterology, Shanghai Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

Yao-Jun Wang, Department of Gastroenterology, General Hospital of Jinan Military Region, Jinan 250031, Shandong Province, China

Author contributions: Ge WS, Wu JX, Fan JG, Wang YJ and Chen YW designed research; Ge WS, Wang YJ and Chen YW performed research; Wang YJ and Chen YW contributed new reagents/analytic tools; Ge WS and Chen YW analyzed data; and Ge WS, Wang YJ and Chen YW wrote the paper.

Supported by The Select and Train Outstanding Young Teachers Foundation of Shanghai, No. jdy08086 and WUJieping Experimental Diagnosis of Liver Disease Medical Foundation, No. LDWMF-SY-2011B009

Correspondence to: Ying-Wei Chen, Vice-professor, Department of Gastroenterology, Shanghai Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China. way_01chen@hotmail.com

Telephone: +86-21-25076431 Fax: +86-21-25071316

Received: July 5, 2011 Revised: September 5, 2011

Accepted: September 12, 2011

Published online: September 28, 2011

Abstract

AIM: To explore the role of high-mobility group box 1 (HMGB1) protein during liver fibrogenesis and investigate the functional effects of *HMGB1* gene silencing in hepatic stellate cells (HSCs) using siRNA.

METHODS: Hepatic fibrosis in rats was induced through serial subcutaneous injections of dimethylnitrosamine, and expression of HMGB1 was detected by immunohistochemistry. HMGB1 siRNAs were developed and transiently transfected into HSC-T6 cells using Lipofectamine 2000. HMGB1 expression was evaluated by real-time polymerase chain reaction (PCR) and Western blotting analysis. Expression of α -smooth muscle actin (α -SMA) and collagen types I and III was evaluated by real-time PCR. Cell proliferation and the cell cycle were determined

using the methyl thiazolyl tetrazolium method. Finally, collagen content in HSC supernatant was evaluated by an enzyme-linked immunosorbent assay.

RESULTS: The results showed that HMGB1 was up-regulated during liver fibrosis and that its expression was closely correlated with the deposition of collagen. siRNA molecules were successfully transfected into HSCs and induced inhibition of HMGB1 expression in a time-dependent manner. Moreover, HMGB1 siRNA treatment inhibited synthesis of α -SMA and collagen types I and III in transfected HSCs.

CONCLUSION: This study suggests a significant functional role for HMGB1 in the development of liver fibrosis. It also demonstrates that downregulation of HMGB1 expression might be a potential strategy to treat liver fibrosis.

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Key words: Hepatic fibrosis; High-mobility group box 1; Hepatic stellate cells; RNA interference

Peer reviewers: Ekihiro Seki, MD, PhD, Department of Medicine, University of California San Diego, Leichag Biomedical Research Building Rm 349H, 9500 Gilman Drive MC#0702, La Jolla, CA 92093-0702, United States; London Lucien Ooi, Professor, Chairman, Division of Surgery, Singapore General Hospital, 1 Hospital Drive, 169608, Singapore

Ge WS, Wu JX, Fan JG, Wang YJ, Chen YW. Inhibition of high-mobility group box 1 expression by siRNA in rat hepatic stellate cells. *World J Gastroenterol* 2011; 17(36): 4090-4098 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4090.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4090>

INTRODUCTION

Hepatic fibrosis is a major medical problem associated with significant morbidity and mortality. Regardless of

the underlying aetiology^[1], hepatic fibrosis is characterized by the accumulation of excess extracellular matrix (ECM). The amount of matrix deposition depends on the balance between its synthesis and degradation. When synthesis of ECM exceeds its degradation, the pathological accumulation of ECM leads to liver fibrosis. Therefore, a critical balance must be achieved between maintaining the proper amount of ECM for homeostasis, while at the same time, providing a means of ensuring that excess or improper accumulation does not occur.

High-mobility group box 1 (HMGB1) protein was originally identified as a nuclear nonhistone protein with DNA-binding domains, and it has been implicated as an important endogenous danger signaling molecule. In addition, it can be secreted from cells and exert extracellular functions as a proinflammatory cytokine^[2,3]. Increasing evidence now points to multiple functions of HMGB1 in infection, tissue injury, inflammation, apoptosis, and the immune response^[4]. HMGB1 can be released both through active secretion from various cells, including activated monocytes/macrophages, neutrophils, and endothelial cells, and through passive release from necrotic cells^[5-7]. HMGB1 can directly promote the secretion of proinflammatory cytokines [tumor necrosis factor (TNF), interleukin (IL)-1A/B, IL-6 and IL-8] and chemokines (macrophage inflammatory protein-1A/B) by peripheral blood mononuclear cells (PBMCs)^[8,9]. In turn, PBMCs also produce different cytokines that are potentially involved in virus-induced liver damage. HMGB1 acts as a chemoattractant for fibroblasts and endothelial and smooth muscle cells, which are cell types that significantly contribute to wound repair^[9,10]. Consequently, HMGB1 can directly stimulate fibroblast proliferation and participate in fibrogenesis^[4]. Additionally, inhibitors of HMGB1 significantly reduce tissue damage^[5,6]. Moreover, Hamada *et al*^[4] have reported that inhibition of HMGB1 may be beneficial in pulmonary fibrosis. Therefore, we postulated that inhibiting the up-regulation of HMGB1 during liver fibrogenesis could be a potential strategy for treating liver fibrosis.

RNA interference is known as a powerful tool for post-transcriptional gene silencing^[11] and has opened new avenues in gene therapy. In this study, we induced hepatic fibrosis in rats through serial subcutaneous injections of dimethylnitrosamine (DMN) for 4 wk and evaluated the expression of HMGB1 during the process of hepatic fibrogenesis. Additionally, siRNA molecules targeting the sequences within the rat *HMGB1* gene were transfected into hepatic stellate cell (HSC)-T6 cells. The results show that the expression of HMGB1 was correlated with collagen deposition during hepatic fibrosis and that down-regulating HMGB1 expression could prohibit collagen production and enhance collagen degradation.

MATERIALS AND METHODS

Animal models

Thirty-two 6-wk-old male Sprague-Dawley rats (230-260 g)

were purchased from the Shanghai Laboratory Animal Centre of Chinese Academy of Sciences and fed *ad libitum* with standard laboratory chow. All rats received humane care according to the Guide for the Care and Use of Laboratory Animals by the Chinese Academy of Sciences. Hepatic fibrosis was induced by intraperitoneal injections of 1% DMN (1 mL/kg body weight) for three consecutive days per week for up to 4 wk^[11]. Rats were sacrificed at 1, 2 and 3 wk from the first DMN injection. Liver tissues were either snap-frozen in liquid nitrogen or fixed in 10% formalin for histology and immunostaining.

Histological and immunohistochemical examination

Liver tissue sections were stained with hematoxylin-eosin (HE) for histopathological examination. Immunohistochemical examination was performed to detect the expression of HMGB1 and collagen types I and III in liver tissues. Briefly, the paraffin sections of left median hepatic lobes were incubated with 3% H₂O₂ in methanol at 37 °C for 10 min to quench endogenous peroxidase activity. After blocking at room temperature for 20 min, the sections were incubated with antibodies against HMGB1 (R and D Systems, Germany), collagen type I or collagen type III (Boster, Wuhan, China) overnight at 4 °C followed by incubation with horseradish-peroxidase-conjugated secondary antibody (Dako, Kyoto, Japan) at 37 °C for 20 min. Finally, the signals were detected using the Diaminobenzidine Substrate Kit (Vector Laboratories, Burlingame, CA, United States), and a positive outcome was indicated by brown staining in the cytoplasm or nucleus. For the semiquantitative analysis of HMGB1 and collagen expression, the brown-stained tissues in immunohistostaining sections were measured on an image analyzer by a technician blinded to the samples. Five fields were selected randomly from each of two sections, and six rats from each group were examined.

Double immunostaining of HMGB1 and α -smooth muscle actin

Liver sections were blocked with 5% normal goat serum after fixing and then simultaneously incubated with both monoclonal anti-HMGB1 (R and D Systems, Germany) and polyclonal α -smooth muscle actin (α -SMA) (Fremont, CA, United States) antibodies prepared in phosphate-buffered saline (PBS). The sections were incubated overnight at 4 °C or 1 h at room temperature and then washed with PBS. Sections were then simultaneously incubated with fluorescein-isothiocyanate-conjugated secondary antibody and rhodamine-conjugated secondary antibody for 30 min at 37 °C in the dark. Both primary antibodies were produced in different species. Antibody labeling was examined under a Zeiss LSM-510 laser scanning confocal microscope.

Cell culture

The HSC-T6 cell line, an immortalized rat HSC line, which has a stable phenotype and biochemical characteristics, was kindly provided by Dr. SL Friedman (Divi-

sion of Liver Diseases, Mount Sinai School of Medicine, New York, NY, United States). All cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and 5% antibiotics and incubated at 37 °C in a humidified atmosphere of 5% CO₂. Cells were seeded at 2 × 10⁵ per well in six-well plates 24 h before transfection. The amount of siRNA and transfection reagent was calculated according to the manufacturer's instructions.

Immunofluorescence study

HSC-T6 cells were cultured for 24 h on glass coverslips and fixed in 4% formaldehyde for 30 min at room temperature prior to detergent extraction with 0.1% Triton X-100 for 10 min at 4 °C. Coverslips were saturated with PBS containing 2% bovine serum albumin (BSA) for 1 h at room temperature. Next, cells were incubated with the specific primary antibody for HMGB1 (R and D Systems, Germany) in 1% BSA for 1 h, washed, and incubated with secondary antibody (TRITC AffiniPure Goat Anti-Rabbit IgG, EarthOx, LLC, United States). Finally, cells were stained for 30 min at room temperature with 4,6-diamidino-2-phenylindole. Slides were viewed with a Zeiss LSM-510 laser scanning confocal microscope.

Preparation of siRNA, construction of siRNA expression vector and transfection assay

The siRNAs for rat HMGB1 mRNA were designed and synthesised by Invitrogen Life Technologies. We prepared three siRNAs, and the most effective one was selected for construction of the siRNA expression vector. The siRNA sequences used are shown in Table 1. Negative control siRNAs were used to assess non-specific gene silencing effects, and the mock group was the non-transfection group. Cells were transfected with a mixture of plasmid DNA and Lipofectamine 2000 (Invitrogen) in Opti-MEM I medium without serum as recommended by the manufacturer. The medium was then replaced with standard RPMI medium (containing 10% FBS and gentamicin) 24 h post-transfection.

Real-time quantitative polymerase chain reaction

Total RNA was extracted at different time points after siRNA transfection using the Trizol kit (Gibco/Life Technologies) according to the manufacturer's protocol. The mixture of RNA and primers was loaded into the polymerase chain reaction (PCR) amplifier. The PCR protocol was as follows: predenaturate setting at 95 °C for 5 min, 94 °C for 45 s, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. The PCR was performed for 40 cycles followed by a final extension at 72 °C for 10 min. We then visualized the PCR product by running it on a 1.5% agarose gel and quantitatively analysed it with Lab Works 4.5 analysis software.

Western blotting

The same quantities of cells were collected from the four groups, and the protein was extracted from the cells at the 24, 48 and 72 h after transfection. The pro-

Table 1 Design of small interfering RNA sequences for high-mobility group box 1

Plasmid constructs	Target sequence in mRNA(5'-3')
HMGB1-1 (shRNAH1)	GCAAATGACTCAATCTGATT
HMGB1-2 (shRNAH2)	AATAGGAAAAGGATATTGCT
HMGB1-3 (shRNAH3)	ACCCGGATGCTTCGTCAAC

HMGB1: High-mobility group box 1.

tein content in the supernatant was detected using the bicinchoninic acid method. An equal amount of protein was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was incubated overnight at 4 °C with monoclonal anti-human HMGB1 (1:300) and was then incubated for 2 h with a secondary antibody (1:5000). Finally, after staining and fixing, the film was analyzed using the Image Analysis System.

Enzyme-linked immunosorbent assay

Commercial kits (Sigma, St. Louis, MO, United States) were used to quantitate the amount of collagen types I and III in the culture supernatant of HSCs at different time points after siRNA transfection.

Methyl thiazolyl tetrazolium used for observing cell proliferation

The cell suspension was inoculated into 96-well plates at 1000 cells per well with eight ambi-wells and incubated for 1, 2, 3, 4 and 5 d after transfection. Cells were incubated with 20 μL methyl thiazolyl tetrazolium for 4 h. After centrifugation, 150 μL dimethyl sulfoxide was added to the precipitate, and the absorbance of the enzyme was measured at 490 nm. Cell growth rates (average absorbance of each transfected group/non-transfected group) were then calculated.

Statistical analysis

Continuous data were expressed as the mean ± SD and were analyzed using the Student's *t* test. Correlations among the study variables were tested using Pearson's correlation coefficients. *P* < 0.05 were considered statistically significant. All calculations were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Histological and immunohistochemical assessment

To investigate the expression of HMGB1 during liver fibrosis, liver sections were analysed by HE staining and immunohistochemistry. We localized HMGB1 and collagen types I and III in liver specimens by immunohistochemistry. None of these proteins were observed in control rat livers. In fibrotic rat livers, HMGB1 was markedly increased during liver fibrogenesis and was correlated with

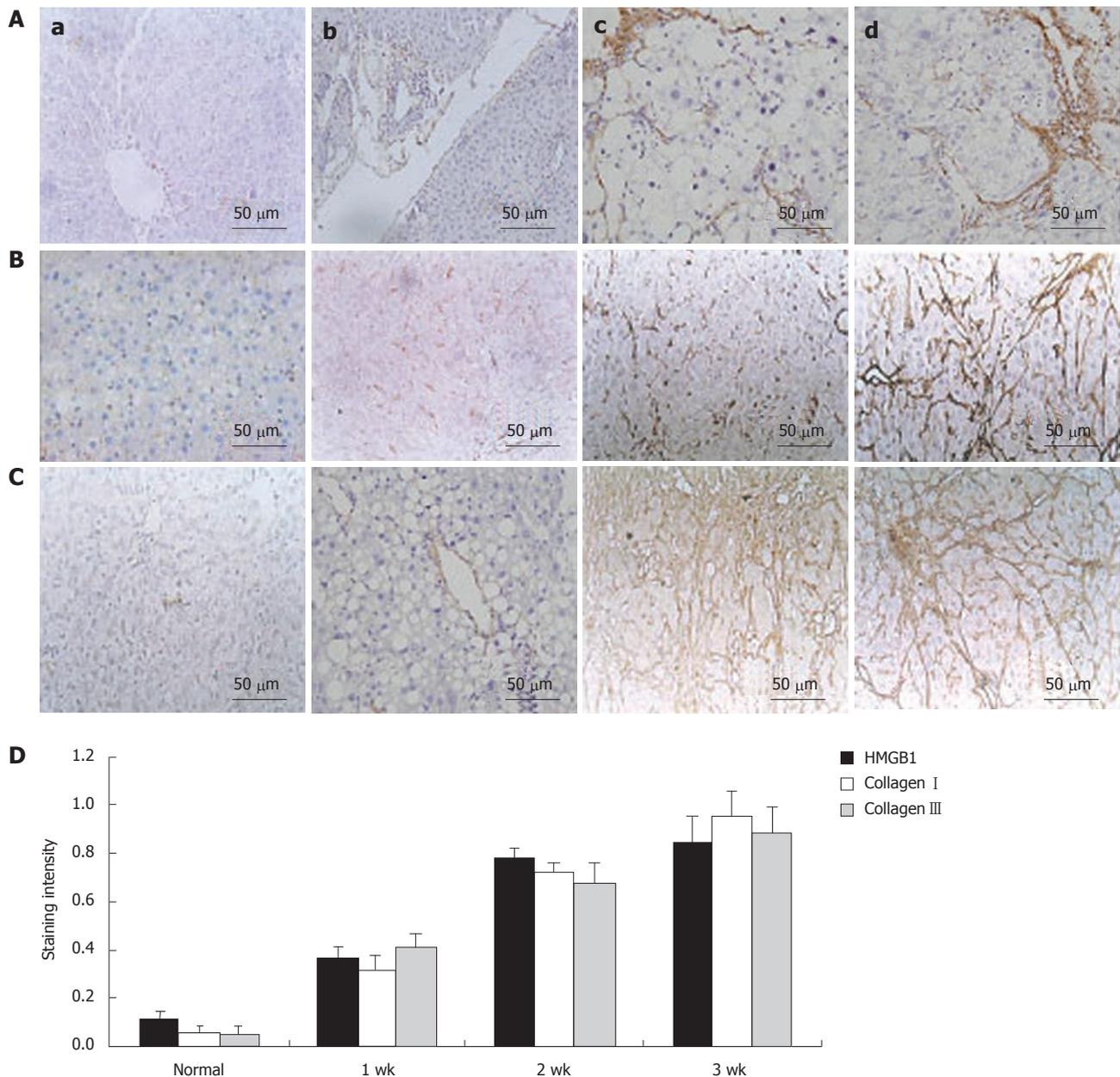


Figure 1 High-mobility group box 1 protein was upregulated after dimethylnitrosamine injection. A: Immunohistochemical study of high-mobility group box 1 (HMGB1) distribution and expression in liver fibrosis specimens (original magnification, $\times 400$). Brown color displays the positive expression. a: There was no immunoreactivity in the normal liver tissue; b: Weak staining in liver fibrosis tissue at 1 wk after the first dimethylnitrosamine (DMN) injection; c: Moderate staining in liver fibrosis tissue at 2 wk after the first DMN injection; d: Strong staining in liver fibrosis tissue at 3 wk after the first DMN injection; B: Immunohistochemical study of collagen type I in liver fibrosis specimens (original magnification, $\times 400$). Brown color displays the positive expression. Collagen type I was markedly increased during liver fibrogenesis; C: Immunohistochemical study of collagen type III in liver fibrosis specimens (original magnification, $\times 400$). Brown color displays the positive expression. Collagen type III was markedly increased during liver fibrogenesis; D: The amount of HMGB1, collagen types I and III staining in liver tissue was measured using an image analyzer during liver fibrosis. HMGB1 was markedly increased during liver fibrogenesis, correlated with the expression of collagen types I and III ($r = 0.90$, $P < 0.05$ and $r = 0.89$, $P < 0.05$).

the expression of collagen types I and III. Immunohistochemistry indicated that the intensity of HMGB1 immunostaining was stronger in the fibrotic samples (DMN week 1) than in the control group. After DMN injection for 2-3 wk, greater HMGB1 staining was found around the portal tracts and fibrotic septa (Figure 1A). With the development of hepatic fibrosis, there was an enhanced expression of HMGB1, correlating with collagen types I and III expression, which was mainly located within

the mesenchymal (Figure 1B and C). Statistical analysis showed that the expression of HMGB1 was completely correlated with the expression of collagen types I and III during the development of hepatic fibrosis (Figure 1D) ($P < 0.05$).

Cellular localization of HMGB1 in DMN-treated rats

α -SMA, a typical marker of activated HSCs, was selected to determine the cellular localization of HMGB1 in hepat-

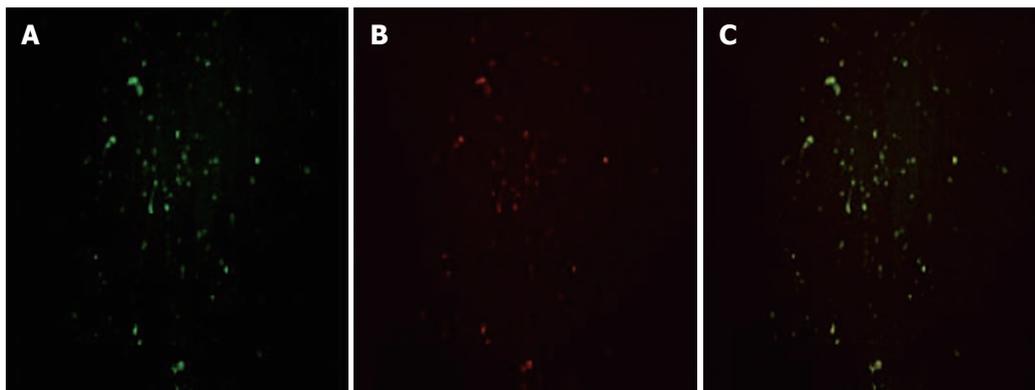


Figure 2 Double immunostaining was used to analyze the cellular localization of high-mobility group box 1 protein and α -smooth muscle actin in hepatic fibrosis tissue (original magnification, $\times 200$). A: α -smooth muscle actin (α -SMA) was stained with polyclonal α -SMA antibody and secondarily by rhodamine-conjugated anti-rabbit antibody (green); B: High-mobility group box 1 (HMGB1) was stained with monoclonal anti-HMGB1 antibody and secondarily by fluorescein isothiocyanate-conjugated anti-rabbit antibody (red); C: The yellow areas on the merged image show co-localization of α -SMA and HMGB1.

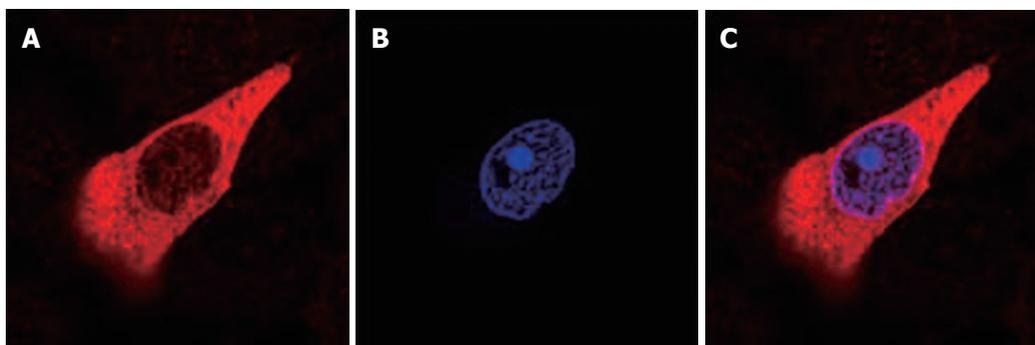


Figure 3 High-mobility group box 1 protein expression in hepatic stellate cell-T6 cells by immunofluorescence staining (original magnification, $\times 200$). A: High-mobility group box 1 (HMGB1) protein was stained with monoclonal anti-HMGB1 antibody and secondarily by fluorescein isothiocyanate-conjugated anti-rabbit antibody (red); B: Nuclei were labeled with 4',6-diamidino-2-phenylindole (blue); C: The merge picture.

ic fibrosis tissue. The localization of HMGB1 and α -SMA was visualized by immunofluorescent double labeling and laser scanning confocal microscopy. The image analysis showed a diffused distribution of HMGB1 throughout the hepatic fibrosis tissue (Figure 2A), and a similar distribution was observed for α -SMA (Figure 2B). When the two images were merged, there was a very high degree of co-localization of HMGB1 with α -SMA throughout the hepatic fibrosis tissue (Figure 2C).

Intracellular localization of HMGB1 in activated HSC-T6 cells

An immunofluorescence study of HSC-T6 cells after 24 h of culture demonstrated the intracellular localization of HMGB1. We evaluated the subcellular localization of HMGB1 by separating bulk nuclei and cytosolic fractions, and HMGB1 was detected primarily within the cytosol of activated HSC-T6 cells (Figure 3).

Selection of HMGB1 mRNA sequence target

As shown in Table 1, a total of three candidate siRNA sequences were chosen to be complementary to various regions of the rat *HMGB1* gene. In a set of preliminary experiments designed to identify the most appropriate sequence for further study, these sequences were transfected

into HSC-T6 using Lipofectamine. Forty-eight hours after transfection, HMGB1 transcript and protein levels were reduced in transfected cells. This *HMGB1* gene-silencing effect was reproducible and was specific in that it failed to knock down the expression of an unrelated protein, β -actin. All three HMGB1 shRNAs tested in this study were able to reduce the HMGB1 expression in HSC-T6 cells compared with the negative control (NC) siRNA transfectants. Although all three HMGB1 shRNA constructs were effective, shRNAH3 was more efficient in reducing the HMGB1 transcript levels than shRNAH2 and shRNAH1 (Figure 4A). Western blotting analysis (Figure 4C) further confirmed the shRNAH3 silencing of the HMGB1 protein in HSC-T6 cells. Semiquantitative analysis of the real-time (RT)-PCR and Western blot results (Figure 4B and D) also showed that HMGB1 shRNAH3 decreased the expression of HMGB1 in HSC-T6 cells more efficiently than shRNAH2 and shRNAH1. Accordingly, we chose shRNAH3 for the subsequent experiments.

HMGB1 siRNA downregulated mRNA expression of α -SMA and types I and III collagen in HSC-T6

To investigate the effect of HMGB1 siRNA on HSCs and its potential molecular mechanisms, we detected the

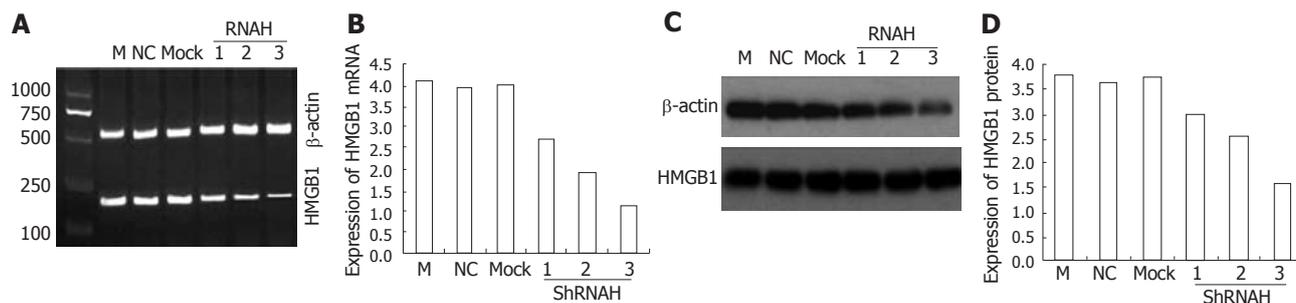


Figure 4 Screening the most effective high-mobility group box 1 siRNA sequence. Total RNA and protein were obtained from hepatic stellate cell-T6 transfected with negative control (NC), mock and three different high-mobility group box 1 (HMGB1) siRNA molecules (shRNAH1, shRNAH2 and shRNAH3). A: Real-time polymerase chain reaction (RT-PCR) for the effect of three different HMGB1 siRNA molecules on HMGB1 mRNA level 48 h after transfection. The expression was normalized against β -actin; B: Semiquantitative analysis of the RT-PCR result; C: Western blotting analyzed HMGB1 protein expression 48 h after transfection; D: Semiquantitative analysis of the western blotting results. Data represent results from one of three similar experiments. Results show that all three HMGB1 shRNA constructs were effective, but shRNAH3 was more efficient in reducing the HMGB1 mRNA and protein levels than shRNAH2 and shRNAH1.

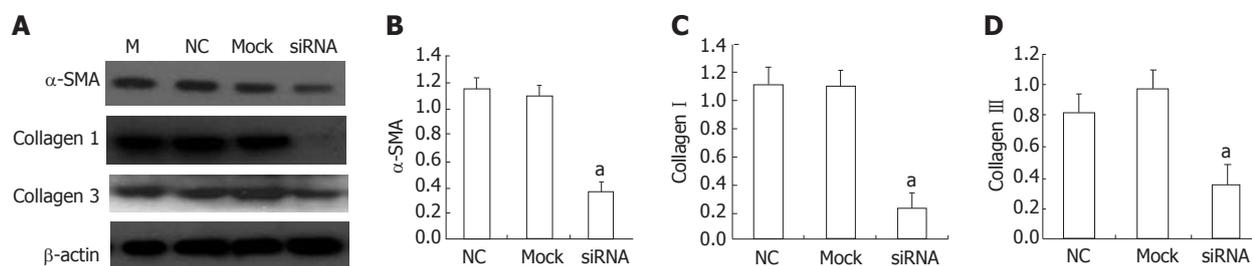


Figure 5 High-mobility group box 1 siRNA inhibited α -smooth muscle actin, collagen types I and III mRNA expression in hepatic stellate cell-T6 cells. A: Real-time polymerase chain reaction (RT-PCR) analysis for α -smooth muscle actin (α -SMA), collagen types I and III mRNA expression in hepatic stellate cell-T6 cells after siRNA high-mobility group box 1 transfection. β -actin was used as the internal loading control; B-D: Semiquantitative analysis of the RT-PCR result. ^a $P < 0.05$ vs negative controls (NC) or mock.

Table 2 Effect of high-mobility group box 1 siRNA on the cell cycle

Cell cycle phases(%)	ShRNAH3 group	NC group
G0/G1 phase	58.31% \pm 0.48% ^a	44.25% \pm 0.63%
S phase	29.12% \pm 1.26% ^a	41.32% \pm 1.58%
G2/M phase	12.57% \pm 1.04%	14.53% \pm 1.28%

^a $P < 0.05$ vs negative controls (NC) group.

mRNA expression of some profibrogenic markers, including α -SMA and collagen types I and III, in transfected HSC-T6. As shown in Figure 5, HMGB1 siRNA reduced the mRNA levels of α -SMA and collagen types I and III.

HMGB1 siRNA reduced the collagen content in the HSC-T6 supernatant

To confirm the effect of HMGB1 siRNA on collagen secretion and degradation, we examined the amount of collagen types I and III in HSCs 48 and 72 h after transfection with shRNAH3 using an ELISA. The results reveal that the content of both collagen types I and III was decreased after transfection with HMGB1 siRNA. Compared with the NC group, the content of collagen types I and III was reduced to 63% and 61%, respectively, 72 h after shRNAH3 transfection (Figure 6).

HMGB1 siRNA inhibited HSC-T6 cells proliferation

The trypan blue dye test showed that there were no sig-

nificant differences in the number of cells in the three-groups 2 d after transfection ($P > 0.05$), but the proliferation in the shRNAH3 group was less than that in the NC group and non-transfection group (Mock group) 3, 4 and 5 d after transfection ($P < 0.05$, Figure 7). A cell cycle study also indicated that cells were arrested in the G0/G1 phase and that the proportion of cells in the S phase was significantly reduced after downregulation of HMGB1 in HSCs (Table 2).

DISCUSSION

Liver fibrosis is highly associated with chronic hepatocellular injury and the subsequent inflammatory response that produces inflammatory cytokines and recruits inflammatory leukocytes to the injured site. This inflammatory circumstance in the liver drives the activation of HSCs through various fibrogenic mediators^[12,13]. Activated HSCs transdifferentiate into myofibroblasts, which then produce excessive amounts of ECM proteins, including collagen types I, III and IV. This leads to irreversible collagen deposition, resulting in liver fibrosis^[12,13]. Many studies have suggested that enhancement of matrix degradation may prove particularly valuable in response to injury caused by matrix deposition^[14-17]. Some studies have shown that HMGB1 can stimulate proinflammatory cytokine synthesis and directly stimulate fibroblast proliferation and participate in fibrogenesis^[8-10].

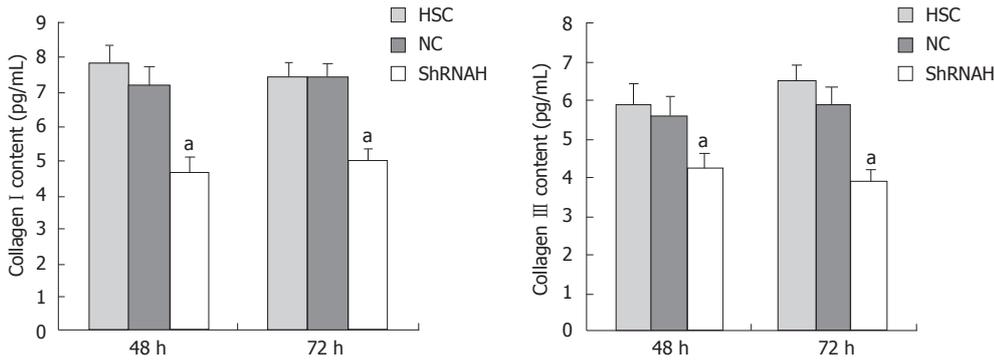


Figure 6 Determination of content of collagen types I and III after shRNAH3 transfection. Enzyme-linked immunosorbent assays were used for quantitative determination of collagen types I and III content hepatic stellate cells (HSCs) culture supernatant at 48 and 72 h after shRNAH3 transfection using Lipofectamine 2000. Values are presented as mean \pm SD. ^a $P < 0.05$ vs negative controls (NC) and HSC group.

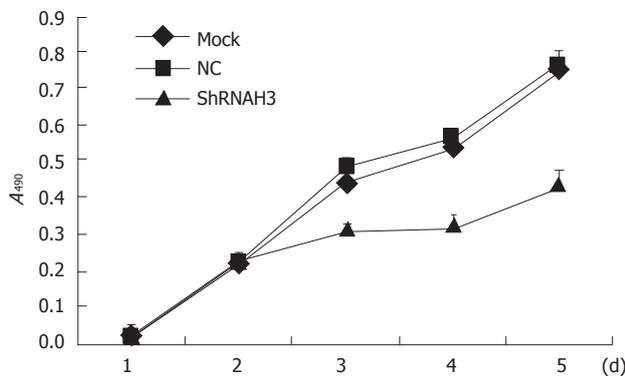


Figure 7 High-mobility group box 1 siRNA suppressed hepatic stellate cell-T6 proliferation. Cell growth curves of hepatic stellate cell-T6 transfected with shRNAH3 were analyzed by methyl thiazolyl tetrazolium conversion. Each sample was tested in triplicate and error bars were included. Compared with negative controls (NC) group and non-transfection group, proliferation of shRNAH3 group was less at the 3-5 d after transfection ($P < 0.05$).

Increased expression of HMGB1 has been reported in several liver diseases, including Con A-induced hepatitis^[18], hepatic ischemia^[2], and orthotopic liver transplantation (OLT)^[19]. In the present study, we evaluated HMGB1 expression in the DMN rat model. We found that the level of HMGB1 was upregulated during DMN injection. Moreover, the expression of HMGB1 was closely correlated with the expression of collagen types I and III and was mainly localized to the nonparenchymal cells, especially HSCs. These results suggest that HMGB1 is involved in hepatic fibrogenesis and may play a critical role in the reversal process of liver fibrosis.

HMGB1 was originally identified as a nuclear non-histone protein with DNA-binding domains and was implicated as an important endogenous danger signaling molecule. Although predominantly located in the nucleus of quiescent cells, HMGB1 can be actively secreted in response to exogenous and endogenous inflammatory stimuli such as endotoxin, TNF- α , IL-1, and interferon- γ ^[20,21]. In addition, extracellular HMGB1 mediates a wide range of inflammatory responses and promotes cell proliferation, migration, and differentiation^[10,22]. The cytoplasmic localization of HMGB1 in our

study may suggest that HMGB1 plays extra nuclear roles in liver fibrosis and that HSC-T6 cells may even secrete HMGB1 to promote extracellular functions. The subcellular location of HMGB1 in monocytic cells is known to be dependent on the acetylation status of the nuclear localization signal (NLS) of the HMGB1 protein^[23]. Inflammatory signals promote acetylation of the NLS, leading to cytoplasmic accumulation of HMGB1 in secretory lysosomes in the monocytic cells^[24]. These secretory lysosomes are subsequently exocytosed when the monocytic cells are triggered by a second inflammatory stimulus. Whether the subcellular location of HMGB1 in HSC-T6 cells is regulated in a similar way remains to be investigated.

It has become apparent in recent years that HMGB1 is instrumental in mediating a response to tissue damage and infection. HMGB1 released from necrotic or damaged cells not only triggers inflammation as a non-specific proinflammatory cytokine but also triggers the adaptive immune response^[25,26]. Extracellular HMGB1 functions as a damage-associated molecular pattern molecule and activates proinflammatory signaling pathways by activating pattern-recognition receptors including toll-like receptor 4 (TLR4) and the receptor for advanced glycation end-products (RAGE)^[27,28]. A previous report showed that RAGE expression in fibrotic livers is restricted to HSCs; its expression is up regulated during cellular activation and transition to myofibroblasts^[29], strongly suggesting that HMGB1 is involved in the pathogenesis of liver fibrosis. TLR4 has been suggested to be a receptor for extracellular HMGB1^[30,31], and previous studies have indicated that the interaction of HMGB1 with TLR4 plays a critical role in hepatic fibrosis^[32]. To date, little has been reported about the pathogenic interactions between HMGB1 and HSCs in terms of profibrogenic propensity. Kao provided evidence that HMGB1 up regulates α -SMA expression and suppresses the activity of the collagen-degrading enzyme matrix metalloproteinase-2^[33]. That study also implied that HMGB1, once it is released during rejection of OLT, activates HSCs and exhibits profibrogenic effects either by increasing the HSC population and ECM deposition

in liver grafts or by transforming HSCs into myofibroblasts. In contrast, neutralization with an anti-HMGB1 antibody may be a therapeutic modality to prevent fibrogenesis in post-OLT liver grafts^[33].

siRNA has become a powerful tool for functional genetic studies and gene therapy in mammals^[34,35]. Although gene knockdown by siRNA is highly effective, the off-target effect of siRNA may represent a major obstacle for therapeutic applications. However, the potential off-target effects could be minimized by choosing an siRNA with maximal sequence divergence from the list of genes with partial sequence identity to the intended mRNA target^[36]. Software was used to choose a maximal sequence identity of HMGB1 siRNA, and three siRNA sequences were designed. In preliminary experiments, we identified the fact that shRNA3 had certain interference effects. Our results show that this sequence was more efficient in reducing the HMGB1 transcript levels.

In the present study, we found that after HMGB1 was downregulated in HSCs by siRNA, there was an inhibitory effect on the mRNA levels of α -SMA and collagen types I and III, suggesting that inhibition of HMGB1 could directly result in suppression of HSC activation and collagen production. We also discovered that HMGB1 siRNA prohibited HSC proliferation, and a cell cycle analysis revealed that downregulation of HMGB1 arrested cells at the G0/G1 phase, which confirmed the effect of HMGB1 on cell proliferation; however, the definitive mechanism responsible is still uncertain because HMGB1 is multifunctional and has multiple molecular interactions.

In conclusion, HMGB1 was upregulated during liver fibrogenesis, and downregulating HMGB1 expression in HSCs by siRNA prohibited the activity of HSCs and collagen synthesis and enhanced collagen degradation. The results of our study indicate a significant functional role for HMGB1 in the development of liver fibrosis, and downregulating HMGB1 expression with siRNA could be an effective way to treat liver fibrosis.

COMMENTS

Background

Hepatic fibrosis is a response to injury in the liver. It is characterized by both a quantitative and qualitative change in the extracellular matrix (ECM). The activated hepatic stellate cell (HSC) is primarily responsible for excessive ECM deposition during liver fibrosis. It has been shown that high-mobility group box 1 (HMGB1) expression is up regulated during myofibroblast cellular activation and involved in the pathogenesis of hepatic fibrosis. This suggests that HMGB1 is a promising molecular target for hepatic fibrosis gene therapy. Inhibition of abnormal expression of HMGB1 may be an effective strategy for biological therapy of hepatic fibrosis.

Research frontiers

HMGB1 is a major component of mammalian chromatin endowed with an architectural function. Increasing evidence now points to multiple functions of HMGB1 in infection, tissue injury, inflammation, apoptosis and the immune response. It has been reported in several liver diseases, including hepatitis, hepatic ischemia, and orthotopic liver transplantation. HMGB1 has been implicated in the pathogenesis of several liver diseases, including Con-A-induced hepatitis, hepatic ischemia, and orthotopic liver transplantation. However, the role of HMGB1 and how to inhibit its expression in hepatic fibrosis has yet to be fully elucidated. In this study, the authors demonstrate that the overexpres-

sion of HMGB1 could be a potential mechanism for mediating collagen expression and downregulating HMGB1 expression might present as a potential strategy to treat liver fibrosis.

Innovations and breakthroughs

Studies of targeting *in vitro* and *in vivo* over expressed genes in hepatic fibrosis by RNA interference, including transforming growth factor- β , connective tissue growth factor and p90RSK, have been reported. However, there has been still no report about targeting HMGB1 by siRNA in hepatic fibrosis. In the present study, the authors used siRNA approach to block HMGB1 expression in HSC-T6 cells, to determine the role of constitutively activated HMGB1 during hepatic fibrosis pathogenesis, and to explore the role and molecular mechanism of targeting HMGB1 in hepatic fibrosis therapy.

Applications

By investigating the effect of silencing HMGB1 expression by siRNA on the collagen synthesis and proliferation of HSC-T6 cells, this study may provides a new strategy for biological therapy of liver fibrosis by targeting HMGB1.

Terminology

HMGB1 was originally identified as a nuclear nonhistone protein with DNA-binding domains and implicated as an important endogenous danger signaling molecule. But it can also be secreted from cells and exert extracellular functions as a proinflammatory cytokine. HSCs are a minor and quiescent cell type in the liver that usually reside in the space of Disse, but which undergo activation after hepatic injury to produce large quantities of fibrillar collagens.

Peer review

The authors demonstrated the increase of HMGB1 expression in fibrotic livers. Then, they investigated the effect of HMGB1 silencing by siRNA on stellate cell activation and proliferation. The results show that siRNA for HMGB1 significantly inhibits collagen expression and stellate cell proliferation.

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S- Editor Tian L L- Editor Kerr C E- Editor Li JY

Antioxidative potential of a combined therapy of anti TNF α and Zn acetate in experimental colitis

Michela Barollo, Valentina Medici, Renata D'Inca, Antara Banerjee, Giuseppe Ingravallo, Marco Scarpa, Surajit Patak, Cesare Ruffolo, Romilda Cardin, Giacomo Carlo Sturniolo

Michela Barollo, Renata D'Inca, Antara Banerjee, Surajit Patak, Cesare Ruffolo, Romilda Cardin, Giacomo Carlo Sturniolo, Department of Surgical and Gastroenterological Sciences, University of Padova, Padova 35128, Italy

Valentina Medici, Department of Internal Medicine, Division of Gastroenterology and Hepatology, University of California, Davis, CA 95616, United States

Giuseppe Ingravallo, Department of Pathology, University of Bari, Bari 70121, Italy

Marco Scarpa, Department of Oncological Surgery, Veneto Oncological Institute, Padova 35128, Italy

Author contributions: Barollo M and Medici V designed the study and wrote the manuscript; Scarpa M and Ruffolo C performed the experiments and the statistical analysis; Banerjee A, Patak S revised the manuscript; D'Inca R and Sturniolo GC designed the study and revised the manuscript; Cardin R performed the biochemical analysis; Ingravallo G carried out the histological analysis.

Supported by MIUR 40% University of Padova

Correspondence to: Giacomo Carlo Sturniolo, MD, Professor of Gastroenterology, Department of Gastroenterology, Via Giustiniani 2, Padova 35128, Italy. gc.sturniolo@unipd.it
Telephone: +39-49-8218726 Fax: +39-49-8760820

Received: December 15, 2010 Revised: February 19, 2011

Accepted: February 26, 2011

Published online: September 28, 2011

Abstract

AIM: To evaluate whether combination therapy with anti-tumour necrosis factor α (TNF α) antibody and Zn acetate is beneficial in dextran sodium sulphate (DSS) colitis.

METHODS: Colitis was induced in CD1-Swiss mice with 5% DSS for 7 d. The experimental mice were then randomised into the following subgroups: standard diet + DSS treated (induced colitis group); standard diet + DSS + subcutaneous 25 μ g anti-TNF α treated group; Zn acetate treated group + DSS + subcutaneous 25 μ g anti-TNF α ; standard diet + DSS + subcutaneous 6.25 μ g

anti-TNF α treated group and Zn acetate treated group + DSS + subcutaneous 6.25 μ g anti-TNF α . Each group of mice was matched with a similar group of sham control animals. Macroscopic and histological features were scored blindly. Homogenates of the colonic mucosa were assessed for myeloperoxidase activity as a biochemical marker of inflammation and DNA adducts (8OH-dG) as a measure of oxidative damage.

RESULTS: DSS produced submucosal erosions, ulcers, inflammatory cell infiltration and cryptic abscesses which were reduced in both groups of mice receiving either anti-TNF α alone or combined with zinc. The effect was more pronounced in the latter group (*vs* Zn diet, $P < 0.02$). Myeloperoxidase activity (*vs* controls, $P < 0.02$) and DNA adducts, greatly elevated in the DSS fed colitis group (*vs* controls, $P < 0.05$), were significantly reduced in the treated groups, with a more remarkable effect in the group receiving combined therapy (*vs* standard diet, $P < 0.04$).

CONCLUSION: DSS induces colonic inflammation which is modulated by the administration of anti-TNF α . Combining anti-TNF α with Zn acetate offers marginal benefit in colitis severity.

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Key words: Anti tumor necrosis factor α ; Experimental colitis; Inflammatory bowel disease; Oxidative damage; Zinc

Peer reviewers: Dr. Tamara Vorobjova, MD, PhD, Scimed. Senior Researcher in Immunology, Department of Immunology, Institute of General and Molecular Pathology, University of Tartu, Ravila, 19, Tartu 51014, Estonia; Jay Pravda, MD, Inflammatory Disease Research Center, West Palm Beach, FL 33420, United States

Barollo M, Medici V, D'Inca R, Banerjee A, Ingravallo G,

Scarpa M, Patak S, Ruffolo C, Cardin R, Sturniolo GC. Antioxidative potential of a combined therapy of anti TNF α and Zn acetate in experimental colitis. *World J Gastroenterol* 2011; 17(36): 4099-4103 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4099.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4099>

INTRODUCTION

Ulcerative colitis and Crohn's disease are chronic diseases of the gastrointestinal tract characterized by activation of the immune system with production of several inflammatory cytokines^[1,2]. Altered T cell apoptosis^[3,4] and abnormal production of the pro-inflammatory cytokine tumour necrosis factor α (TNF α) play a central role in intestinal inflammation of inflammatory bowel disease patients^[5].

Novel treatment strategies based on the inhibition of TNF α have shown to be effective both in experimental models of colitis^[6] and in inducing and maintaining remission in humans affected with inflammatory bowel disease^[7]. However, as these therapies are very expensive they may represent an important and unaffordable economic burden in the near future.

Trace element metabolism is altered during inflammatory processes of the gastrointestinal tract. Zinc is essential for intestinal homeostasis, since there are several zinc-dependent antioxidant enzymes such as superoxide dismutase which converts superoxide to hydrogen peroxide and metallothionein which can neutralize free radical production. Moreover, zinc status affects gene expression of the inflammatory cytokines TNF, IL-1B and IL-8. Zinc deficiency causes functional defects in T cells, neutrophils and macrophages, and positive modulatory responses are produced following zinc supplementation^[8]. In the model of acetic acid-induced ulcerations, zinc reduced mucosal damage^[9]. In models of experimental colitis both oral and topical zinc treatment were found to decrease intestinal inflammation, to favour mucosal healing and to improve immune function^[10]. We therefore thought that zinc may be useful if added to conventional anti-TNF α therapy in modulating the symptoms of dextran sodium sulphate (DSS)-induced colitis in mice and in decreasing oxidative stress.

MATERIALS AND METHODS

Animals

Male CD1 Swiss mice, 4 wk old, weighing 20-25 g purchased from Charles River (Calco, Italy) were used in this study. The animals were kept in plastic platform cages in a temperature controlled room (22 °C) under a 12-h light-dark cycle, with free access to water and standard chow containing 125 mg/kg zinc oxide. The experimental protocol was approved by the Veterinary and Health Committee of the University of Padua.

Experimental protocol

Mice were fed 5% DSS (5% dextran sulphate solution purchased from ICN Pharmaceuticals, SRL, Italy) dissolved in drinking water in one single cycle to induce acute colitis. The cycle consisted of administering 5% DSS for 7 d which caused loose stools in all animals and the presence of gross rectal bleeding in about 50% of the animals.

The animals were randomised into the following six groups each with 6 mice: (1) **healthy untreated mice** receiving standard diet; (2) **induced colitis group**, i.e., mice receiving standard diet + 5% DSS for 7 d; (3) **mice** receiving standard diet + 675 mg/kg Zn acetate supplement starting 7 d before induction of colitis; (4) **mice** receiving standard diet + 25 μ g anti-TNF α intraperitoneally after 1 wk of DSS administration; (5) **mice** receiving standard diet + 675 mg/kg Zn acetate supplement + 25 μ g anti-TNF α intraperitoneally after 1 wk of DSS administration; and (6) **mice receiving standard diet + 675 mg/kg Zn acetate supplement + 6.25 μ g anti-TNF α intraperitoneally after 1 wk of DSS administration**. The three groups receiving anti-TNF α treatment were sacrificed 48 h after initiation of treatment. Anti-TNF α monoclonal antibody (rat anti-mouse TNF α) was purchased from Biosource International Inc. (United States) and Zn Acetate 675 mg/kg diet, from Mucedola SRL, (Milano, Italy).

Macroscopic and histologic features of colitis

Damage was assessed macroscopically by scoring the number and extent of ulcers, adhesions, and thickness of the colonic wall^[11] and histologically by scoring cryptitis, crypt abscesses and epithelial injury. Colonic tissue samples were obtained and processed for myeloperoxidase and 8-hydroxydeoxyguanosine (8-OHdG) in order to quantify inflammation and DNA damage.

Colonic samples were immediately fixed in buffered formalin (10%). After fixation, the specimens were routinely processed and embedded in paraffin. Serial histology sections of 4 μ m thickness were obtained from each paraffin block and mounted on poly-L-lysine coated slides. Sections were stained with haematoxylin-eosin and examined blindly.

Cryptitis was defined as the presence of polymorphonuclear cells within crypt epithelium, while crypt abscesses were defined as the presence of polymorphonuclear cells within the crypt lumens. Epithelial injury included changes such as crypt regeneration, mucodepletion, cuboidal shape, nuclear enlargement, loss of surface cells, erosion, and ulceration. Each of the features, defined above, was scored on a 0 to 3+ scale based on the severity and degree of involvement^[12,13].

Mean colonic activity scores for cryptitis, crypt abscesses and epithelial injury were marked for each slide on the following basis: 0 (no activity); 1-2 (mild activity); 3-4 (moderate activity); 5-6 (severe activity).

Assessment of myeloperoxidase activity

Assessment of myeloperoxidase (MPO) activity was as-

Table 1 Biochemical and morphological parameters of colitis severity among the study groups

	Macroscopic score	Colonic activity index	Myeloperoxidase activity (U/g)
Controls	0 (0-0)	0 (0-0)	1.9 (1.34-1.1)
Colitis	1 (1-1) ^b	4 (1-1) ^a	5.69 (0.04-0.21) ^b
Colitis + Zinc	2 (2-2) ^b	5 (0-0) ^a	7.8 (0-0.8) ^e
Colitis + anti-TNF α (25 μ g)	0.5 (0-1)	4 (1-3) ^a	4.85 (1.81-3) ^d
Colitis + Zinc + anti-TNF α (25 μ g)	0 (0-0) ^c	1 (2-0) ^{ad}	3.88 (2.9-2.87)
Colitis + anti-TNF α (6.25 μ g)	1 (0.25-1)	5 (0-2)	5.44 (0.63-0)
Colitis + Zinc + anti-TNF α (6.25 μ g)	0.5 (0-1)	3 (2-0)	4.42 (0.34-0.33)

^a $P < 0.01$ vs controls; ^b $P < 0.03$ vs controls; ^c $P < 0.03$ vs colitis; ^d $P < 0.02$ vs colitis+Zinc; ^e $P < 0.02$ vs controls. TNF: Tumour necrosis factor.

essed according to the method previously described^[14]. Briefly, colonic tissue samples were minced in 1 mL of 50 mmol/L potassium phosphate buffer (pH = 6.0) containing 14 mmol/L hexadecyltrimethylammonium bromide (Fluka), homogenized and sonicated. The lysates were frozen and thawed thrice, then centrifuged for 2 min in cold at 15000 *g*. Aliquots of the supernatants were mixed with potassium phosphate buffer containing *o*-dianisidine-HCl (Sigma-Aldrich, St. Louis, MO, United States) and 0.0005% H₂O₂. MPO activity was expressed as units/g of wet tissue. The enzyme unit was defined as the conversion of 1 mol of H₂O₂ per min at 25.

8-OHdG determination

Oxidative DNA damage was assessed following previously described methods^[15]. Briefly, colonic biopsy specimens were thawed, homogenized in a separation buffer and approximately 20 μ g of purified DNA per sample was injected into the HPLC system (Shimadzu, Kyoto, Japan). The 8-OHdG was detected using an electrochemical detector (ESA Coulochem II 5200A, Bedford, MA, United States). The levels of 8-OHdG were expressed as the number of 8-OHdG adducts per 10⁵ dG bases. The coefficient of variation was < 10%; 100 μ g of DNA were required for the determination.

Statistical analysis

Data are expressed as median (interquartile range). Statistical data were analyzed with Mann-Whitney *U* test for comparison of the groups and Spearman's rank correlation test. *P* values less than 0.05 were considered significant.

RESULTS

Macroscopic evaluation of colitis

The macroscopic score was increased significantly in untreated colitic mice. Groups treated with anti-TNF α or anti-TNF α and zinc acetate showed a decreased macroscopic score which was more evident in the combined diet. Chronic feeding of DSS significantly increased the colonic activity score. The administration of anti-TNF α alone or combined with zinc acetate significantly reduced this index. The effect appeared to be significantly more evident in the group receiving anti-TNF α and zinc acetate than in the group receiving anti-TNF α alone. The

administration of a reduced dose of anti-TNF α (6.25 μ g) was effective only if combined with zinc acetate (Table 1).

Myeloperoxidase activity

Myeloperoxidase activity was increased in all colitic mice. However, there was a significant reduction in this activity in the groups treated with anti-TNF α alone and anti-TNF α + Zn supplementation, with a slightly better effect in the group receiving the combination therapy. A lower dose of anti-TNF α was associated with reduced MPO activity only in the group receiving both zinc and anti-TNF α (Table 1).

Determination of oxidative damage as measured by 8-OHdG mucosal levels

Oxidative damage was significantly increased in colitic mice. Anti-TNF α significantly reduced DNA adducts, OH-dG levels were similar in the group receiving both anti-TNF α and zinc acetate (Figure 1). Anti-TNF α treatment significantly reduced DNA adducts at both doses used. In both groups receiving the combination therapy, DNA adducts were reduced compared to anti-TNF α therapy alone, but no significant effect was demonstrated with respect to the groups receiving anti-TNF α alone (Figure 1).

DISCUSSION

Chemically induced models of intestinal inflammation are widely used as surrogate models of chronic inflammatory bowel disease and oral DSS administration effectively resembles human inflammatory bowel disease with similar clinical features (bloody diarrhoea) and endoscopic/histological findings (**ulcerations and neutrophil infiltration**). DSS is believed to be directly toxic to gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier.

Zinc metabolism has been reported to be reduced in about 65% of patients with Crohn's disease. In an experimental model of colitis we also reported that zinc supplementation induced metallothionein expression, while having little effect on the short-term course of colitis^[16]. Zinc has several potential mechanisms of action which can benefit the inflammatory process. It regulated tight junction permeability in an experimental model of colitis^[17] and in Crohn disease^[18]. Sturniolo *et al*^[19] reported

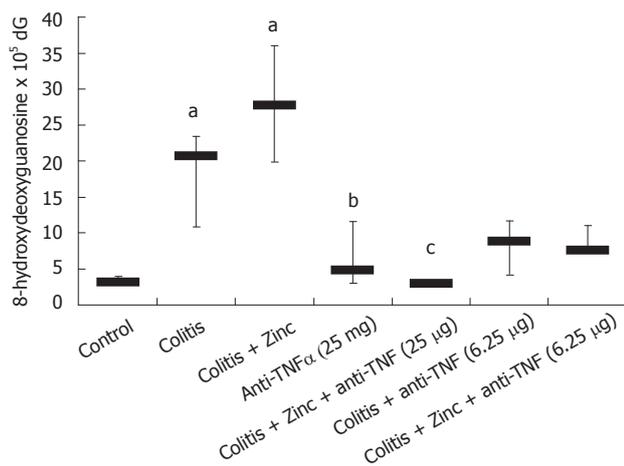


Figure 1 8-hydroxydeoxyguanosine. ^a $P < 0.05$ vs controls; ^b $P < 0.02$ vs colitis; ^c $P < 0.04$ vs colitis. TNF: Tumour necrosis factor.

that zinc sulphate enemas exert an anti-inflammatory action on experimental colitis.

In the last few years, biological therapies have changed the pharmacological armamentarium of inflammatory bowel disease therapy: the first and still most widely used drug is the anti-TNF α monoclonal antibody, infliximab^[20]. Even in experimental models of colitis, the subcutaneous administration of infliximab reduced the inflammatory activity as well as tissue TNF α concentration^[21]. Our experimental approach which added zinc acetate to the diet while administering anti-TNF α monoclonal antibody, aimed to examine the effects on DSS-induced colitis in mice.

The mucosa did not show complete healing probably because the treatment effects were evaluated 48 h after treatment. Nevertheless, therapy with anti-TNF α ameliorated the macroscopic and histological aspects and decreased myeloperoxidase concentration. Similar results were reported by Videla *et al.*^[22] who found that anti-TNF α significantly reduced the release of inflammatory mediators and induced histopathological remission in a model of experimental colitis. Zinc alone had little effect in ameliorating the severity of acute colitis induced by intra-rectal instillation of dinitrobenzene-sulphonic acid in rats, even though Tran *et al.*^[23] and Luk *et al.*^[24] recently reported some therapeutic effects of zinc supplementation in DSS-induced colitis in mice.

Zinc supplementation alone worsened the histopathological and biochemical aspects of colitis compared to colitis alone and this can be explained by the fact that superoxide dismutase by itself is a pro-oxidant enzyme by virtue of its ability to generate hydrogen peroxide^[25,26]. This may explain why a worsening of colitis was recorded when zinc was added alone. However, in our study zinc allowed us to reduce the dose of anti-TNF α maintaining the same biochemical and morphological effects.

Acute colitis is characterised by an increased production of free radicals which contribute to protein, DNA chain and lipid damage. As the antioxidant potential of colonic epithelial cells is quite low, this results in tissue

injury^[27]. The administration of antioxidants thus has the potential to improve the outcome of experimental colitis by scavenging free radicals. In our experimental conditions, the oxidative damage, expressed by DNA adducts was significantly reduced in the groups treated with anti-TNF α confirming the findings of Popivanova *et al.*^[28]. In our study, the effect of anti-TNF α on oxidative stress appeared to be dose-dependent with the highest dose having the strongest effect in reducing oxidative damage, and the combination of anti-TNF α and zinc supplementation added little effect.

Obermeier *et al.*^[29] reported that excess nitric oxide formation occurs in experimental colitis and can be decreased by treatment with rat anti-mouse TNF and interferon gamma monoclonal antibodies. In several studies, zinc supplementation ameliorated antioxidant concentrations thus reducing the production of oxidative species^[27-30]. In the present study, zinc supplementation allowed a reduction in the dose of anti-TNF α antibody, while maintaining the same level of reduced intestinal inflammation observed with a higher dose of anti-TNF α antibody alone, as quantified by the four parameters of tissue inflammation utilized in the study. This effect is in accordance with the described capability of zinc to increase antioxidant concentration and reduce oxidative species.

In conclusion, the combined administration of zinc acetate in the diet along with the systemic administration of anti-TNF α had a positive effect in reducing the severity of DSS-induced colitis in mice, with reduced production of DNA adducts. Moreover, the same effect was demonstrated with the reduced anti-TNF α dose combined with zinc. This experimental approach offers the advantage of reducing the potential side effects of anti-TNF α and costs, while ameliorating oxidative stress and inflammation in patients with inflammatory bowel disease.

COMMENTS

Background

Dextran sodium sulphate (DSS) colitis is a well-known model of inflammatory bowel disease in which the authors tested the effect of the well-known drug anti-tumour necrosis factor α (TNF α) combined with zinc with the aim of evaluating the possibility of lowering the dose of anti-TNF α .

Research frontiers

In this article the authors explore the possibility of a combination therapy in inflammatory bowel disease (IBD) in order to reduce potential side effects and costs.

Innovations and breakthroughs

In this article, zinc was added to a biological therapy in order to evaluate the effects of this combination therapy. **There are no articles in the literature exploring this combination therapy.**

Applications

The potential applications include the possibility of adding zinc to anti-TNF α therapy. Moreover, future perspectives include the application of other combination therapies in inflammatory bowel disease.

Peer review

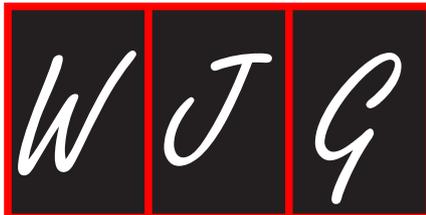
This study considers the investigation of the role of combined administration of Zinc acetate in the diet with systemic administration of anti TNF alpha on the effect of the severity of experimental colitis in mice induced by DSS. The study is set up correctly. The paper is written sufficiently good, the Introduction give a good overview about the study background and the authors raised clearly the hypoth-

esis of the study. The description of methods used is accurate. The results are presented clearly and have been discussed well, the table and figure give good overview about the results.

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S- Editor Sun H L- Editor Webster JR E- Editor Xiong L



Helicobacter species and gut bacterial DNA in Meckel's diverticulum and the appendix

Peren H Karagin, Unne Stenram, Torkel Wadström, Åsa Ljungh

Peren H Karagin, Torkel Wadström, Åsa Ljungh, Department of Medical Microbiology, Lund University, Sölvegatan 23, SE-223 62 Lund, Sweden

Unne Stenram, Department of Pathology, Lund University, SE-22185 Lund, Sweden

Author contributions: Karagin PH did the all molecular analyses and wrote most of the manuscript; Stenram U did the pathological analyses and wrote part of the manuscript; Stenram U, Ljungh Å, and Wadström T designed the project and helped in writing the manuscript.

Supported by A grant from the University Hospital of Lund (ALF) to Torkel Wadström and a grant from the John Forssman's foundation, the Royal Physiographic Society in Lund to Peren Karagin

Correspondence to: Peren H Karagin, PhD, Department of Medical Microbiology, Lund University, Sölvegatan 23, SE-223 62 Lund, Sweden. perenbaglan@yahoo.com

Telephone: +46-46-173298 Fax: +46-46-189117

Received: February 1, 2011 Revised: March 4, 2011

Accepted: March 11, 2011

Published online: September 28, 2011

Abstract

AIM: To analyse the possible association of various *Helicobacter* species and certain common gut bacteria in patients with Meckel's diverticulum and appendicitis.

METHODS: A nested-polymerase chain reaction (PCR), specific to 16S rRNA of the *Helicobacter* genus, was performed on paraffin embedded samples, 50 with acute appendicitis, 50 normal appendixes, and 33 Meckel's diverticulum with gastric heterotopia and/or ulcer. *Helicobacter* genus positive samples were sequenced for species identification. All samples were also analysed for certain gut bacteria by PCR.

RESULTS: *Helicobacter pullorum* DNA was found in one out of 33 cases and *Enterobacteria* in two cases of Meckel's diverticulum. *Helicobacter pylori* (*H. pylori*) was found in three, *Enterobacter* in 18, and *Bacteroides*

in 19 out of 100 appendix samples by PCR. *Enterococcus* was not found in any MD or appendix samples. All *H. pylori* positive cases were from normal appendixes.

CONCLUSION: *Helicobacter* is not an etiological agent in the pathogenesis of symptomatic Meckel's diverticulum or in acute appendicitis.

Key words: Meckel's diverticulum; *Helicobacter*; Appendix; Polymerase chain reaction

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Peer reviewers: Tamara Vorobjova, Senior Researcher in Immunology, Department of Immunology, Institute of General and Molecular Pathology, University of Tartu, Ravila, 19, Tartu 51014, Estonia; Gopal Nath, MD, PhD, Professor, Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India

Karagin PH, Stenram U, Wadström T, Ljungh Å. *Helicobacter* species and gut bacterial DNA in Meckel's diverticulum and the appendix. *World J Gastroenterol* 2011; 17(36): 4104-4108 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4104.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4104>

INTRODUCTION

Although the stomach is the most frequent site of *Helicobacter pylori* (*H. pylori*) infection, *H. pylori* and enterohepatic *Helicobacter spp.* (*EHS*) have also been associated with extragastric diseases^[1].

Meckel's diverticulum (MD) is the most common developmental anomaly of the gastrointestinal tract and is present in 1%-2% of the general population. It often contains ectopic tissue, notably gastric and pancreatic tissue^[2]. Gastric mucosa is found in 10%-25% of MD and may be associated with inflammation, ulceration, gastrointestinal bleeding, and perforation^[3-5]. *H. pylori*

has been demonstrated in the ectopic gastric epithelium within MD^[6]. *Campylobacter-like* organisms in MD were first reported in 1989^[7-9]. However, conflicting results were reported concerning colonisation by *H. pylori* of such ectopic mucosa^[10,11]. There has been no study that investigated EHS in MD.

Acute appendicitis is the most common abdominal surgical emergency and can be seen in all ages, especially in those younger than 30 years^[12]. However, the aetiology of acute appendicitis is uncertain, and diagnosis is often difficult. There have been some investigations of *H. pylori* in appendix tissue^[13-15], but none that investigated non-*pylori Helicobacters*.

We hypothesized that non-*pylori Helicobacters*, such as enterohepatic *Helicobacters*, might be associated with these diseases. Most studies have investigated only *H. pylori* in MD and the appendix and mostly used non-molecular biological techniques; therefore, we aimed to analyse gastric, EHS and certain common gut bacteria in appendicitis and MD patients by genus specific polymerase chain reaction (PCR) and sequencing.

MATERIALS AND METHODS

Patients and histology

We re-examined all MD patients from 1990-2009 taken from the files of the Department of Pathology, Lund University Hospital. Thirty-three MD patients (two cases of ulcer without heterotopia, 31 cases with gastric heterotopia, of which seven also had an ulcer) (mean age: 11 years; range: 4 wk-73 years; 26 male, 7 female) were included in our study. Abdominal pain was the reason for operation in 16 cases, two of whom had acute appendicitis and one enlarged lymph nodes, nine were operated upon because of gastrointestinal bleeding and six for other abdominal diseases. No indication was given in two of the cases. Histological sections from stored paraffin blocks were stained with Alcian blue-periodic acid Schiff (AB-PAS) pH 2.5, Whartin-Starry silver stain and immunostained with an anti-*H. pylori* antibody (DAKO, Glostrup, Denmark, diluted 1:300).

We also re-examined mucosa from 50 cases of acute appendicitis (26 male, 24 female, median age: 30 years; range: 9-87 years) and 50 cases of normal appendix (16 male, 34 female, median age: 34 years; range: 10 d-80 years) from 2008-2009. Of the latter patients 26 were operated for a suspected appendicitis (8 male, 18 female, median age: 21 years; range: 8-77 years), 12 for intestinal diseases (8 male, 4 female, median age: 59 years; range: 10 d-80 years), and 12 for female genital disorders (median age: 38 years; range: 11-75 years). Histological sections from the *Helicobacter* positive cases were stained as mentioned above.

From the cases of MD, heterotopic mucosa of the gastric as well as the antral type were obtained from the paraffin blocks for PCR-assay with the tip of a scalpel. Ulcers were examined separately. In the case positive for *Helicobacter* DNA, the intestinal type mucosa surrounding

the heterotopia was also studied. Corresponding areas from appendix samples of mucosa, or of necrotic appendicitis were sampled for PCR. It was not possible to avoid including material from the appendical lumen in these samples. In the cases positive for *Helicobacter* DNA, other tissues removed at the same operation were also examined by PCR.

DNA extraction

DNA was extracted from the paraffin-embedded tissue samples by de-embedding, as previously described^[1]. DNA was extracted by a QIAamp DNA Mini Kit Tissue protocol (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Helicobacter specific PCR

DNA extracts were amplified in a GeneAmp 2700 Thermocycler (Applied Biosystems, Foster City, CA, United States) using a semi-nested PCR assay specific for *Helicobacter spp.* 16S rDNA, as previously described^[1]. *H. pylori* (CCUG 17874) was used as a positive control in all PCR reactions. The 416-bp PCR products were visualized by 1.3% agarose gel electrophoresis.

Amplification of non-Helicobacter bacteria

PCR specific for *Enterobacteriaceae*, the *Bacterioides-Prevotella* group, and *Enterococcus* were performed. The reaction mixture and amplification conditions, except for annealing temperatures, for non-*Helicobacter* PCR assays were the same as in the first step of the semi-nested *Helicobacter* PCR. The annealing temperatures and primers used for detection of *Enterobacteriaceae*, *Bacterioides-Prevotella* group, and *Enterococcus* were as described by Karagin *et al.*^[1] 2010. As positive controls, *Escherichia coli* (CCUG 17620), *Bacterioides fragilis* (CCUG 4856), and *Enterococcus faecalis* (CCUG 9997) were used in all PCR reactions. The 112-bp PCR product of *Enterococcus*, the 418-bp product of *Bacterioides*, and the 195-bp product of *Enterobacteriaceae* were visualized by 1.3% agarose gel electrophoresis.

DNA sequence analysis

Helicobacter specific PCR products were purified from agarose gels using the Montage DNA Gel Extraction Kit (Millipore, Bedford, MA, United States), according to the manufacturer's instructions. DNA sequence reactions were performed using the ABI PRISMTM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit version 3.0 (Applied Biosystems) as described by Tolia *et al.*^[16]. Products of the sequencing reaction were aligned and the closest homologous DNA was identified by BLASTn-analysis.

The study was approved by the Research Ethics Committee at Lund University, permit number 588/2006.

RESULTS

Histology

The most dominant heterotopia seen in MD was of the

corpus type with, in most cases, small areas of antral heterotopia. It was therefore easy to include both types of heterotopia, if present, in the same sample. No *Helicobacter* was found by histological and immunohistological examinations, neither in heterotopia nor in ulcers. In 3/33 of the MD cases, a mild chronic inflammation in the heterotopic area with slightly increased amounts of lymphocytes was seen. The ulcer was infiltrated with polymorphonuclear cells but there was no general, active gastritis. The normal intestinal mucosa in the MD outside the heterotopia did not have an increased amount of lymphatic tissue, in except the *Helicobacter pullorum* (*H. pullorum*) positive case. The nine ulcers and their surrounding mucosa were negative for *Helicobacter* DNA.

One of the heterotopic mucosa specimens was positive for *Helicobacter* DNA, namely that from a 44-year-old male. He was operated on for acute appendicitis. The appendix was not sent for histological analysis. The MD was also removed. Histology displayed a few gastric glands of the corpus type and a small strip of surface epithelium of the gastric type. There were a moderate number of lymphocytes and plasma cells in the heterotopic area. The surrounding mucosa of the intestinal type displayed an unusually well developed lymphatic tissue with germinal centres, a predominance of lymphocytes, and very few polymorphonuclear cells (Figure 1). There was no *Helicobacter* DNA detected by PCR in this sample.

Three normal appendixes were positive for *Helicobacter* DNA: from one an 18-year-old female with suspicion of appendicitis, one from a 63-year-old male with colon adenoma, one from a 55-year-old male with colon diverticulitis. Adenoma, diverticulitis, and normal colon tissue removed from the two latter patients were negative for *Helicobacter*. No tissue other than the appendix was removed from the first patient. All cases revealed *H. pylori* on sequence analysis. There was no gastric metaplasia in any of the appendixes, and no immunopositive *H. pylori* structures in the mucosa of the samples that were PCR-positive for *Helicobacter*.

Helicobacter specific PCR assay and sequencing results

Using the *Helicobacter* specific PCR assay and agarose gel electrophoresis, *Helicobacter spp.* was detected in 1/33 (3%) of specimens from patients with MD by genus specific nested-PCR. The sequenced PCR amplicon showed 98% similarity to *H. pullorum*. There were 3/50 (6%) samples that were positive for *Helicobacter spp.*, among normal appendixes. All of them showed 98%-99% sequence similarity to *H. pylori*. However *Helicobacter spp.* was not found in any samples of acute appendicitis.

PCR detection of bacterial DNA other than Helicobacter

Using the *Enterobacteria* specific PCR assay and agarose gel electrophoresis, *Enterobacteria spp.* was detected in 10/50 (20%) acute appendicitis cases and 8/50 (16%) normal appendixes. There were 7/50 (14%) and 12/50 (24%) samples that were positive for *Bacteroides spp.*,

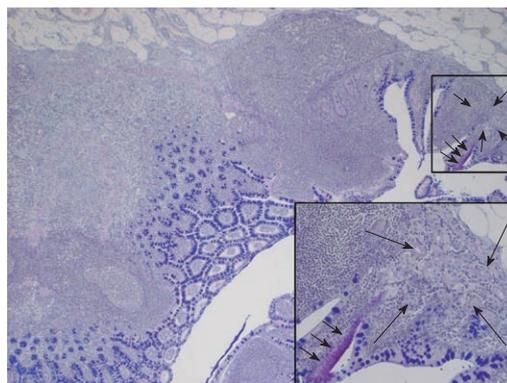


Figure 1 Microscopic analysis of the *Helicobacter pullorum* positive Meckel's diverticulum case. Low power view of a histological section from Meckel's diverticulum, positive for *Helicobacter pullorum* (*H. pullorum*) DNA, in the heterotopic area. There is an unusually well developed lymphatic tissue with germinal centres and a predominance of lymphocytes in the non-heterotopic area. Plasma cells were mainly found in the heterotopic area. The small arrows in the big square, point to a small strip of gastric heterotopia. This area displays at higher magnification in the inset. Large arrows point to gastric glands of corpus type. Blue without arrows in the photo indicates intestinal mucus. Alcian blue-PAS staining pH 2.5.

among the acute appendicitis and normal appendix sample, respectively. However, all MD and appendix samples were negative for *Enterococcus spp.* MD samples were also negative for *Bacteroides*; however, 2/33 (6%) were positive for *Enterobacteria spp.*

DISCUSSION

In this study, we screened for the presence of DNA of *Helicobacter spp.* and certain common intestinal bacteria by PCR in MD with gastric heterotopia and in appendix samples. We detected *H. pullorum* DNA in one out of 33 MD cases (3%) and *Enterobacteria* in two (6%). No *Enterococcus* or *Bacteroides* were found in the MD cases.

The *H. pullorum* case was positive only in the heterotopic area, not in the surrounding diverticulum mucosa of the intestinal type. No luminal contents were seen in these samples. This argues for the interpretation that the *H. pullorum* DNA originated from the heterotopic mucosa and not from the lumen. This assumption is further strengthened by the very low prevalence of other bacterial DNA in the MD samples. *H. pullorum* has, however, been described in stools from cases with gastroenteritis^[17], but our patient did not have such symptoms.

No *Helicobacter* was seen by immunohistochemistry. However, PCR is a more sensitive method and does not require intact bacteria. Our PCR technique is considered to be highly reliable for genus identification of *Helicobacter spp.*^[18,19]. Some authors have found *H. pylori* by immunohistochemistry in MD with active gastritis, implying the presence of polymorphonuclear cells; the prevalence varied between 2 and 28%^[6-8,20-23]. We had no cases with such inflammation and found no *H. pylori* DNA.

Interestingly, there was an increased amount of lymphatic tissue in the intestinal type mucosa of the *H.*

pullorum positive case. However, no conclusions can be drawn from just one case.

EHS are known to cause inflammatory bowel diseases^[24,25]. We have previously found *H. pullorum* DNA in cholecystitis samples with gastric metaplasia^[1]. Perhaps *H. pullorum* has some preference for the gastric epithelium.

We found *H. pylori* DNA in three out of 50 normal appendixes (6%) and none in the 50 cases of acute appendicitis. Other bacterial DNA was found in up to 24% of samples. We could not avoid including some luminal material in the appendix samples and therefore *H. pylori* DNA in the appendixes might be a contamination. Pavlidis et al^[14] found *H. pylori* by PCR in two out of 46 samples (4%) of acute appendicitis. However, most authors have failed to demonstrate the presence of *H. pylori* in the appendix^[13,15,26]. *H. pylori* commonly colonises the gastrointestinal tract. However, our results suggest that *Helicobacter* is without importance in the etiology of acute appendicitis.

In conclusion, *H. pullorum* has, for the first time, been detected by PCR in MD patients with gastric heterotopias. However, there is no association between *H. pullorum* and MD pathogenesis. Moreover, *H. pylori* has no role in the aetiology of acute appendicitis. Its presence might have been that of a passenger.

ACKNOWLEDGMENTS

Hans-Olof Nilsson's expert knowledge on PCR and specimen handling and PCR inhibitor removal and Ingrid Nilsson's valuable expertise on *Helicobacters* are highly appreciated. We also thank Roland Andersson, MD PhD, Department of Surgery, for contributing the case story of the *H. pullorum* positive patient.

COMMENTS

Research frontiers

Helicobacter-like bacteria in Meckel's diverticulum (MD) have been reported by histological methods. However, no study has reported *Helicobacter* DNA in such specimens by polymerase chain reaction (PCR) and there is some doubt as to the presence of *Helicobacter* in patients with appendicitis.

Innovations and breakthroughs

Most studies have analyzed only *Helicobacter pylori* (*H. pylori*) in Meckel's diverticulum and appendix samples. However, enterohepatic *Helicobacter* species might also be important in the etiology of such diseases. The authors demonstrated the presence of *Helicobacter pullorum* in Meckel's diverticulum for the first time and concluded that *Helicobacter* might be a passenger in such patients.

Applications

By understanding the role of *Helicobacters* in the pathology of Meckel's diverticulum and appendicitis, this study could represent a future strategy for further pathological studies.

Terminology

Enterohepatic *Helicobacter* spp. (EHS) are the species of the genus *Helicobacter* that colonize the hepatobiliary tract and can cause extragastric diseases in humans or in animals.

Peer review

This work has been had the objective of seeking any association between *Helicobacter* species other than *H. pylori* with Meckel's diverticulum by very sensitive method, i.e., Nested PCR. Most previous studies were based on conventional methods, such as culture isolation, whereas in this study, the authors have used

molecular techniques. Although they did not find any association between MD and *Helicobacter* species, it does not undermine the importance of the study.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Zhang DN

Epinephrine plus argon plasma or heater probe coagulation in ulcer bleeding

Ahmet Karaman, Mevlut Baskol, Sebnem Gursoy, Edip Torun, Alper Yurci, Banu Demet Ozel, Kadri Guven, Omer Ozbakir, Mehmet Yucesoy

Ahmet Karaman, Mevlut Baskol, Sebnem Gursoy, Edip Torun, Alper Yurci, Banu Demet Ozel, Kadri Guven, Omer Ozbakir, Mehmet Yucesoy, Department of Gastroenterology, Erciyes University, 38030 Kayseri, Turkey

Author contributions: Karaman A, Ozel BD, Torun E, Yurci A researched the data; Baskol M and Gursoy S analyzed the data; Karaman A and Baskol M wrote the manuscript; Yucesoy M, Ozbakir O, and Guven K edited the manuscript.

Correspondence to: Dr. Ahmet Karaman, Department of Gastroenterology, Erciyes University, 38030 Kayseri, Turkey. drkaraman@hotmail.com

Telephone: +90-533-4834197 Fax: +90-352-4375273

Received: January 4, 2011 Revised: February 28, 2011

Accepted: March 7, 2011

Published online: September 28, 2011

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Key words: Upper gastrointestinal bleeding; Argon plasma coagulation; Heater probe coagulation; Duodenal ulcer; Gastric ulcer

Peer reviewer: Yoshio Yamaoka, MD, PhD, Associate Professor, Department of Medicine/Gastroenterology, Baylor College of Medicine and VA Medical Center (111D), 2002 Holcombe Blvd, Houston, TX 77030, United States

Karaman A, Baskol M, Gursoy S, Torun E, Yurci A, Ozel BD, Guven K, Ozbakir O, Yucesoy M. Epinephrine plus argon plasma or heater probe coagulation in ulcer bleeding. *World J Gastroenterol* 2011; 17(36): 4109-4112 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4109.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4109>

Abstract

AIM: To compare the effectiveness of argon plasma coagulation (APC) and heater probe coagulation (HPC) in non-variceal upper gastrointestinal bleeding.

METHODS: Eighty-five (18 female, 67 male) patients admitted for acute gastrointestinal bleeding due to gastric or duodenal ulcer were included in the study. Upper endoscopy was performed and HPC or APC were chosen randomly to stop the bleeding. Initial hemostasis and rebleeding rates were primary and secondary end-points of the study.

RESULTS: Initial hemostasis was achieved in 97.7% (42/43) and 81% (36/42) of the APC and HPC groups, respectively ($P < 0.05$). Rebleeding rates were 2.4% (1/42) and 8.3% (3/36) in the APC and HPC groups, respectively, at 4 wk ($P > 0.05$).

CONCLUSION: APC is an effective hemostatic method in bleeding peptic ulcers. Larger multicenter trials are necessary to confirm these results.

INTRODUCTION

Upper gastrointestinal bleeding (UGIB) is a common and life-threatening medical emergency. UGIB is defined as bleeding proximal to the ligament of Treitz. At least 80% of patients admitted to hospital because of acute bleeding have an excellent prognosis; generally, bleeding stops spontaneously and circulatory supportive therapy is adequate. Endoscopic therapy has been shown to reduce the rate of rebleeding, blood transfusion and surgery^[1]. Endoscopic therapy is indicated in the following situations: (1) bleeding esophageal varices; (2) peptic ulcer with major stigmata of recent hemorrhage (active spurting bleeding, non-bleeding visible vessel or non-adherent blood clot); (3) vascular malformations including actively bleeding arteriovenous malformation, gastric antral vascular ectasia, and Dieulafoy malformation; and (4) active bleeding from a Mallory-Weiss tear.

Endoscopic hemostasis has significantly improved the outcome of patients with gastrointestinal bleeding.

Contact thermal coagulation with heater probe and argon plasma coagulation (APC) are among the hemostatic methods for bleeding peptic ulcers. Devices are applied directly to the bleeding point to cause coagulation and thrombosis in heater probe coagulation (HPC). The heater probe is pushed firmly on to the bleeding lesion to apply tamponade and deliver defined pulses of heat energy. APC is a non-contact method of delivering high-frequency monopolar current through ionized and electrically conductive argon gas^[2].

The aim of this study was to compare these two methods for UGIB due to gastric and duodenal ulcers. The primary outcome measure was initial hemostasis and secondary outcome measure was recurrence of bleeding. This study was approved by Erciyes University Ethical Committee.

MATERIALS AND METHODS

All patients admitted for acute gastrointestinal bleeding due to gastric and duodenal ulcers were included in the study. Gastrointestinal bleeding was diagnosed only if medical staff witnessed hematemesis or melena, or detected black, tarry material on digital examination of the rectum. Patients with actively bleeding peptic ulcers, ulcers with adherent clots, or ulcers with non-bleeding visible vessels were randomly assigned to epinephrine injection plus HPC or epinephrine injection plus APC. Informed consent was obtained before the procedure and this was solely for the procedure itself. Patients with previous malignant ulcers, and unidentifiable ulcers because of torrential bleeding were excluded.

Randomization of patients was carried out by means of sealed numbered envelopes. Informed consent was obtained for therapeutic endoscopic intervention. Patients were blind to the study. All patients underwent endoscopy within 24 h of admission. All procedures were performed by experienced gastroenterologists (experienced endoscopist as a specialist in gastroenterology with a minimum of 3 years of post-training experience) with a Fujinon-2200 endoscope. An Olympus HPU-20 heater probe system with 10 F probes was used with power settings of 30-40 J. The heater probe was pushed firmly on to the bleeding lesion to apply compression and to cause coagulation and thrombosis. We used an Erbe 200 D APC unit, which consists of an argon gas source, a high-frequency electrosurgical unit, an APC probe, and foot switches to activate the argon gas source and current generator. Operative distance between the probe and tissue was adjusted to 2-10 mm by sense of proportion (Technically, APC cannot be activated unless the tip of the probe is at least 2-10 mm distant from the ulcer region. An automated switch-off system has been integrated into the system to avoid damage to the endoscope when this distance increases). Power/gas flow settings were 50 W and 2 L/min. All endoscopists used the same settings on the instrument. Epinephrine injection (5-6 mL, 1/10 000 dilution) was applied around the ulcer

Table 1 Demographic data and randomization of the groups

	APC	HPC	Total	P value
Male	33	34	67	0.418
Female	10	8	18	0.418
Duodenal ulcer	23	25	48	0.366
Gastric ulcer	20	17	37	0.366
Age (yr)	57	52	54	0.19
Forrest				
1a	4	9	13	
1b	39	33	72	0.291

Randomization of the groups was homogeneous (All *P* values > 0.05). APC: Argon plasma coagulation; HPC: Heater probe coagulation.

in all patients, before both of these two methods.

Initial hemostasis was defined as cessation of active bleeding. All the patients were treated with the same protocol after the endoscopic procedure. A policy of early feeding was adopted, and intravenous omeprazole was prescribed at a dose of 40 mg/d. Primary failure was defined as failure to stop bleeding during initial endoscopy. Recurrent bleeding was defined by one of the following: 2 g/dL drop in hemoglobin value compared to that when the patient was discharged from hospital; fresh hematemesis; hypotension (systolic blood pressure < 90 mm Hg) with tachycardia (pulse > 110 beats/min); or melena after endoscopic treatment. Patients who did not have initial hemostasis were excluded during evaluation of rebleeding rates. Patients were followed for the next 4 wk after initial hemostasis to monitor rebleeding. Distribution of bleeding focus and severity of bleeding (by using Forrest Classification) was found to be similar by χ^2 analysis (Table 1).

Analysis of data was performed using SPSS version 16.0 (SPSS, Chicago, IL, United States). *P* < 0.05 was regarded as significant. We calculated the power of the study with PASS 2008 software, with $\alpha = 0.05$, $n = 85$, degrees of freedom = 1, and power of study = 99.9%.

RESULTS

Eighty-five (18 female, 67 male) patients were included in the study between February 2008 and November 2009 in the Gastroenterology Department, Erciyes University School of Medicine. Forty-two patients received HPC and 43 received APC. Forty-eight bleeding duodenal ulcers (25 received HPC therapy and 23 APC) and 37 bleeding gastric ulcers (17 received HPC therapy and 20 APC) were included. A consort flow diagram was designed and is presented in Figure 1.

Initial hemostasis was achieved in 97.7% (42/43) and 81% (36/42) of the APC and HPC groups, respectively (*P* < 0.05). There were significant differences in initial hemostasis rates (*P* = 0.015). One patient died, two had surgery, and three had hemoclips applied in the HPC group; one patient had hemoclips applied in the APC group, who did not have initial hemostasis. Rebleeding rates were 2.4% (1/42) and 8.3% (3/36) in the APC and

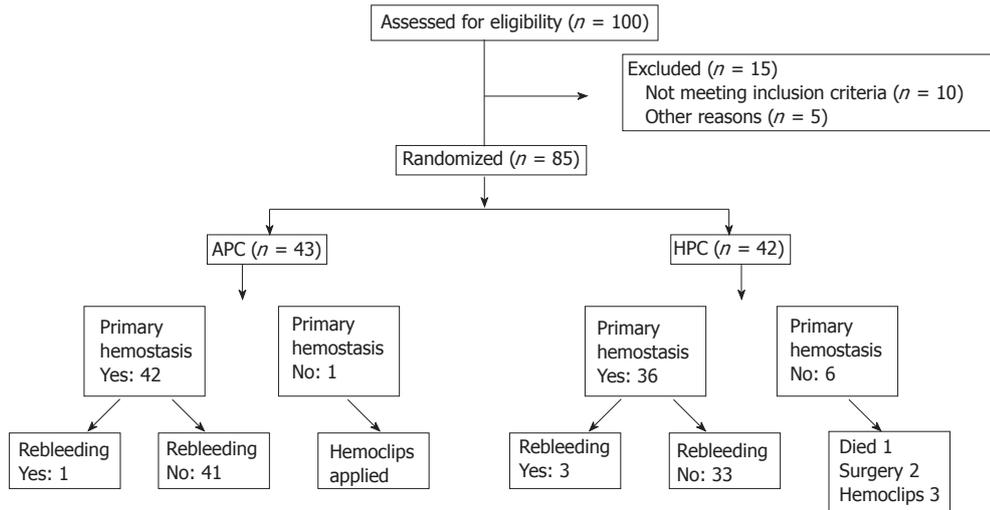


Figure 1 Consort flow diagram of the study. APC: Argon plasma coagulation; HPC: Heater probe coagulation.

Table 2 Initial hemostasis and rebleeding rates of the groups

	No. of cases (gastric/duodenal ulcer)	Initial hemostasis (%)	Rebleeding (%)
APC	43 (20-23)	42/43 (97.7)	1/42 (2.4)
HPC	42 (17-25)	36/42 (81)	3/36 (8.3)
Total	85 (37-48)	78 (91.7)	4/78 (5.1)

There was a significant difference ($P < 0.05$) in initial hemostasis but not in rebleeding rates between the groups. APC: Argon plasma coagulation; HPC: Heater probe coagulation.

HPC groups, respectively at 4 wk after index bleeding ($P = 0.25$). Although rebleeding rate was greater in the HPC group, there was no significant difference between the groups (Table 2).

DISCUSSION

Acute UGIB is a common medical emergency that carries hospital mortality in excess of 10%^[3]. Bleeding stops spontaneously in about 80% of patients with non-variceal UGIB. Various modalities of endoscopic therapy have been used to reduce recurrent bleeding, surgery, and mortality rates, and therefore, endoscopic intervention is a preferable procedure in acute gastrointestinal (GI) bleeding. In the past few decades, several endoscopic methods have been developed for hemostasis of gastrointestinal bleeding. Some of these are HPC, direct injection of fluids (e.g., diluted epinephrine or distilled water) into the bleeding lesion using disposable needles, mechanical devices such as endoclips, and APC. There have been several studies about the efficiency of these methods in gastrointestinal bleeding. Although APC is used especially for chronic radiation proctitis^[4], watermelon stomach and ablation of Barrett's esophagus, there are no current clinical studies about the application of APC in bleeding ulcers and for other causes of UGIB. APC therapy has various theoretical merits over contact-type thermal coagulation. First, depending on different power/flow

settings, the burn depth can be preset between 0.5 mm and 3 mm, which is a particularly appropriate range for hemostasis in thin-walled duodenum and colon. Second, some bleeding points, such as those in the posterior wall or lesser curvature of the upper gastric body or the posterior wall of the duodenal bulb, may be difficult to approach. The no-touch technique in APC can make the approach easier by the arcing effect^[2,5]. Wang *et al*^[6] have compared APC and distilled water injection in treating high-risk bleeding ulcers, and they have reported that bleeding recurrence was 11% in the APC group and 27% in the distilled water injection group. They have concluded that endoscopic therapy is more effective than distilled water injection for preventing rebleeding in these patients, and no significant differences were observed between the two groups in terms of surgery and mortality. There have been a few studies in which APC and HPC were compared for treatment of peptic ulcer bleeding^[7]. Although HPC is the more commonly used method in active bleeding lesions^[8], APC is not used as much as HPC for stopping gastrointestinal bleeding. In our study, APC appeared to be more effective in initial hemostasis, but rebleeding rates were similar with both techniques. Bleeding recurrence has consistently been identified as the most important prognostic factor for mortality^[9]. Arresting recurrence of bleeding can decrease the rate of morbidity and mortality from UGIB. Cipolletta *et al*^[7] have reported that initial hemostasis rates were 95% and 95.2%, and recurrent bleeding rates were 21% and 15% in HPC and APC group, respectively, and there was no significant difference between the groups in the rate of recurrent bleeding^[2]. Although rebleeding rates were much lower in the APC group, we found no significant difference in the rebleeding rates between the groups. Skok *et al*^[10] have reported that clinically and endoscopically diagnosed bleeding recurred in 14% of patients in the APC group, and 18% of patients in the sclerotherapy group. Although these controlled trials had similar hemostatic efficacy, the patients treated

with APC had noticeably lower rebleeding rates.

The theoretical advantages of APC include its ease of application, speedy treatment of multiple lesions in the case of arteriovenous malformations or wide areas (the base of resected polyps or tumor bleeding), and safety due to reduced depth of penetration^[11]. Superficial ulceration occurs following APC, which typically heals within 2-3 wk. Despite theoretical safety advantages due to reduced depth of penetration, all of the complications that have been reported with other thermal hemostasis techniques may occur. The first series of clinical applications of APC in gastrointestinal endoscopy was published in 1994^[12]. Although no specific data were provided to assess the outcome for GI bleeding, the authors have described the technique as successful^[12]. Several centers have subsequently reported experience with this technique in the management of GI bleeding^[12,12]. However, few randomized studies comparing with APC with other hemostasis techniques have been performed, and our study is believed to be the first comparison of these two techniques in UGIB in recent years. APC application had better rates of initial hemostasis than HPC in our study.

The heater probe has a thermocouple at the tip of the probe that can heat up quickly and achieve tissue coagulation. As a result, deep coagulation is feasible with the heater probe, but this effect may risk perforation. As mentioned above, few studies have directly compared APC to other methods, especially HPC, for achieving hemostasis. In conclusion, APC is one of the effective hemostatic methods in bleeding peptic ulcers. Larger multicenter trials are necessary to confirm these data.

COMMENTS

Background

Contact thermal coagulation with heater probe coagulation (HPC) and argon plasma coagulation (APC) are the hemostatic methods used for the treatment of bleeding peptic ulcers. Few studies have directly compared the use of epinephrine injection plus APC versus epinephrine injection plus HPC for achieving hemostasis.

Research frontiers

Upper gastrointestinal bleeding (UGIB) is a common and life-threatening medical emergency. Contact thermal coagulation with HPC and APC are among the hemostatic methods for treatment of bleeding peptic ulcers. In this study, the authors demonstrate that there is a higher initial hemostasis rate in APC when compared with HPC in ulcer bleeding.

Innovations and breakthroughs

Few randomized studies have compared APC with other hemostasis techniques, and the study is believed to be the first comparison of these two techniques with additional use of epinephrine injections for UGIB in recent years. This study suggests that APC could be used instead of HPC, and that APC could provide clinicians with an effective alternative method to stop bleeding

ulcers, due to its high rate of primary hemostasis and low rate of rebleeding.

Applications

This study may encourage clinicians without experience in APC to use the technique for treatment of ulcer bleeding.

Terminology

APC is a non-contact method of delivering a high-frequency monopolar current through ionized and electrically conductive argon gas. Devices are applied directly to the bleeding point to cause coagulation and thrombosis in HPC. The heater probe is pushed firmly onto the bleeding lesion to apply tamponade and deliver defined pulses of heat energy.

Peer review

The authors have compared the effectiveness of APC and HPC for ulcer bleeding. They have concluded that APC is superior to HPC for initial hemostasis. This was a well-designed study because the patients were assigned to two groups at random.

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S- Editor Tian L L- Editor Kerr C E- Editor Zhang DN

Role of *cyclooxygenase-2* gene polymorphisms in pancreatic carcinogenesis

Renata Talar-Wojnarowska, Anita Gasiorowska, Marek Olakowski, Pawel Lampe, Beata Smolarz, Hanna Romanowicz-Makowska, Ewa Malecka-Panas

Renata Talar-Wojnarowska, Anita Gasiorowska, Ewa Malecka-Panas, Department of Digestive Tract Diseases, Medical University, 22 Kopcinskiego, 90-153 Lodz, Poland

Marek Olakowski, Pawel Lampe, Department of Digestive Tract Surgery, Silesian Medical University, 14 Medykow, 40-752 Katowice, Poland

Beata Smolarz, Hanna Romanowicz-Makowska, Laboratory of Molecular Genetics, Institute of Polish Mother's Memorial Hospital, 281/289 Rzgowska, 93-338 Lodz, Poland

Author contributions: Talar-Wojnarowska R and Gasiorowska A provided the protocols and performed the research; Olakowski M and Lampe P provided the collection of human material and analyzed the data; Smolarz B and Romanowicz-Makowska H carried out the molecular analysis; and Talar-Wojnarowska R and Malecka-Panas E designed the study and wrote the paper.

Correspondence to: Renata Talar-Wojnarowska, MD, PhD, Department of Digestive Tract Diseases, Medical University of Lodz, Kopcinskiego 22, 90-153 Lodz,

Poland. renata.talar-wojnarowska@umed.lodz.pl

Telephone: +48-42-6786480 Fax: +48-42-6786480

Received: December 20, 2010 Revised: March 26, 2011

Accepted: April 2, 2011

Published online: September 28, 2011

Abstract

AIM: To evaluate the clinical significance of *-765G/C* and *-1195G/A cyclooxygenase-2 (COX-2)* gene polymorphisms in patients with pancreatic cancer (PC).

METHODS: The study included 201 patients: 85 with PC and 116 healthy controls. *-765G/C* and *-1195G/A COX-2* gene polymorphisms were studied in DNA isolated from blood samples. The associations of the analyzed genotypes and clinical data at diagnosis were evaluated.

RESULTS: We found an increased frequency of the homozygous *-1195AA COX-2* genotype in patients with PC (53.7%) compared with the control group (21%) ($P < 0.01$). In contrast, the distribution of genotype

and allele frequencies of the *-765G/C COX-2* polymorphism in the PC patients were not different from those in control groups. A correlation between presence of homozygous *-1195AA COX-2* genotype and tumor size > 3 cm was observed ($P < 0.05$). Analyzed polymorphisms were unrelated to the patients' sex and age, nor to the presence of regional or distant metastases.

CONCLUSION: These preliminary results indicate that the *-1195G/A COX-2* polymorphism may play an important role in PC prognosis and carcinogenesis.

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Key words: *Cyclooxygenase-2*; Polymorphisms; Pancreatic cancer; Carcinogenesis

Peer reviewer: Kotaro Miyake, MD, PhD, Department of Surgery, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8503, Japan

Talar-Wojnarowska R, Gasiorowska A, Olakowski M, Lampe P, Smolarz B, Romanowicz-Makowska H, Malecka-Panas E. Role of *cyclooxygenase-2* gene polymorphisms in pancreatic carcinogenesis. *World J Gastroenterol* 2011; 17(36): 4113-4117 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4113.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4113>

INTRODUCTION

Cyclooxygenase-2 (COX-2), also known as prostaglandin endoperoxide synthase, is a key enzyme in the arachidonic acid pathway, initiating the synthesis of biologically important prostanoids and eicosanoids. *COX-2* is involved in many biologic processes, such as cell proliferation, invasion, angiogenesis and inhibition of apoptosis, which are all relevant to cancer development and progression^[1].

COX-2 is expressed under certain extracellular or intracellular stimuli, such as mitogens, growth factors, hormones, infectious agents and proinflammatory cytokines^[1,2]. Numerous studies have demonstrated increased expression of *COX-2* in various cancers, including pancreatic tumors^[3-6]. In several studies, the overexpression of *COX-2* in pancreatic cancer (PC) cells has been shown to be an independent prognostic factor and to increase the clinical aggressiveness of this disease^[3,4,7].

The *COX-2* gene was demonstrated to be genetically polymorphic, which may affect the expression or activity of this enzyme and consequently contribute to variation in individual susceptibility to cancer through aberrant arachidonic acid metabolism. Genetic variants represented by single nucleotide polymorphisms (SNPs) of the *COX-2* gene, among them *-765G/C* and *-1195G/A* in the promoter region, have been identified. The functional effects of these SNPs have been recently confirmed^[8,9]. The *-765G* allele is associated with heightened *COX-2* transcription by creating a transcriptional factor c-MYB-binding site and with significantly higher promoter activity compared with *-765C* allele^[8,9]. In the study of Zhang *et al*^[10], the *-1195A*-containing haplotypes also had significantly increased *COX-2* messenger RNA levels in esophageal tissues compared with the *-1195G*-containing counterparts.

COX-2 has been the object of prevention/intervention strategies in many clinical trials, including being a potential therapeutic target for chemoprevention and therapy of PC^[11,12]. Selective inhibition of *COX-2* results in variable responses in individual patients. Information regarding the functional significance of *COX-2* polymorphisms with risk-modulating ability would have significant implications, not only for risk identification, but also for pharmacological management of the disease.

The purpose of this study was to evaluate the clinical significance of *-765G/C* and *-1195G/A* *COX-2* gene polymorphisms in patients with PC.

MATERIALS AND METHODS

The study included 201 Caucasian patients: 85 with PC (41 men and 44 women, aged 44-84 years) and 116 gender- and age-matched healthy volunteers. Analyzed patients were hospitalized in the Department of Digestive Tract Diseases, Medical University of Lodz Hospital or in the Department of Digestive Tract Surgery of Silesian Medical University in Katowice between 2004 and 2009. Only patients with a confirmed pathological diagnosis of ductal pancreatic adenocarcinoma were included in the study. The pathological diagnosis was confirmed after surgical treatment or after pancreatic tissue biopsy in patients who qualified for palliative chemotherapy. Twenty-nine patients (34.1%) with PC underwent Whipple resection or distal pancreatectomy, 33 patients (38.8%) underwent palliative surgery and 23 patients (27.1%) underwent palliative chemotherapy and/or endoscopic treatment. Tumor grade was classified into G1 (well differentiated), G2 (moderately differentiated) and G3 (poorly differentiated).

The study protocol was approved by the ethical committee of Lodz Medical University.

Peripheral venous blood samples were obtained from all analyzed patients at the time of hospital admission. *-765G/C* and *-1195G/A* *COX-2* gene polymorphisms were studied in DNA isolated from blood samples using the QI-Amp DNA Mini Kit (Qiagen). Polymerase chain reaction (PCR) products for the *COX-2* variants were analyzed by the restriction fragment length polymorphism method. The primers used to amplify the *COX-2* promoter region were 5'-TAT TAT GAG GAG AAT TTA CCT TTC GC-3' and 5'-GCT AAG TTG CTT CAA CAG AAG AAT-3' for the *-765G/C* variant; and 5'-CCC TGA GCA CTA CCC ATG AT-3' and 5'-GCC CTT CAT AGG AGA TAC TGG-3' for the *-1195G/A* polymorphism. PCR amplification was performed in a final volume of 25 μ L containing 30-100 ng of DNA, 10 mmol/L Tris-HCl (pH 8.3), 4 μ L of 25 mmol/L MgCl₂, 50 mmol/L KCl, 0.5 μ L dNTP (10 mmol/L), each primer at 1.0 μ mol/L and 1.0 unit of Taq polymerase (BIOKOM, Takara, Japan) in a GeneAmp PCR system 9700 Thermocycler (Applied Biosystems).

Ten microliters of the PCR product were digested with 2 units of restriction enzymes HhaI or Pvu II (BioLabs, New England) using the manufacturer's recommended protocol. PCR products were visualized on 8% polyacrylamide gels with 10% ethidium bromide. *COX-2* genotypes that could be detected were respectively: *-765CC* (100 bp fragment), *-765GC* (100 and 74 and 26 bp fragments), *-765GG* (74 and 26 bp fragments), *-1195AA* (273 bp fragment), *-1195GA* (273 and 220 and 53 bp fragments) and *-1195GG* (220 and 53 bp fragments).

The serum concentrations of CA19-9 were measured by an enzyme-linked immunoassay (DRG International, United States), according to the manufacturer's recommendations. The associations of the analyzed genotypes and patient characteristics at PC diagnosis were evaluated. The following demographic and clinical data were analyzed: age, gender, tumor size, lymph node involvement, histological grade, CA19-9 levels, weight loss and history of smoking. The cut-off point of CA19-9 was set at 37 U/mL.

Statistics analysis

The results were analyzed using StatSoft Statistica for Windows, release 6.0 (StatSoft, Inc., Tulsa, United States). To determine differences between groups, Mann-Whitney *t* tests were used. Clinical significance of analyzed polymorphisms was determined using logistic regression analysis and presented in tables as odds ratios (OR) with their 95% confidence intervals. The deviations from Hardy-Weinberg equilibrium were analyzed using the χ^2 test. Differences with a *P* value less than 0.05 were considered significant.

RESULTS

All patients involved in the study were Caucasians. Mean ages were not significantly different for patients with PC

Table 1 Distribution of -1195 G/A and -765 G/C COX-2 genotype in the analyzed group of patients *n* (%)

Genotype	PC patients (<i>n</i> = 85)	Control group (<i>n</i> = 116)	OR (95% CI)
-1195 G/A			
GG	13 (15.7)	44 (37.9)	Reference
GA	26 (30.6)	48 (41.4)	1.83 (0.84-4.00)
AA	46 (53.7)	24 (21.0)	6.48 (2.93-14.31)
-765 G/C			
GG	47 (55.4)	44 (37.9)	Reference
GC	27 (31.7)	40 (34.5)	0.63 (0.33-1.19)
CC	11 (12.9)	32 (27.6)	0.32 (0.14-1.71)

PC: Pancreatic cancer; OR: Odds ratios.

Table 2 Relationship between -1195 G/C COX-2 polymorphism and clinical data of patients with pancreatic cancer *n* (%)

	Group	G ⁺ allele (GA and GG) (<i>n</i> = 39)	<i>P</i> value	G ⁻ allele (AA) (<i>n</i> = 46)	<i>P</i> value
Age	< 65	20 (51.3)	NS	19 (41.3)	NS
	≥ 65	19 (48.7)		27 (58.7)	
Gender	Male	18 (46.2)	NS	23 (50.0)	NS
	Female	21 (53.8)		23 (50.0)	
Tumor size	≤ 3 cm	21 (53.8)	NS	13 (28.3)	<i>P</i> < 0.05
	> 3 cm	18 (46.2)		33 (71.7)	
Tumor	G1 + G2	21 (53.8)	NS	27 (58.7)	NS
Differentiation	G3	17 (43.8)	NS	18 (39.2)	NS
Lymph node	Absent	23 (58.9)	NS	23 (50.0)	NS
Metastases	Present	16 (41.1)	NS	23 (50.5)	NS
Weight loss	< 10 %	19 (48.7)	NS	25 (54.4)	NS
	≥ 10 %	20 (51.3)		21 (45.6)	
Smoking	Yes	21 (53.8)	NS	19 (41.3)	NS
	No	18 (46.2)		27 (58.7)	
CA19-9	< 37 U/mL	10 (25.6)	NS	13 (28.3)	NS
	≥ 37 U/mL	29 (74.4)		33 (71.7)	

NS: Not significant.

Table 3 Relationship between -765 G/C COX-2 polymorphism and clinical data of patients with pancreatic cancer *n* (%)

	Group	G ⁺ allele (GC and GG) (<i>n</i> = 47)	<i>P</i> value	G ⁻ allele (CC) (<i>n</i> = 38)	<i>P</i> value
Age	< 65	23 (48.9)	NS	16 (42.1)	NS
	≥ 65	24 (51.1)		22 (57.9)	
Gender	Male	22 (46.8)	NS	19 (50.0)	NS
	Female	25 (53.2)		19 (50.0)	
Tumor size	≤ 3 cm	17 (40.4)	NS	15 (39.5)	NS
	> 3 cm	28 (59.6)		23 (60.5)	
Tumor	G1 + G2	27 (57.4)	NS	21 (55.3)	NS
Differentiation	G3	20 (40.4)	NS	16 (42.1)	NS
Lymph node	Absent	28 (59.8)	NS	18 (47.4)	NS
Metastases	Present	19 (40.4)	NS	20 (52.6)	NS
Weight loss	< 10 %	30 (63.8)	NS	21 (55.3)	NS
	≥ 10 %	17 (36.2)		17 (44.7)	
Smoking	Yes	23 (48.9)	NS	17 (44.7)	NS
	No	24 (51.1)		21 (55.3)	
CA19-9	< 37 U/mL	12 (25.5)	NS	12 (31.6)	NS
	≥ 37 U/mL	35 (74.5)		26 (68.4)	

NS: Not significant.

(mean 66.8 ± 4.1 years) and controls (63.1 ± 4.7 years, $P > 0.05$). In patients with pancreatic adenocarcinoma, the tumor size ranged from 2 cm to 7 cm (mean 3.7 ± 2.3). As for histological differentiation, 19, 29 and 35 patients were classified into G1, G2 and G3 respectively, whereas 2 patients had missing data. Lymph node metastases were observed in 39 patients with PC (45.9%) and liver metastases in 16 of them (18.8%). Serum levels of CA19-9 were higher in patients with PC compared to control group ($P < 0.001$; respectively 101.2 ± 21.4 U/mL *vs* 17.6 ± 3.2 U/mL; data shown in our previously published work^[22]).

The genotype distributions of analyzed -765G/C and -1195G/A COX-2 gene polymorphisms are summarized in Table 1. We found an increased frequency of the homozygous -1195AA COX-2 genotype in patients with PC compared with control group [OR 6.48 (2.93-14.31), $P < 0.01$]. In contrast, the distribution of genotype and allele frequencies of the -765G/C COX-2 polymorphism in the PC patients did not differ from those in control groups (Table 1). Each of the COX-2 polymorphisms in the controls was consistent with Hardy-Weinberg equilibrium.

The potential relationship between COX-2 genotype distribution and clinical data of the PC patients was investigated. COX-2 -1195AA genotype showed a significant association with tumor size > 3 cm in patients with PC ($P < 0.05$, Table 2). This analyzed polymorphism was unrelated to the patients' sex and age, weight loss, history of smoking, CA19-9 levels, nor with the presence of regional or distant metastases. In contrast, the -765G/C COX-2 polymorphism was not associated with any clinical data (Table 3).

DISCUSSION

The overexpression of COX-2 has been shown to induce angiogenesis by increased synthesis of vascular endothelial growth factor and to inhibit apoptosis by activation of proto-oncogene Bcl-2^[2]. It is known that the expression of COX-2 is increased in the majority of PC cells and may represent a target for adjuvant therapy of PC. However, little is known about the role of COX-2 gene polymorphisms in pancreatic carcinogenesis. We investigated the clinical significance of the -765G/C and -1195G/A COX-2 gene polymorphisms, as well as their potential association with the risk of developing PC.

In our study, the presence of the -1195AA genotype was found more frequently in patients with PC compared to the control group. Similarly, Zhao *et al*^[8] observed that subjects carrying the COX-2 -1195A allele had significantly increased risk for developing PC compared with subjects carrying the -1195G allele. In previous studies, the association of the -1195A allele with an increased risk of lung, oral and esophageal cancers was also demonstrated^[12-14]. In the study of Bi *et al*^[13], -1195AA genotype was significantly correlated with worse overall survival (15.7 mo *vs* 20.2 mo, $P = 0.006$) and with shorter

progression-free survival (9.5 mo *vs* 11.9 mo, $P = 0.0034$) in patients with unresectable locally advanced non-small cell lung cancer.

However, others authors observed opposite results. In the study of Kristinsson *et al*^[15], the -1195GG genotype resulted in a higher risk of developing esophageal adenocarcinoma. On the other hand, Pereira *et al*^[16] observed that men carrying the -1195G allele appeared to have a nine-fold increased risk for colorectal cancer. Racial and ethnic differences in the studies' populations may explain these contradictory results, because the distribution of COX-2 polymorphisms may differ considerably between populations.

The published data about clinical significance of the second analyzed polymorphism of COX-2 gene, -765G/C, are also controversial. In the study of Hoff *et al*^[17], the -765GG genotype was present more often in patients with colorectal cancer compared to control group. Similarly, Coskunpinar *et al*^[14] observed increased risk of lung carcinoma in Turkish patients carrying the -765G allele. In contrast, the -765C allele was associated with an increased risk for developing PC and urinary bladder cancer^[8,18]. This is not in line with our findings, since we could not demonstrate a significant difference in -765G/C genotype distribution in patients with PC. Similarly, Dong *et al*^[19] in a meta-analysis of 47 case-control studies did not find a convincing association between -765G/C COX-2 gene polymorphism and the risk of cancer in diverse populations.

Another important aspect of our analysis was to assess the potential association of -765G/C and -1195G/A COX-2 gene polymorphisms with clinical data of patients with PC. In the current study, the homozygous -1195AA was found to be present more frequently in patients with larger tumor size. To the best of our knowledge there are no available data about relationships between -1195G/A COX-2 polymorphism and clinical characteristics of patients with PC. Earlier, Tan *et al*^[20] demonstrated that the COX-2 -1195A allele was associated with the presence of distant metastases in patients with colorectal cancer. They suggested that COX-2 may play a role not only in colorectal tumorigenesis but also in cancer progression by stimulating cell proliferation and spread.

According to our data, the second analyzed COX-2 polymorphism, -765G/C, was not associated with clinical parameters. Similarly, in other studies the -765G/C variant was not correlated with clinical stage of patients with cervical and colorectal cancers^[17,21].

Overexpression of COX-2 may be an important cellular mechanism in smoking-related PC development. Numerous studies have shown that smoking induces COX-2 expression, but the exact signal pathways remain to be elucidated. Zhao *et al*^[8] suggested that COX-2 genetic polymorphisms may determine interindividual variation in the inducibility of COX-2 expression. They observed that smoking remarkably increased COX-2 promoter activity, especially in patients with PC carrying the -765C allele. In contrast, in our study, there was no

association between analyzed polymorphisms and smoking. Similarly, Pandey *et al*^[21] did not find a correlation between smoking and COX-2 polymorphisms in patients with cervical cancer. This lack of association could be due to a relatively small number of subjects and certainly needs further validation.

In summary, we found a significant difference in the -1195G/A COX-2 gene polymorphism distribution between patients with PC and the control group. The presence of -1195AA genotype was associated with an increased PC risk; however, further studies are needed to investigate its possible association with PC prognosis. Our results are consistent with the biological function of the polymorphisms and support the hypothesis that aberrant arachidonic acid metabolism may play an important role in pancreatic carcinogenesis.

COMMENTS

Background

Despite improved diagnostic and therapeutic capabilities, pancreatic cancer (PC) still has a very poor prognosis. Numerous studies suggest a role for cyclooxygenase-2 (COX-2) in pancreatic carcinogenesis. COX-2 is involved in many biologic processes, such as cell proliferation, invasion, angiogenesis and inhibition of apoptosis, which are all relevant to cancer development and progression.

Research frontiers

The COX-2 gene was demonstrated to be genetically polymorphic, which may affect the expression or activity of this enzyme and consequently contribute to variation in individual susceptibility and aggressiveness of PC.

Innovations and breakthroughs

This study analyzed the clinical significance of -765G/C and -1195G/A COX-2 gene polymorphisms in patients with PC. In the study, the presence of the -1195AA genotype was associated with an increased risk of PC; however, further studies are needed to investigate its possible association with PC prognosis.

Applications

The results are consistent with the biological function of the polymorphisms and support the hypothesis that aberrant arachidonic acid metabolism may play an important role in pancreatic carcinogenesis.

Terminology

COX-2 is a key enzyme in the arachidonic acid pathway, initiating the synthesis of biologically important prostaglandin H₂ (the precursor of other prostaglandins), prostacyclin and thromboxanes.

Peer review

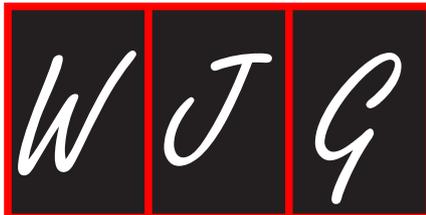
In this manuscript, the authors demonstrate that COX-2 gene polymorphisms might be associated with carcinogenesis of PC. A series of experiments are well-planned and well-performed and this manuscript is well written.

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S- Editor Tian L L- Editor Logan S E- Editor Xiong L



Does the bile duct angulation affect recurrence of choledocholithiasis?

Dong Beom Seo, Byoung Wook Bang, Seok Jeong, Don Haeng Lee, Shin Goo Park, Yong Sun Jeon, Jung Il Lee, Jin-Woo Lee

Dong Beom Seo, Byoung Wook Bang, Seok Jeong, Jung Il Lee, Jin-Woo Lee, Division of Gastroenterology, Department of Internal Medicine, Inha University School of Medicine, Incheon 400-711, South Korea

Don Haeng Lee, Division of Gastroenterology, Department of Internal Medicine and Center for Advanced Medical Education by BK21 Project, Inha University School of Medicine, and Utah-Inha DDS and Advanced Therapeutics Research Center, Incheon 400-711, South Korea

Shin Goo Park, Department of Occupational and Environmental Medicine, Inha University School of Medicine, Incheon 400-711, South Korea

Yong Sun Jeon, Department of Radiology, Inha University School of Medicine, Incheon, 400-711, South Korea

Author contributions: Seo DB and Bang BW wrote the paper; all authors performed research and collected the data; Park SG analyzed the data; Jeong S and Lee DH reviewed the paper; Jeon YS, Lee JI, Lee JW provided technical support and advice. Supported by An Inha University Research Grant

Correspondence to: Don Haeng Lee, MD, Department of Internal Medicine, Inha University Hospital, 7-206, 3-Ga, Sinheung-Dong, Jung-Gu, Incheon 400-711, South Korea. ldh@inha.ac.kr

Telephone: +82-32-8902548 Fax: +82-32-8902549

Received: September 26, 2010 Revised: March 24, 2011

Accepted: March 31, 2011

Published online: September 28, 2011

Abstract

AIM: To investigate whether bile duct angulation and T-tube choledochostomy influence the recurrence of choledocholithiasis.

METHODS: We conducted a retrospective study including 259 patients who underwent endoscopic sphincterotomy and cholecystectomy for choledocholithiasis between 2000 and 2007. The imaginary line was drawn along the center of the bile duct and each internal angle was measured at the two angulation sites of

the bile duct respectively. The values of both angles were added together. We then tested our hypothesis by examining whether T-tube choledochostomy was performed and stone recurrence occurred by reviewing each subject's medical records.

RESULTS: The overall recurrence rate was 9.3% (24 of 259 patients). The mean value of sums of angles in the recurrence group was $268.3^\circ \pm 29.6^\circ$, while that in the non-recurrence group was $314.8^\circ \pm 19.9^\circ$ ($P < 0.05$). Recurrence rate of the T-tube group was 15.9% (17 of 107), while that of the non T-tube group was 4.6% (7 of 152) ($P < 0.05$). Mean value of sums of angles after T-tube drainage was $262.5^\circ \pm 24.6^\circ$ and that before T-tube drainage was $298.0^\circ \pm 23.9^\circ$ in 22 patients ($P < 0.05$).

CONCLUSION: The bile duct angulation and T-tube choledochostomy may be risk factors of recurrence of bile duct stones.

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Key words: Choledocholithiasis; Common bile duct; Cholecystectomy; Recurrence; Endoscopic retrograde cholangiopancreatography

Peer reviewers: Giuseppe Currò, MD, University of Messina, Via Panoramica, 30/A, 98168 Messina, Italy; Beata Jolanta Jabłońska, MD, PhD, Department of Digestive Tract Surgery, University Hospital of Medical University of Silesia, Medyków 14 St. 40-752 Katowice, Poland

Seo DB, Bang BW, Jeong S, Lee DH, Park SG, Jeon YS, Lee JI, Lee JW. Does the bile duct angulation affect recurrence of choledocholithiasis? *World J Gastroenterol* 2011; 17(36): 4118-4123 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4118.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4118>

INTRODUCTION

Since its introduction in 1974, endoscopic sphincterotomy (EST) has become a well established therapeutic method for the extraction of common bile duct (CBD) stones^[1,2]. In previous studies of EST, 4%-24% of patients experienced recurrent CBD stones during follow-up intervals of up to 15 years^[3-5]. The suggested causes of recurrent bile duct stones after EST are bile duct inflammation, papillary stenosis, dilated common bile duct, peripapillary diverticulum, reflux of the duodenal contents into the bile duct, and foreign bodies within the bile duct^[6,7]. After EST, the biliary sphincter is rendered permanently insufficient^[8]. The loss of this physiologic barrier between duodenum and biliary tract results in duodenocholedochal reflux and bacterial colonization of the biliary tract^[9,10]. The presence of bacteria in the biliary system might lead to late complications after EST. These complications may include recurrence of CBD stones from deconjugation of bilirubin by bacterial enzymes^[11], inflammatory changes of the biliary and/or hepatic system^[12,13], recurrent ascending cholangitis^[14], and even malignant degeneration^[13,15].

Bile stasis may be one of the possible mechanisms of stone recurrence^[16]. If bile flow can be decreased by the bile duct angulation, it may be a risk factor of stone recurrence. In a previous study, it was disclosed that in treatment of CBD stones, a T-tube drainage group had a higher recurrence rate of stones than either a choledochoduodenostomy group or an EST group, although their accurate mechanism was not elucidated^[17]. It was also reported that the angulation of the extrahepatic bile duct—so called “the elbow sign”—occurred as a sequela of T-tube drainage^[18]. Therefore, we investigated whether draining the T-tube changes configuration of the extrahepatic bile duct significantly and then whether bile duct deformity affects recurrence of CBD stones.

MATERIALS AND METHODS

Patient selection

All patients who were treated for choledocholithiasis by means of endoscopic retrograde cholangiopancreatography (ERCP) with EST between January 2000 and December 2007 in our institution were recruited. Their medical records were retrospectively reviewed. Among them, patients who met the following criteria were selected: (1) complete clearance of the bile duct stones was achieved; (2) cholecystectomy performed; (3) absence of bile duct stricture; (4) absence of concurrent hepatolithiasis; (5) absence of coexisting malignant neoplasm; and (6) absence of coexisting severe medical disease. Consequently, in total 259 patients were enrolled in the study.

For all the patients, the following data were collected: gender, age, recurrence, presence of perampullary diverticulum, maximal CBD diameter and presence of T-tube drainage. We divided them into two groups; recurrent and non-recurrent groups, and the sum of extrahepatic bile

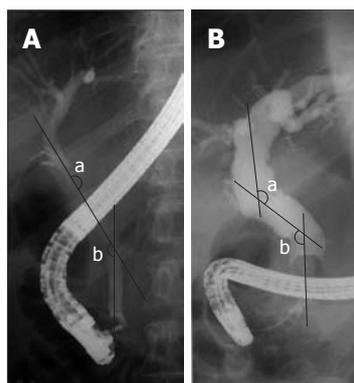


Figure 1 Cholangiographic configuration of (A) usual extrahepatic bile duct and (B) deformed bile duct. The imaginary line was drawn along the center of the bile duct and each internal angle was measured at the angulation of the proximal (a) and distal (b) bile duct level respectively. The values of both angles were added together (a + b).

duct angles and maximal CBD diameter between both groups was compared. EST was performed completely by traction-type or needle-knife sphincterotome and the bile duct stones were extracted with basket and/or stone-retrieval balloon catheters. After extraction of CBD stones, we performed a regular check-up on each patient at the outpatient department base, and considered absence of symptoms of biliary colic or cholangitis episode as absence of recurrence by using telephone interviews in patients who were missed.

Periampullary diverticulum

Periampullary diverticulum was defined as the presence of a diverticulum within a 2-cm radius from ampulla of Vater. We could identify whether periampullary diverticulum was present or not in 231 of the subjects. The information was missing from the medical records of the remaining patients. We divided patients into two groups: non-periampullary diverticulum and periampullary diverticulum groups, and the recurrence rate between the two groups was compared.

Measurement of bile duct angulation

There were films of acceptable quality, taken in the prone position during ERCP. When the extrahepatic bile duct was deformed, the number of its angulation site was one or two in most patients. The angles were measured at the intersection of imaginary lines drawn down the center of the bile duct. Its intersection did not necessarily occur within the lumen of the duct, particularly where angulation took the form of a gentle curve. Each internal angle was measured at the two angulation sites of the proximal and distal bile duct level respectively by one experienced radiologist (Figure 1). **The values of both angles were added together to estimate the grade of angulation.** The smaller sum of angles implies more angulation.

T-tube choledochostomy

T-tube drainage was performed during the cholecystectomy when the bile duct was injured surgically or when a

Table 1 Univariate analysis of risk factors for recurrent bile duct stones *n* (%)

Variable		Non recurrence group (<i>n</i> = 235)	Recurrence group (<i>n</i> = 24)	<i>P</i> value
Age (yr)		57.5 ± 15.2	62.4 ± 11.6	0.064
Gender (M/F)		116/119	11/13	0.742
Common bile duct diameter (mm) ¹		15.8 ± 6.2	20.6 ± 7.6	0.001
Sum of angle (°) ¹		314.9 ± 20.0	268.3 ± 29.6	0.001
T-tube drainage	No	145 (95.4)	7 (4.6)	0.002
	Yes	90 (84.1)	17 (15.9)	
Periampullary diverticulum ²	No	111 (93.3)	8 (6.7)	0.091
	Yes	97 (86.6)	15 (13.4)	

M: Male; F: Female. ¹Values expressed as mean ± SD; ²231 patients identified by whether periampullary diverticulum was present or not were included.

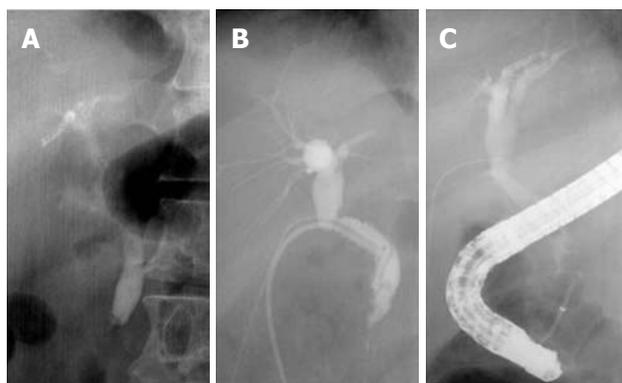


Figure 2 The bile duct angulation caused by T-tube choledochostomy. A: Extrahepatic bile duct configuration before T-tube choledochostomy; B: Bile duct configuration during T-tube drainage; C: Bile duct configuration altered after T-tube drainage. This change of cholangiographic findings showed that T-tube drainage would have induced bile duct angulation.

bile duct stone remained after EST. We divided patients into two groups; T-tube drainage (107 patients) and non-T-tube drainage group (152 patients). The recurrence rate in each group and the sum of angles, especially the sum of the angles before and after T-tube drainages in 22 patients whose films of ERCP before and after T-tube drainage were available among T-tube drainage group, were determined. We compared the recurrence rate and sum of angles between the T-tube drainage group and the non-T-tube drainage group, and evaluated the change of the sum of angles before and after the T-tube drainages in 22 patients to find the influence of T-tube drainage on bile duct configuration (Figure 2).

Maximal transverse common bile duct diameter

We defined transverse diameter of the CBD as a diameter measured in the line which forms a right angle to an imaginary line drawing down along the center of the CBD lumen. We measured the maximal transverse diameter of the CBD in all subjects and corrected for the magnification, with the external diameter of the distal end of the duodenoscope as a reference. We also evaluated the recurrence rate according to the CBD diameter.

Statistical analysis

Categorical variables (gender, presence of periampul-

lary diverticulum and presence of T-tube drainage) were compared by chi-square test or Fisher exact test. Continuous variables (age, CBD diameter, sum of angle) were analyzed using the Student *t* test when the variables were normally distributed or by using the Mann Whitney *U* test for non-normal distribution. Significant predictors for choledocholithiasis recurrence identified by univariate analyses were included in a multiple logistic regression model to determine the most significant risk factors for recurrence of the bile duct stones. For multivariate analysis, we categorized continuous variables into two subgroups: age (< 65 years vs ≥ 65 years), sum of angle (< 270° vs ≥ 270°), and maximal transverse CBD diameter (< 13 mm vs ≥ 13 mm).

Statistical analysis were conducted using SPSS (Statistical Package for the Social Sciences) computer program (version 14.00).

RESULTS

Two hundred and fifty-nine patients were included in the study. Median age was 58 years (range, 17-85 years). There were 132 (50.9%) women and 127 (49.1%) men. Median follow-up period was 47.9 mo (6-101 mo). The results of univariate analyses for recurrence of bile duct stones in relation to each factor were presented in Table 1.

Recurrence rate

The overall recurrence rate of CBD stones was 9.3% (24 of 259 patients). The recurrence rate was 8.7% (11 of 127 patients) in males and 9.9% (13 of 132 patients) in females respectively.

A total of 13.4% (15 of 112) of the patients with periampullary diverticulum developed recurrent stones, while 6.7% (8 of 119) of those without diverticulum showed recurrence. There was no evidence that the periampullary diverticulum exerted a significant influence on the recurrence of bile duct stones statistically (*P* = 0.091). Seventeen of 107 patients (15.9%) who underwent T-tube drainage had a recurrence, compared with 7 of 152 patients (4.6%) without T-tube drainage, and the presence of T-tube drainage was shown to be statistically associated with the recurrence of CBD stones (*P* = 0.002).

Bile duct angulation

The mean value of the sum of angles in the non-recur-

Table 2 Adjusted odds ratio of risk factors for recurrent bile duct stones

Variables	Recurrence of bile duct stones		
	Odds ratio	P value	95% CI ¹
Periampullary diverticulum	2.648	0.107	0.809-8.668
Common bile duct diameter	3.011	0.152	0.564-11.700
T-tube drainage	2.578	0.119	0.785-8.470
Sum of angle (°)	17.897	0.000	5.083-63.015
Age ≥ 65 years	0.372	0.102	0.113-1.219

¹95% CI: Expressed as 95% confidence interval.

recurrence group was $314.9^\circ \pm 20.0^\circ$, while the mean sum of angles in the recurrence group was $268.3^\circ \pm 29.6^\circ$ ($P = 0.001$). These results mean that the more angulation in the extrahepatic bile duct could develop, the greater recurrence of the stone.

common bile duct diameter

The mean maximal transverse CBD diameter in the non-recurrence group was 15.8 ± 6.2 mm while the mean value of CBD in the recurrence group was 20.6 ± 7.6 mm. CBD diameter was a significant risk factor for recurrence on univariate analysis ($P = 0.001$).

T-tube drainage

The mean value of sums of angles at the cholangiogram before T-tube choledochostomy was $311.7^\circ \pm 22.4^\circ$ in T-tube group, while that in the non T-tube group was $314.9^\circ \pm 19.3^\circ$ ($P = 0.232$). On the other hand, the mean value of sums of angles before T-tube drainage was $298.0^\circ \pm 3.9^\circ$, while after T-tube drainage, it was $262.5^\circ \pm 24.6^\circ$ in some patients ($n = 22$) who underwent ERCP after removal of the T-tube among members of the T-tube group ($P = 0.001$). Consequently, these results suggested that T-tube drainage may affect bile duct angulation.

Multivariate analysis

A univariate analysis revealed that CBD diameter, T-tube drainage and sum of angle were significant risk factors for the recurrence of choledocholithiasis ($P < 0.05$). But multivariate analyses of all variables that reached a P value of less than 0.1 in univariate analysis were performed. On multivariate analysis, angulation was the only independent risk factor for the recurrence of choledocholithiasis (Table 2).

DISCUSSION

ERCP with EST is the therapeutic procedure of choice for CBD stones. Early complications of EST include acute pancreatitis, bleeding, duodenal perforation, and acute cholangitis. On the other hand, its late complications include recurrence of stones and papillary stenosis^[19,20]. In long-term follow-up studies after EST, the recurrence rate of CBD stones in patients with cholecystectomy has been in the range of 4% to 24%^[3-5].

However, the theory that loss of sphincter function after EST leads to formation of recurrent CBD stones seems to need validation. Several authors have demonstrated that the biliary tree becomes infected with bacteria after EST^[9,10] and there is substantial evidence that bacteria play an essential role in the formation of brown pigment stones. Some bacterial species, especially *Escherichia coli*^[21], produce enzymes such as β -glucuronidase that are known to precipitate bilirubin and calcium^[11], the main components of brown pigment stone^[22]. Furthermore, electron microscopy studies have demonstrated that bacteria are present in the core of brown pigment stone whereas they are absent in cholesterol and black pigment stone^[14,23,24].

Apart from bacterial infection, biliary stasis is thought to be an important factor in the pathogenesis of recurrent bile duct stones^[6,16,25]. The markedly dilated bile duct is often contaminated with bacteria and also leads to bile stasis, which is thought to be an important factor in the pathogenesis of recurrent stones. The association between dilated CBD and stone recurrence has been reported in previous studies of post-EST patients^[26,27]. In the univariate analysis of our study, in the recurrence group ($n = 21$, mean CBD diameter = 20.6 mm \pm 7.6 mm) there was greater dilation in the extrahepatic bile duct than in the non-recurrence group ($n = 200$, mean CBD diameter = 15.8 mm \pm 6.2 mm) ($P = 0.001$). Therefore dilated bile ducts may be another important risk factor of CBD stone recurrence by causing impaired bile flow.

Previous studies of the peripapillary diverticulum in relation to recurrence of bile duct stone have disclosed inconsistent results^[28-31]. Biliary stasis in patients with diverticulum could be caused by either mechanical factors or the presence of coexisting motility disorders involving the sphincter of Oddi^[28-31]. Theoretically, the effect of the diverticulum on bile flow might not completely disappear after the EST. In our study, the recurrence rate of the diverticulum group ($n = 112$, 13.4%) was higher than that of the non-diverticulum group ($n = 119$, 6.7%), but the result was not statistically significant ($P = 0.91$). Hence further studies on periampullary diverticulum as a risk factor of stone recurrence might be needed.

Bile duct angulation may cause stasis of bile flow. Warren suggested that angulation of CBD is associated with choledocholithiasis^[32]. Mean cholangiographic angulation of CBD differed significantly between patients with cholelithiasis only and those with choledocholithiasis in this previous series. The degree of ductal angulation may be a useful consideration in development of bile duct stones. Recently two reports showed angulation of the CBD contributes to biliary stasis, and hence predisposes to recurrent choledocholithiasis^[33,34]. In the current study, the recurrence group ($n = 24$, mean of sums of angles = $268.3^\circ \pm 29.6^\circ$) was more angulated in the extrahepatic bile duct than the non-recurrence group ($n = 235$, mean of sum of angles = $314.9^\circ \pm 20.0^\circ$) ($P < 0.05$). In multivariate analysis, the sum of angles was the only independent risk factor of CBD stone recurrence (Table 2).

There was a previous study describing long-term results of medical or surgical treatment in patients with choledocholithiasis^[17]. Two hundred and thirteen patients were treated for CBD stones, and then the patients were divided into 3 groups based on the treatment modality: group 1, choledocholithotomy and T-tube choledochostomy; group 2, choledochoduodenostomy; and group 3, endoscopic sphincterotomy. Recurrence of choledocholithiasis was examined for each type of treatment modality. This study suggested that patients treated by T-tube drainage had more recurrent bile duct stones than those in the choledochoduodenostomy or EST groups, but accurate mechanisms were not clearly demonstrated.

Lee and Burhenne suggested that extrahepatic bile duct angulation was caused by T-tube drainage^[18]. They observed lateral distortion in the shape of the bile ducts in a considerable number of patients with an indwelling T-tube such that an angle measured between the proximal and distal parts of the duct, centered at the site of T-tube drainage insertion, decreased to a range of 60 to 158. They have called this finding the “elbow sign”. In the current study, the mean sum of angles before T-tube drainage was $298.0^\circ \pm 23.9^\circ$, while the mean value of angles after T-tube drainage was $262.5^\circ \pm 24.6^\circ$ in the same patients. The sum of the angles in the CBD changed after T-tube drainage by 35.5° and was statistically significant ($P < 0.05$). Furthermore, the extrahepatic bile duct deformity caused by T-tube drainage influenced the recurrence rate of CBD stones. The recurrence rate of the non-T-tube drainage group was 4.6%, while the recurrence rate of the T-tube drainage group was 15.9% ($P < 0.05$). It is presumed that T-tube placement could introduce local adhesion^[33], which, in turn, influences the angulation of the bile duct and then leads to recurrence of bile duct stones as one of the mechanisms.

Our study has some limitations. First, this study is retrospective. Second, we did not prove recurrence with repeat ERCP or cholangiogram in every patient. It may have ascertainment bias. Third, we should estimate the three dimensional image, but we measure bile duct angulation by two-dimensional fluoroscopic imaging. This may show some difference from real angulation. Practically, it is difficult to get three dimensional bile duct images. Fourth, there may be several possible confounders which we did not consider. Medication such as ursodeoxycholic acid and the time of drainage of contrast from the bile duct were not reported in our series. On account of these limitations, it is difficult to be absolutely certain that our specific risk factors were solely responsible for the recurrence of choledocholithiasis.

In conclusion, subsequent to endoscopic biliary sphincterotomy and clearance of bile duct stones, three significant risk factors for the recurrence of the stone were identified on univariate analyses, although bile duct angulation was the only risk factor for recurrence of choledocholithiasis in the multivariate analysis. Patients with a more angulated and dilated bile duct, and a history of T-tube choledochostomy developed stone recurrence more frequently. T-tube choledochostomy performed af-

ter cholecystectomy were prone to recurrence of stones by an influence on bile duct angulation.

COMMENTS

Background

After endoscopic sphincterotomy (EST), 4%-24% of patients might experience recurrent choledocholithiasis. The risk of stone recurrence is an important issue, especially for relatively young patients. Bile duct angulation may be a risk factor of stone recurrence by decreasing bile flow.

Research frontiers

The risk factors for true recurrence of bile duct stones after EST are suboptimally defined. Until now, infection along the bile duct and bile stasis were thought to contribute to the recurrence.

Innovations and breakthroughs

Recent several studies showed the association between bile duct angulation and recurrence of stones. The authors added more information about that. In addition, the authors suggested that T-tube choledochostomy influences the recurrence of choledocholithiasis by angulating the bile duct.

Applications

This article provides important data about the risk factors of choledocholithiasis recurrence. By identifying risk factors for stone recurrence, people can improve outcomes by prophylactic treatments or earlier intervention.

Peer review

This is an interesting study that investigated the risk factors of recurrence of common bile duct stones. This study suggested an association between T-tube drainage and recurrence of choledocholithiasis by analyzing the angulation of the common bile duct before and after T-tube drainage.

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S- Editor Sun H L- Editor O'Neill M E- Editor Zhang DN

Assessment of participant satisfaction with upper gastrointestinal endoscopy in South Korea

Hoo-Yeon Lee, Sun Mi Lim, Mi Ah Han, Jae Kwan Jun, Kui Son Choi, Myung-Il Hahm, Eun-Cheol Park

Hoo-Yeon Lee, Department of Social Medicine, College of Medicine, Dankook University, 201, Manghyang-ro, Dongnam-gu, Cheonan-si, Chungcheongnam-do 330-714, South Korea
Hoo-Yeon Lee, Sun Mi Lim, Jae Kwan Jun, Kui Son Choi, National Cancer Control Institute, National Cancer Center, Gyeonggi-do 410-769, South Korea

Mi Ah Han, Department of Preventive Medicine, College of Medicine, Chosun University, Seoseok-dong, Dong-gu, Gwangju 501-759, South Korea

Myung-Il Hahm, Department of Health Administration and Management, College of Medical Science, Soonchunhyang University, Shinchang-myun, Asan-si, Chungcheongnam-do 336-745, South Korea

Eun-Cheol Park, Department of Preventive Medicine and Institute of Health Services Research, College of Medicine, Yonsei University, Seoul 120-752, South Korea

Author contributions: Lee HY and Park EC contributed to the concept and study design; Lee HY and Lim SM contributed to execution of the work; Lee HY, Han MA and Hahm MI drafted the article and revise it critically for important intellectual content; Lee HY, Jun JK and Choi KS conducted the data analysis and interpretation; all authors approved the final version of the article.

Supported by The National Cancer Center Research Fund (grant No. 1010200)

Correspondence to: Eun-Cheol Park, MD, PhD, Department of Preventive Medicine and Institute of Health Services Research, College of Medicine, Yonsei University, 250 Seongsan-no, Seodaemun-gu, Seoul 120-752, South Korea. ecpark@yuhs.ac
Telephone: +82-2-22281862 Fax: +82-2-3928133

Received: November 12, 2010 Revised: May 19, 2011
Accepted: May 26, 2011

Published online: September 28, 2011

Abstract

AIM: To measure the perceived satisfaction with gastric cancer screening as part of the National Cancer Screening Program (NCSP) in South Korea.

METHODS: Data were derived from the participants

in a satisfaction survey of the Quality Evaluation of National Cancer Screening in 2009. This is a population-based nationwide telephone survey of participants who were screened by the NCSP between May and October 2009. This study included 4412 participants who provided full sets of data and who had upper endoscopies for the purpose of gastric cancer screening.

RESULTS: The negative appraisal percentages for each of the seven questions were as follows: explanation in preparation for the upper endoscopy, 12.3%; explanation about the process and procedure of the upper endoscopy, 13.8%; explanation about any pain or discomfort related to the upper endoscopy, 27.5%; level of pain during the procedure, 30.3%; physical environment, 16.2%; manner of the staff, 11.2%, and privacy protection, 8.8%.

CONCLUSION: The critical issues identified by the Pareto analysis include the adequacy of the explanation about any pain or discomfort associated with the upper endoscopy and the level of pain experienced during the procedure.

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Key words: Gastric cancer; Cancer screening; Upper endoscopy; Satisfaction

Peer reviewer: Sung Kim, MD, PhD, Professor, Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-Dong, Gangnam-Gu, Seoul, South Korea

Lee HY, Lim SM, Han MA, Jun JK, Choi KS, Hahm MI, Park EC. Assessment of participant satisfaction with upper gastrointestinal endoscopy in South Korea. *World J Gastroenterol* 2011; 17(36): 4124-4129 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4124.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4124>

INTRODUCTION

In recent years, patient satisfaction with endoscopic procedures has become an important outcome measure of gastrointestinal (GI) endoscopies^[1]. Patient satisfaction not only establishes performance standards, but also increases the accountability of physicians and staff, and most importantly, can lead to improvement in the quality of care. Few studies have assessed the factors associated with patient satisfaction with upper endoscopies. Factors that have been significantly and positively associated with patient satisfaction included the adequacy of analgesia during the procedure, low patient anxiety before the endoscopic procedure, respectful personal manner of endoscopists, respectful personal manner of nurses, patient positive perceptions of endoscopists' technical skills, pleasant physical environment in the endoscopy unit, and adequate time spent by physicians explaining the procedure^[1-3].

Understanding the level of satisfaction with upper endoscopies performed for gastric cancer screening may provide information necessary for optimising adherence to screening protocols. The satisfaction or procedure-related discomfort in upper endoscopies has rarely been given priority because the procedure can be done quickly and is associated with few complications. Despite a low rate of medical complications, the procedure is associated with substantial pre-procedural anxiety and procedure-related discomfort. Endoscopists tend to underestimate patient discomfort or dissatisfaction^[4].

Gastric cancer is the second most common type of cancer worldwide. With estimated 934 000 new cases in 2002 (8.6%), gastric cancer fell to the 4th place behind cancers of the lung, breast, and colon and rectum^[5]. However, gastric cancer remains the most frequently diagnosed cancer in South Korea^[6-8]. Gastric cancer screening is an increasingly important activity in the effort to early detect and control gastric cancer^[9,10]. Countries, such as Japan and South Korea, where gastric cancer is highly prevalent, have conducted gastric cancer screenings for people at average risk. Although there is debate over the value and risk of screening asymptomatic individuals, interest has shifted to determining the preferred screening strategy and discerning the most effective ways of implementing screening procedures for the general population^[11].

In South Korea, screening for gastric cancer started in 1999 as a part of the National Cancer Screening Program (NCSP) for low-income groups. Currently, the NCSP provides Medical Aid recipients and National Health Insurance beneficiaries in the 50% of income brackets with free screening services for five common cancers: gastric, liver, colorectal, breast, and cervical. The NCSP recommends a biennial upper gastrointestinal series (UGIS) or an upper endoscopy for men and women over 40 years of age. In recent years, mass screenings using upper endoscopies have replaced upper GI X-rays in several cities in South Korea. In 2002, 75.0% of the participants underwent upper-GI X-rays, and 25.0% received upper

endoscopies. In 2008, 50.9% received upper-GI X-rays, and 49.1% received upper endoscopies^[12].

The objectives of this study were to analyze the data obtained by questionnaires measuring the perceived satisfaction with gastric cancer screening as part of the NCSP and to identify and ameliorate the issues that contribute most to patient dissatisfaction.

MATERIALS AND METHODS

Data source

Quality Evaluation of National Cancer Screening (QENCS) programs to improve the quality of NCSP were established in 2008. QENCS programs in an upper endoscopy evaluate all aspects of cancer screening, including the structure, process and outcome. It also includes the general items, such as the indication for the endoscopic examination, informed consent, patient risk stratification, and sedation practice. Outcome indicators are participant satisfaction and accuracy such as cancer detection rate, false positive rate, and interval cancer.

Based on a previously validated questionnaire^[13], we conducted a population-based nationwide telephone survey among participants who were screened by the NCSP between May and October 2009. A sample of 43 157 participants was randomly chosen and stratified according to age, gender and gastric cancer unit. We evaluated participants' satisfaction with gastric cancer screening performed in hospitals. In total, 12 922 calls were successful, and 9090 participants (70.3%) agreed to complete the survey. Participants who received gastric cancer screening with an upper gastrointestinal series were excluded from the study. Finally, 4412 participants who provided full sets of data and who had upper endoscopies for purposes of gastric cancer screening were included in this study. This research was approved by the Institutional Review Board Committee.

Questionnaire

The questionnaire addressed six specific aspects of participant satisfaction with the screening experience: the adequacy of explanations (questions 1, 2 and 3), the manner adopted by doctors and nurses (question 4), privacy protection (question 5), physical surroundings (question 6), pain or discomfort during the procedure (question 7), and overall satisfaction (question 8). The items and scoring method are presented in Table 1. Participants answered each question, except the question regarding overall satisfaction, with a numerical score on a Likert scale, ranging from 1 (poor) to 4 (excellent). Overall satisfaction was scored on a 10-point scale.

Statistical analysis and graphic representation

We defined the problem rate as the percentage of "fair" or "poor" responses given by all participants to all questions^[14]. The problem rate was calculated by adding all poor or fair responses on all questionnaires, dividing this figure by the total number of questions, and multiplying

Questions	Poor	Fair	Good	Excellent						
I received adequate information/explanations in preparation for the upper endoscopy	1	2	3	4						
I received adequate information/explanations about the process and procedure of the upper endoscopy	1	2	3	4						
I received adequate information/explanations about any pain or discomfort related to the upper endoscopy	1	2	3	4						
I was satisfied with the respectfulness of the staff and the manner of doctors and nurses	1	2	3	4						
I had adequate privacy during the procedure	1	2	3	4						
I was satisfied with the pleasantness of the physical environment	1	2	3	4						
I did not experience too much pain/discomfort during the procedure	1	2	3	4						
	Strongly disagree			Strongly agree						
I am satisfied with my overall experience with gastric cancer screening	1	2	3	4	5	6	7	8	9	10

Variable group	Frequency (%)
Gender	
Male	40.1
Female	59.9
Age	
≥ 40 yr, < 50 yr	49.9
≥ 50 yr, < 60 yr	33.6
≥ 60 yr	17.5
Education level	
Elementary school or none	17.3
Middle school	19.7
High school	44.3
University or above	18.7
Sedation	
Yes	48.0
No	52.0

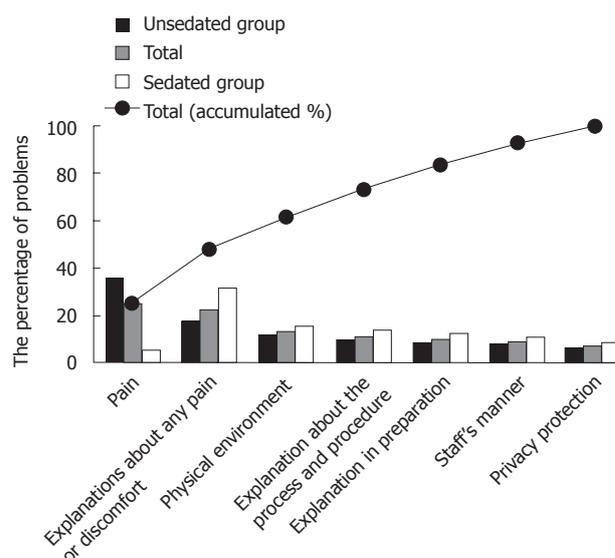


Figure 1 Relative importance of each question for the problem rate among all participants.

this result by 100. This calculation can be expressed by the following formula:

$$\frac{\sum \text{poor and fair answers}}{\sum \text{times each question was evaluated}} \times 100$$

The percentage of poor or fair responses was also calculated for each question.

Quality assessment uses special methods for graphic representation and analysis, and is considered to be as important as the statistical analysis itself. Of the various graphic analysis methods used currently, the Pareto chart was adopted in this study^[14]. This chart presents the relative importance assigned by all participants to each question included in the calculation of the problem rate and produces a bar chart representing the percentage of problems for each question over the total number of problems emerging from the data. Bars are shown from the left to right in order of decreasing level of importance. Figures in the column represent the percentages of poor and fair responses over the total of poor and fair responses. In addition, we drew a line over the bars to represent the accumulated percentage of problems

RESULTS

Of the total 4412 participants in the study population, men accounted for 40.1%, and those aged 40-49 years

accounted for 49.9%. Additionally, 48.0% underwent upper endoscopies while sedated (Table 2). In general, the mean and median satisfaction scores were high (> 3) for all questions, with the exception of the question addressing the level of pain (Table 3). We observed a trend toward higher mean satisfaction scores among the sedated group with respect to seven of the eight questions; the exception was the question addressing the adequacy of the explanation of any pain or discomfort associated with the procedure.

The critical few issues identified by the Pareto analysis included the question about the adequacy of the explanation about any pain or discomfort associated with the procedure and the question regarding the level of pain experienced during the procedure (Table 3, Figure 1). The problem rate was 17.2% (5298 poor or fair responses to a total of 30 884 questions) in the total study population. The critical few with a 30.3% problem rate, included the question about the level of pain experienced during the procedure (Table 3). The problem rate was 12.8% (1900 poor or fair responses to a total of 14 812 questions) in the sedated group and 21.1% (3398 poor or fair responses to a total of 16 072 questions) in the unsedated group.

Table 3 Comparison of satisfaction scores between sedated and unsedated groups

Question label	Total (n = 4412)			Sedated group (n = 2116)			Unsedated group (n = 2296)		
	Mean (SD)	Median (25Q-75Q)	Poor and fair (%)	Mean (SD)	Median (25Q-75Q)	Poor and fair (%)	Mean (SD)	Median (25Q-75Q)	Poor and fair (%)
Adequacy of explanations in preparation for upper endoscopy (%)	3.3 (0.8)	3 (3-4)	12.3	3.4 (0.8)	4 (3-4)	11.4	3.3 (0.8)	3 (3-4)	13.2
Adequacy of explanations about process and procedure of upper endoscopy (%)	3.3 (0.8)	3 (3-4)	13.8	3.3 (0.8)	4 (3-4)	12.6	3.2 (0.8)	3 (3-4)	14.9
Adequacy of explanations about any pain or discomfort related to upper endoscopy (%)	3.0 (1.0)	3 (2-4)	27.5	3.0 (1.0)	3 (2-4)	28.7	3.0 (0.9)	3 (2-4)	26.3
Staff's manner (%)	3.3 (0.7)	3 (3-4)	11.2	3.4 (0.7)	3 (3-4)	10	3.3 (0.7)	3 (3-4)	12.2
Privacy protection (%)	3.4 (0.7)	3 (3-4)	8.8	3.4 (0.7)	4 (3-4)	7.9	3.3 (0.7)	3 (3-4)	9.6
Physical environment (%)	3.2 (0.7)	3 (3-4)	16.2	3.2 (0.7)	3 (3-4)	14	3.1 (0.7)	3 (3-4)	18.3
Level of pain experienced during the procedure (%)	3.0 (1.0)	3 (2-4)	30.3	3.7 (0.6)	4 (4-4)	5.2	2.4 (0.8)	2 (2-3)	53.5
Overall satisfaction	7.8 (1.8)	8 (7-10)		8.0 (1.8)	8 (7-10)		7.6 (1.7)	8 (7-8)	
Problem rate			17.2			12.8			21.1

DISCUSSION

We used the overall score on questions 1-8 as an indicator of participants' satisfaction with the upper endoscopy offered by the NCSP. In general, the mean and median satisfaction scores were high (> 3) for all questions, with the exception of the question concerning the level of pain. We observed a trend toward higher mean satisfaction scores in the sedated group. Our research shows that the level of pain experienced during the procedure and the adequacy of the explanation of any pain or discomfort associated with the procedure constituted the factors that contributed most to the problem rate among participants.

In terms of quality, these two concerns comprise the critical few. The implementation of measures to improve these two main problem areas would probably reduce the rate of problems among participants. The level of pain was an important determinant of satisfaction with the upper endoscopic procedure used for gastric cancer screening, especially in the unsedated group. This finding is consistent with the results of previous studies, which reported that higher levels of pain or discomfort were correlated with lower patient satisfaction with the procedure^[15,16]. A second reason for dissatisfaction identified by our study was the adequacy of explanations of any pain or discomfort associated with the procedure. A large percentage of complaints in both sedated and unsedated groups was related to communication problems^[14]. The total problem rates identified by the three questions regarding the adequacy of explanations were greater than 50% in both sedated and unsedated groups, indicating that dissatisfaction with explanations and communication is the most important contributor to the problem rate. This suggests that screenings are often performed without sufficient explanation, even though participants acquire information before this procedure^[17,18]. Before screening, both the preparation for and the actual process of the endoscopy should be explained. It would also be helpful to explain the possible

discomfort associated with this procedure. Additionally, efforts to ensure privacy and improve the interactional style of staff members may improve population screening programmes.

The total gastric cancer-screening rates have been increasing steadily in South Korea, and the rate of upper endoscopic examination has also been increasing^[12]. However, the rate of participation in gastric cancer screening programs is still not optimal^[19]. Previous results of a population-based survey have identified the upper endoscopy as the preferred gastric cancer screening method. Interestingly, respondents with higher income levels were more likely to have had an endoscopic examination compared with those in lower income levels. Under the South Korean NCSP, endoscopic examination, like UGI tests, are free of charge. Despite these programmes, the use of endoscopy varies with household income, suggesting the possible impact of barriers other than the cost of endoscopy *per se*^[6]. Indeed, under this NCSP, participants have to pay for all procedure-related costs associated with sedation, which may represent one of the barriers associated with income disparities.

Patient satisfaction is a crucial parameter in the management of the quality of endoscopies because it directly reflects patient acceptance of procedures and possibly reflects patient compliance with screening and monitoring^[20], thus, dissatisfaction with the screening experience may lead to non-compliance^[13,21-24]. Satisfaction is particularly important when targeting asymptomatic individuals because they have no obvious reason to seek the services of a screening program. Moreover, levels of satisfaction are also important indicators of the quality of care, and feedback from participants can be used to modify program operations^[21]. To increase compliance with the NCSP, we addressed issues related to improving satisfaction with screening services.

Upper endoscopy is a safe and quick procedure, and can be performed without sedation^[25]. However, it can also evoke anxiety, feelings of vulnerability, embarrass-

ment, and discomfort, and the fears and concerns associated with endoscopic procedures decrease patient compliance. Conscious sedation is the method most widely used, and good tolerance or conscious sedation has been related to the acceptance of and higher satisfaction with endoscopic procedures^[1,15,16,26,27]. Although usually safe, gastric cancer screening tests have tradeoffs in terms of efficacy, complications, discomfort, time, and cost^[6,12,28]. The performance of upper endoscopies using sedation is more costly but remains an efficacious strategy because it increases the rates of successful endoscopies, patient satisfaction, and willingness to undergo repeat procedures^[29].

For these reasons, the NCSP should support the cost of sedation for upper endoscopies and let patients choose whether to undergo this procedure with or without sedation. Such decisions should be based on patient preferences and the clinical judgment of endoscopists, which, in turn, are based on patient age, sex, and tolerance for the procedure^[6,16]. Providers' assessment of individuals' screening preferences, in combination with intervention strategies to promote the preferred screening method, may increase compliance with gastric cancer screening recommendations^[28,30]. It will be imperative to consider these results when making decisions about population-based screening strategies^[31].

This study has several limitations, including the possibility of a degree of participant bias. Respondents who completed the follow-up questionnaire were often those who reported discomfort on the post-procedure questionnaire; thus, this group may have been more likely to respond. The study was conducted among healthy individuals (i.e., participants in the National Gastric Cancer Screening Program), and the results may not be applicable to other patient populations. Indeed, participants might have different values, expectations about the procedure, and pain tolerance, which would potentially influence their satisfaction with the endoscopic procedure. Additionally, our study was conducted at a hospital, which may influence the extent to which our findings are generalizable to non-hospital settings such as clinics.

Despite these limitations, the present study used a reliable and valid survey methodology to evaluate satisfaction with the NCSP. The assessment of satisfaction with the NCSP is useful, as the degree of satisfaction with screening programmes is correlated with adherence patterns. The present study serves as a basis for future interventions to improve satisfaction with upper endoscopic procedures, including using sedation or establishing a program for training staff in communication skills and interpersonal interactions. These findings may be used to develop strategies to promote participation in and adherence to the South Korean Gastric Cancer Screening Program.

an important outcome measure of gastrointestinal (GI) endoscopies. Patient satisfaction not only establishes performance standards, but also increases the accountability of physicians and staff, and most importantly, can lead to improvement in the quality of care.

Research frontiers

National gastric cancer-screening rates have been increasing steadily in South Korea. However, the rate of participation in gastric cancer screening programs is still not optimistic. The authors suggest measuring the perceived satisfaction with gastric cancer screening as part of the National Cancer Screening Program so as to identify and ameliorate the issues that contribute most to participant dissatisfaction.

Innovations and breakthroughs

This study shows that the level of pain experienced during the endoscopic procedure and the adequacy of the explanation of any pain or discomfort associated with the procedure constituted the factors that contributed most to the problem rate among participants. The implementation of measures to improve these two main problem areas would probably reduce the rate of problems among participants.

Applications

This study serves as a basis for future interventions to improve the satisfaction with upper endoscopic procedures, including using sedation or establishing a program for training staff in communication skills and interpersonal interactions.

Peer review

The study is well done both number and selection of cases, and the results quite clear.

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COMMENTS

Background

In recent years, patient satisfaction with endoscopic procedures has become

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S- Editor Sun H L- Editor Ma JY E- Editor Zhang DN

Anti-hepatitis A seroprevalence among chronic viral hepatitis patients in Kelantan, Malaysia

Fazlina Ahmad, Nor Aizal Che Hamzah, Nazri Mustaffa, Siew Hua Gan

Fazlina Ahmad, Nor Aizal Che Hamzah, Nazri Mustaffa, Department of Medicine, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

Siew Hua Gan, Human Genome Centre, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

Author contributions: Che Hamzah NA, Ahmad F, Mustaffa N and Gan SH designed the study; Ahmad F performed the research and analysed the data; Ahmad F and Che Hamzah NA wrote the paper.

Supported by Short term grant No. 304/PPSP/61310014 from the Universiti Sains Malaysia

Correspondence to: Nor Aizal Che Hamzah, MRCP (UK), Senior Lecturer in Internal Medicine and Gastroenterology, Department of Medicine, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia. aizalchehamzah@hotmail.com

Telephone: +60-9-7676590 Fax: +60-9-7673949

Received: February 14, 2011 Revised: April 15, 2011

Accepted: April 22, 2011

Published online: September 28, 2011

Abstract

AIM: To determine the seroprevalence of anti-hepatitis A virus (HAV) antibodies in patients with chronic liver disease (CLD) and to justify the need for hepatitis A vaccination.

METHODS: Patients ($n = 119$) were enrolled between July and September 2009. The diagnosis of CLD was based on the presence of viral markers for more than 6 mo. The diagnosis of liver cirrhosis was based on clinical, biochemical and radiological profiles. Patient serum was tested for anti-HAV IgG.

RESULTS: The overall anti-HAV seroprevalence was 88.2%. The aetiology of CLD was hepatitis B in 96 patients (80.7%) and hepatitis C in 23 patients (19.3%). Mean age was 44.4 ± 14 years. Patients were grouped according to age as follows: 24 (20.2%) patients in the 21-30 years age group, 22 (18.5%) in the 31-40 years age group, 31 (26.1%) in the 41-50 years age group, 23

(19.3%) in the 51-60 years age group and 19 (16.0%) patients aged greater than 60 years, with reported seroprevalences of 66.7%, 95.5%, 93.5%, 91.3% and 94.7%, respectively. There was a marked increase of seroprevalence in subjects older than 30 years ($P = 0.001$).

CONCLUSION: Our study demonstrated that patients aged greater than 30 years of age were likely to have natural immunity to hepatitis A. Therefore, hepatitis A vaccination may not be routinely required in this age group.

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Key words: Hepatitis A seroprevalence; Chronic viral hepatitis; Malaysia; Hepatitis A vaccination

Peer reviewer: Yoshiaki Iwasaki, MD, PhD, Associate Professor, Health Service Center, Okayama University, 2-1-1, Tsushima-Naka, Kita-ku, Okayama 700-8530, Japan

Ahmad F, Che Hamzah NA, Mustaffa N, Gan SH. Anti-hepatitis A seroprevalence among chronic viral hepatitis patients in Kelantan, Malaysia. *World J Gastroenterol* 2011; 17(36): 4130-4134 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4130.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4130>

INTRODUCTION

Hepatitis A remains a significant problem in Malaysia. Malaysia is among those countries reported to be of intermediate endemicity, along with Thailand and Sri Lanka^[1-3]. The hepatitis A virus (HAV) has been reported to be the main cause of symptomatic clinical hepatitis (up to 66.4% in 1996) in the Eastern region of Peninsular Malaysia when compared to other causes of viral hepatitis^[4]. Kelantan is one of the states situated in the Eastern region of Peninsular Malaysia. However, over the last 20 years, pat-

terns of endemicity in South-East Asia have changed due to improvements in living standards, with some countries shifting from high to intermediate or intermediate to low endemicities^[1,3,5-7]. The prevalence of HAV in Malaysia is expected to fall with time; this means, however, that reintroduction of the virus to a non-immune population could produce a community-level outbreak, which may lead to an increase in morbidity and mortality^[2,8].

HAV super-infections in patients with underlying chronic liver disease (CLD) may lead to decompensation of the liver. Acute HAV super-infection is associated with higher morbidity and mortality than are isolated cases of acute HAV infection, leading to an increase in the likelihood of developing a fulminant liver failure^[9-14]. Based on epidemiological studies of large hepatitis A outbreaks in Shanghai in the late 1980s, acute hepatitis A in patients with chronic hepatitis B has an even more severe clinical course and higher risk of death^[15]. The fatality rates for acute hepatitis A were 5.6 times higher among hepatitis B surface antigen (HBsAg) carriers when compared to HBsAg-negative patients^[16]. A similar scenario may also be true for super-infection of hepatitis A in chronic hepatitis C patients. An observational study conducted over a 7-year period among 432 Italian patients with chronic hepatitis C reported that amongst the 17 patients (3.9%) with acute hepatitis A super-infection, 41% progressed into fulminant hepatic failure^[17]. This emphasises the need for vaccination in CLD patients without natural immunity.

In the West, hepatitis A vaccination has been recommended in all patients with CLD to prevent super-infection with HAV, which may cause high morbidity and mortality in this group of patients^[18-20]. However, in countries where hepatitis A is still endemic, such as Malaysia, the utility of this vaccine must be examined, as it is possible that most patients have already acquired natural immunity^[21-23].

Therefore, we aimed to determine the seroprevalence of anti-HAV antibodies in patients with CLD in our region.

MATERIALS AND METHODS

Sample population

Patients with CLD ($n = 119$) attending the Gastroenterology Clinic of Universiti Sains Malaysia, Kelantan between July and September 2009 were enrolled after having signed written informed consents. Diagnosis of CLD was based on the presence of HBsAg or anti-hepatitis C virus antibody (anti-HCV) in serum for a period of at least 6 mo. The underlying liver diseases were classified into either liver cirrhosis (LC) or non-cirrhotic CLD. LC was evidenced by previous ultrasonography (i.e., coarse liver architecture, nodular liver surface and blunt liver edges) as well as confirmation of hypersplenism (i.e., splenomegaly on ultrasonography with a platelet count $< 100\ 000/\text{mm}^3$)^[3]. Patients with underlying CLD of non-viral origin were excluded.

The patients were classified into the following five groups according to age: (1) **Group A: 21 to 30 years**; (2) **Group B: 31 to 40 years**; (3) **Group C: 41 to 50 years**; (4) **Group D: 51 to 60 years**; and (5) **Group E: greater than 60 years of age**. The study was approved by the local university's Research and Ethics Committee and complied with the Declaration of Helsinki.

Detection of HAV IgG, HBV and HCV infections

Immunity towards hepatitis A was established by detection of anti-HAV IgG using commercially available immunoassay kits for anti-HAV IgG (Abbot Laboratories, Chicago, Illinois, United States) that rely on microparticle enzyme immunoassay methods. Presence of hepatitis B virus (HBV) and hepatitis C virus infection was determined by detection of HBsAg and anti-HCV antibodies, respectively.

Statistical analysis

All data analyses were carried out using SPSS statistical software (Version 12.0.1). Continuous variables were expressed as mean and standard deviation for normally distributed data while categorical variables were expressed as frequency and percentage. A chi-square (χ^2) test was used to determine whether significant differences exist between two categorical variables. Results were reported as significant when $P < 0.05$. For multivariate analyses, a stepwise multivariate logistic regression model was employed to assess the relative importance of variables showing a significant association ($P < 0.05$) or any other clinically important variables in univariate analysis ($P < 0.10$). Results of all multivariable analyses were reported as adjusted odds ratio, 95% CI and exact P value.

RESULTS

The mean age at presentation was 44.4 ± 14 years (range 21-76 years). Males comprised the majority of the study population (62.2%). The Malay constituted the highest proportion of the study subjects (80.7%). This distribution reflects the current ethnic diversity in our population.

The aetiology of the underlying liver disease was chronic HBV infection in 96 (80.7%) patients, while chronic HCV infection was present in the remainder of the population (19.3%). The distribution of disease status was LC (14.3%), while others were in the non-cirrhotic group (Table 1).

The overall prevalence of anti-HAV was 88.2% (105/119), while seroprevalence differed greatly based on age group: 66.7% in Group A, 95.5% in Group B, 93.5% in Group C, 91.3% in Group D and 94.7% in Group E (Figure 1). The anti-HAV prevalence was significantly lower in patients younger than 30 years of age when compared to those who were in the older age groups (Table 2). Multivariate analysis of age category variables also showed a significant difference between patients younger than 30 years when compared to those who were in the older age groups, with the exception of

Table 1 Patient demographic and clinical data

Characteristics	Values (%)
Mean age (yr)	44.4 ± 14.0
Male	74 (62.2)
Aetiology of liver disease	
HBV	96 (80.7)
HCV	23 (19.3)
Status of liver disease	
Non-cirrhotic	102 (85.7)
Liver cirrhosis	17 (14.3)
Prevalence of IgG HAV	105/119 (88.2)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HAV: Hepatitis A virus.

Table 2 Univariate analysis of demographic data and anti-hepatitis A virus IgG positivity

Variables	Anti-HAV IgG n (%)		χ^2 value	P value
	Positive	Negative		
Age (yr), mean ± SD	45.7 ± 13.4	35.1 ± 15.8		
Age less than 30 yr	16 (66.7)	8 (33.3)	13.473	0.001 ¹
Age more than 30 yr	89 (93.7)	6 (6.3)		
Race				
Malay	84 (87.5)	12 (12.5)	0.259	0.611 ¹
Non-Malay	21 (91.3)	2 (8.7)		
Gender				
Male	68 (91.9)	6 (8.1)	2.521	0.112 ²
Female	37 (82.2)	8 (17.8)		
Aetiology of liver disease				
Hepatitis B	83 (86.5)	13 (13.5)	1.511	0.219 ¹
Hepatitis C	22 (95.7)	1 (4.3)		
Status of CLD				
Non cirrhotic	88 (86.3)	14 (13.7)	2.644	0.216 ¹
Liver cirrhosis	17 (100.0)	0 (0.0)		
Education level				
No formal education	5 (71.4)	2 (28.6)	2.024	0.191 ¹
Primary	12 (100.0)	0 (0.0)	1.779	0.356 ¹
Secondary	61 (89.7)	7 (10.3)	0.331	0.565 ²
Tertiary	27 (84.4)	5 (15.6)	0.628	0.522 ¹
Salary category (RM)				
< 1000	48 (84.2)	9 (15.8)	1.707	0.257 ²
1001-2000	31 (88.6)	4 (11.4)	0.005	1.000 ¹
2001-3000	14 (93.3)	1 (6.7)	0.43	1.000 ¹
> 3000	12 (11.4)	0 (0.0)	1.779	0.356 ¹
Comorbidities				
Diabetes	21 (100)	0 (0.0)	3.4	0.127 ¹
Hypertension	19 (100.0)	0 (0.0)	3.015	0.122 ¹
Ischaemic heart	6 (100.0)	0 (0.0)	0.842	1.000 ¹

¹Fisher's exact test; ²Pearson χ^2 Test. P < 0.05 was considered as significant at the 95% confidence level. HAV: Hepatitis A virus; CLD: Chronic liver disease.

group D (P = 0.053) (Table 3).

The overall prevalence of hepatitis A was 80.5% in the 96 patients with chronic HBV infection and 95.7% in the 23 patients with chronic HCV infection. Anti-HAV was more frequently (100%) detected in LC patients when compared to non-cirrhotic patients (86.3%)

DISCUSSION

A study by Ton *et al.*^[24] investigated 100 healthy individu-

Table 3 Multivariate analysis of age category variables

Variables (age)	Walds (df)	Adjusted OR	P value	95% CI	
				Lower	Upper
Group A: 21 to 30 yr	11.021 (4)		0.026 ^a		
Group B: 31 to 40 yr	4.664 (1)	11	0.031 ^a	1.248	96.951
Group C: 41 to 50 yr	5.236 (1)	7	0.022 ^a	1.322	37.066
Group D: 51 to 60 yr	3.741 (1)	5.25	0.053	0.978	28.182
Group E: > 60 yr	3.884 (1)	9	0.049 ^a	1.012	28.182

^aP < 0.05 was considered as significant at the 95% confidence level. OR: Odds ratio.

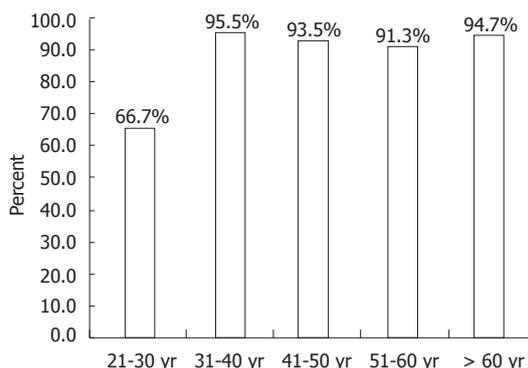


Figure 1 Seropositivity of anti-hepatitis A virus IgG according to age groups.

als from Kuala Lumpur in 1983 and reported a seroprevalence of hepatitis A of 78.2%. In 1985, a study reported that 100% of a Malaysian population were anti-HAV positive by 30 years of age^[25]. However, in 1992, only 45% of the same age group were antibody-positive, indicating a shifting epidemiology, most probably due to improvements in living standards^[25]. In the present study, we found that the overall anti-HAV seropositivity was high, at 88.2%. The higher seroprevalence of hepatitis A in our chronic viral hepatitis patients compared to previously reported seroprevalence for normal individuals was most likely related to the characteristics of the studied population in this region. This study was conducted in Kelantan, which is located in the north-eastern corner of the peninsula facing the South China Sea, with a chiefly agrarian economy. The population has diverse socioeconomic status and large income inequalities. The previous study was conducted in Malaysia's capital, Kuala Lumpur^[24], which has higher living standards, while the other included paediatric age groups with lower expected natural immunity^[26]. Therefore, it is possible that the seroprevalence of HAV in other parts of Malaysia might not be similar to our findings, and we recommend further study in each locality to determine this. It is also possible that CLD patients have a higher prevalence of HAV positivity than the normal population. Contradicting this, Joshi and colleagues have revealed similar hepatitis A seroprevalence differences between CLD and normal populations in India^[21].

We also show that the main factor that influences the rate of positivity is the age group. The seroprevalence

rates in patients in Groups B, C, D and E were greater than 90%, compared to only 66.7% in patients belonging to Group A. These data imply that most patients with chronic viral liver disease who are greater than 30 years of age may have been exposed to HAV infection and have therefore already acquired natural immunity towards the disease. Therefore, routine HAV vaccination cannot be recommended in this age group. However, there remains the need to vaccinate patients aged less than 30 years with chronic liver disease.

Malaysia is a multiracial country consisting of three major ethnic groups: Malay, Chinese and Indian. Because the Indian population is generally very small in Kelantan, we did not manage to enrol any Indian patients, and only the Malay and Chinese races could be compared. There was no significant difference in the seroprevalence rate between these two ethnicities. However, this should be interpreted with caution, as various sociocultural behaviours may also play a role in influencing viral transmission rates.

The anti-HAV seropositive rate was 86.5% in hepatitis B and 95.7% in hepatitis C. Even though chronic hepatitis C is a different disease entity from chronic hepatitis B, there was no significant difference in anti-HAV positivity according to aetiology of underlying chronic liver disease ($P > 0.05$). This could be due to the small sample size of patients with hepatitis C infection ($n = 23$) in our study. Notably, the seroprevalence of hepatitis A was higher in hepatitis C patients than in hepatitis B patients, which is consistent with Korean data, where 100% of chronic hepatitis C patients were anti-HAV IgG positive compared to only 86.1% of hepatitis B patients^[27]. Similarly, the Korean and Indian studies also failed to demonstrate any significance in hepatitis A seropositivity in relation to chronic liver disease aetiology^[21,27].

All cirrhotic patients were anti-HAV positive, compared to only 86.3% in non-cirrhotic liver disease cases. The higher prevalence in cirrhotics was due to these patients falling into an older age group (mean age 52.4 ± 13.4) than non-cirrhotics (mean age 43.06 ± 13.7), as shown by multiple logistic regression analyses. Various studies, particularly those in highly endemic regions such as India, have demonstrated that the majority of cirrhotic patients of any aetiology are positive for anti-HAV IgG. A study from New Delhi revealed that 97.6% of cirrhotics (248/288) were found to be positive for anti-HAV^[28]. Another study from South India demonstrated that 51 out of 52 patients with cirrhosis had antibodies towards HAV^[29]. All these studies proposed that the higher seroprevalence of anti-HAV IgG in cirrhotic patients was actually related to increased age. Our findings are in agreement with the results of these studies.

Our study demonstrated that the overall hepatitis A seroprevalence was higher in CLD patients in Kelantan compared to the previously determined prevalence in normal individuals in other parts of Malaysia. Age was the most important factor in determining anti-HAV positivity, and most patients greater than 30 years of age

were likely to have natural immunity.

ACKNOWLEDGMENTS

We would like to thank Dr. Habsah Hasan for her skilful help in conducting the anti-HAV IgG tests.

COMMENTS

Background

Populations in developed countries may not have had prior exposure to hepatitis A virus (HAV) and, therefore, no natural immunity; thus, there is a need for hepatitis A vaccination in selected high-risk groups. **In view of this, Western guidelines advocate hepatitis A vaccination for those with chronic liver disease (CLD), as infection may lead to further deterioration of liver function, which can then cause significant morbidity and mortality amongst these patients.**

Research frontiers

Over the last 20 years, patterns of endemicity in South East Asia have changed due to improvements in living standards, with some countries shifting from high to intermediate or intermediate to low endemicity. **The question remains as to the importance of hepatitis A vaccination amongst hepatitis B and C CLD patients in Malaysia, a country of intermediate endemicity for hepatitis A. To answer this, a research team from Universiti Sains Malaysia determined the prevalence and associated factors of natural immunity towards hepatitis A amongst these patients in the eastern region of Peninsular Malaysia.**

Innovations and breakthroughs

The study demonstrated that the overall prevalence of natural immunity towards hepatitis A was high (88%). **There was a statistically significant difference when the data were broken down according to age. Results revealed that hepatitis B and C CLD patients less than 30 years of age were significantly less likely to have a natural immunity towards hepatitis A. This implies that although hepatitis A vaccination is not needed for the majority of CLD patients, a subset of patients (particularly patients who are younger than 30 years old) will still benefit from being vaccinated.**

Applications

Study results show that for north-eastern Peninsular Malaysia, there is currently no need for routine hepatitis A vaccination amongst hepatitis B and C CLD patients. **However, CLD patients who are younger than 30 years of age will still benefit from being vaccinated.** Patterns of endemicity in South East Asian countries are expected to change; in Malaysia, the prevalence of hepatitis A viral exposure is expected to fall with time. **Thus, there is a need to repeat this study in the future, as it is expected that the prevalence of natural immunity towards hepatitis A will fall as the country becomes more developed.**

Peer review

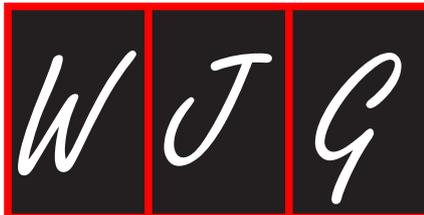
The authors investigated the seroprevalence of anti-HAV antibodies in patients with CLD and the need for vaccination in the region of Kelantan, Malaysia.

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S- Editor Sun H L- Editor Logan S E- Editor Li JY



Viral kinetics of Enterovirus 71 in human rhabdomyosarcoma cells

Jing Lu, Ya-Qing He, Li-Na Yi, Hong Zan, Hsiang-Fu Kung, Ming-Liang He

Jing Lu, Li-Na Yi, Hsiang-Fu Kung, Ming-Liang He, Stanley Ho Center for Emerging Infectious Diseases, and Li Ka Shing Institute of Health Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China

Ya-Qing He, Shenzhen Center for the Disease Control and Prevention, Shenzhen 518055, Guangdong Province, China

Hong Zan, Institute for Immunology, University of California at Irvine, CA 92697, United States

Author contributions: Lu J and He YQ contributed equally to this paper; Lu J, Yi LN, He YQ performed the experiments; Zan H contributed to analysis; He ML designed the research; He ML, Kung HF and Zan H wrote the paper.

Supported by Research Grant Council (RGC, CUHK4428/06M); and a commissioned grant of the Research Fund for Control of Infectious Diseases (CU-09-02-02), Food and Health Bureau, the Government of Hong Kong Special Administration Region (HKSAR)

Correspondence to: Ming-Liang He, Professor, Rm 708, Li Ka Shing Medical Science Bldg, Prince Wales Hospital, The Chinese University of Hong Kong,

Hong Kong, China. mlhe7788@gmail.com

Telephone: +852-37636096 Fax: +852-21458013

Received: January 13, 2011 Revised: May 19, 2011

Accepted: May 26, 2011

Published online: September 28, 2011

Abstract

AIM: To characterise the viral kinetics of enterovirus 71 (EV71).

METHODS: In this study, human rhabdomyosarcoma (RD) cells were infected with EV71 at different multiplicity of infection (MOI). After infection, the cytopathic effect (CPE) was monitored and recorded using a phase contrast microscope associated with a CCD camera at different time points post viral infection (0, 6, 12, 24 h post infection). Cell growth and viability were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in both EV71 infected and mock infected cells at each time point. EV71 replication kinet-

ics in RD cells was determined by measuring the total intracellular viral RNA with real-time reverse-transcription polymerase chain reaction (qRT-PCR). Also, the intracellular and extracellular virion RNA was isolated and quantified at different time points to analyze the viral package and secretion. The expression of viral protein was determined by analyze the levels of viral structure protein VP1 with Western blotting.

RESULTS: EV71 infection induced a significant CPE as early as 6 h post infection (p.i.) in both RD cells infected with high ratio of virus (MOI 10) and low ratio of virus (MOI 1). In EV71 infected cells, the cell growth was inhibited and the number of viable cells was rapidly decreased in the later phase of infection. EV71 virions were uncoated immediately after entry. The intracellular viral RNA began to increase at as early as 3 h p.i. and the exponential increase was found between 3 h to 6 h p.i. in both infected groups. For viral structure protein synthesis, results from western-blot showed that intracellular viral protein VP1 could not be detected until 6 h p.i. in the cells infected at either MOI 1 or MOI 10; and reached the peak at 9 h p.i. in the cells infected with EV71 at both MOI 1 and MOI 10. Simultaneously, the viral package and secretion were also actively processed as the virus underwent rapid replication. The viral package kinetics was comparable for both MOI 1 and MOI 10 infected groups. It was observed that at 3 h p.i., the intracellular virions obviously decreased, thereafter, the intracellular virions began to increase and enter into the exponential phase until 12 h p.i. The total amounts of intracellular virions were decreased from 12 to 24 h p.i. Consistent with this result, the increase of virus secretion occurred during 6 to 12 h p.i.

CONCLUSION: The viral kinetics of EV71 were established by analyzing viral replication, package and secretion in RD cells.

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Key words: Enterovirus 71; Quantitative reverse transcription polymerase chain reaction; Viral kinetics; Western blotting

Peer reviewer: Dr. Mohamed Hassan, Department of Tumour Therapy, University Hospital of Dusseldorf, 40225 Dusseldorf, Germany

Lu J, He YQ, Yi LN, Zan H, Kung HF, He ML. Viral kinetics of Enterovirus 71 in human rhabdomyosarcoma cells. *World J Gastroenterol* 2011; 17(36): 4135-4142 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4135.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4135>

INTRODUCTION

Enterovirus 71 (EV71) is a member of human enterovirus species which belongs to the *Picornaviridae* family. It is one of the major causative agents for herpangina and hand, foot and mouth disease (HFMD)^[1,2]. Among young children, acute EV71 infection may also cause severe neurological diseases such as encephalitis and meningitis that lead to significant mortality^[3,4]. Recently, outbreaks of EV71 infection have been frequently reported throughout the world^[5-10]. In China, a recent outbreak of EV71 infected more than 1.4 million children and caused 760 deaths from January to July, 2010. It is also noted that an adult died due to EV71 infection in Hong Kong in May, 2010 (http://www.hkcd.com.hk/content/2010-05/28/content_2531606.htm).

EV71 is a small RNA virus containing a non-enveloped capsid and a single-stranded positive genomic RNA (about 7400 bp)^[11]. The life cycle of EV71 was speculated according to studies on other enteroviruses. EV71 would attach to the host cell *via* its specific receptors^[12] and then the viral genomic RNA is released into the cytoplasm, where it directly translates into a polyprotein. This precursor protein can subsequently be cleaved into four structural (VP1, VP2, VP3 and VP4) and seven non-structural (2A, 2B, 2C, 3A, 3B, 3C and 3D) proteins^[4]. For virus RNA replication, a complementary minus-strand RNA is synthesised in the cytoplasm and then this minus-strand RNA can serve as a template for new plus-strand RNA molecules. Newly synthesized virus RNA may go into another round of translation and replication, or is packaged into capsid proteins to produce infectious viral particles (see review in Ref.[13]). Studies on other viruses demonstrate that the knowledge of virus kinetics is important for clarifying viral pathogenesis and exploring effective treatments. The knowledge of the viral kinetics on human immunodeficiency virus, hepatitis B virus and hepatitis C virus has greatly improved the understanding of the cell response to these viruses and mechanisms of related antiviral therapy^[14-16]. In the *Picornavirus* family, the kinetics of swine vesicular disease virus (SVDV) and foot-and-mouth disease virus (FMDV) have been described in several studies^[17,18]. However, little information is known about EV71 infec-

tion. In this study, we used rhabdomyosarcoma (RD) cells as an *in vitro* model and intensively investigated the viral kinetics of EV71, including the kinetics of viral replication, viral protein synthesis, packaging and secretion.

MATERIALS AND METHODS

Cell culture and virus propagation

RD cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. EV71 strain SHZH98 (GenBank accession number AF302996.1) was obtained from Shen-zhen Center for Disease Control and Prevention, Shenzhen, China. To prepare virus stocks, viruses were propagated on a 90% confluent cell monolayer in DMEM with 2% FBS. The viral titres were measured by the cytopathic effect (CPE) microtitration assays and expressed as 50% tissue culture infective dose (TCID₅₀) per millilitre (mL) according to the Kärber formula^[19].

Viral infection and cytopathic effects assay

RD cells were cultured in 12-well plates and infected with nil or EV71 at multiplicity of infection (MOI) 1 and 10, respectively. Briefly, plated cells were washed twice with phosphate buffered saline (PBS) and infected with EV71. Time was set as zero after adsorption for 1 h. The culture media were removed and cells were washed twice with PBS to remove unattached virus before adding 1 mL of DMEM medium containing 10% FBS to each well. The cells were cultured at 37 °C in 5% CO₂. The cell morphology was monitored and recorded using a phase-contrast microscope associated with a CCD camera and computer at different time points. The infected cells and culture supernatants were harvested to isolate RNA and proteins.

MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were performed to determine the cell viability upon EV71 infection. Briefly, RD cells were set in 96-well plates at 1×10^4 cells per well 24 h before infection with EV71 at MOI 1 or MOI 10. The medium was then replaced with 0.5 mg/mL MTT medium at different time points and incubated for another 4 h. The MTT solution was removed from the wells and the formazan crystals were dissolved in DMSO. Absorbances of the formazan products were measured at 550 nm with the reference wavelength at 690 nm.

RNA isolation

For intracellular viral RNA quantification, the total cellular RNA was isolated from EV71 infected cells using TRIzol reagent (Invitrogen, United States) according to the manufacturer's instructions. To quantitate the extracellular virions, we first isolated the virions from the culture media of infected cells. The media were harvested and briefly centrifuged to remove cell debris. Viral core

particles were then precipitated with 10% polyethylene glycol 8000 containing 0.5 mol/L NaCl at 4 °C overnight. After centrifuging for 30 min at 16000 *g*, viral particles were pelleted and treated with 100 µg/mL of RNase A (Sigma, United States). To isolate the intracellular virions, EV71 infected cells were lysed with lysis buffer (1% Triton 100 and 1 × Roche protease inhibitor cocktail in PBS). Then the cell lysates were used to isolate viral particles as described above. The virion-associated RNA was then isolated by using TRIzol reagent. To set up the standard curve of infectious viruses, the viral titres were first determined by CPE assay. Then the viral RNA was extracted from those infectious EV71 viruses. RNA was diluted at ten-fold serial and used to reflect the calculated PFU from 10 to 1 × 10⁷ live virions.

Quantitative reverse transcription-polymerase chain reaction

The one-step quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using the ABI 7500 Real-Time PCR system (Applied Biosystems) with QuantiTect SYBR Green RT-PCR Kit (Qiagen) and specific forward EV71-VP1F (5'-GCAGCCCCAAA-GAAGTTTCAC-3') and reverse EV71-VP1R (5'-ATTTCAGCAGCTTGGAGTGC-3') primers targeting a conserved region of the VP1 gene^[20]. PCR assay was carried out in a 20 µL volume consisting of 10 µL of 2 × QuantiTect SYBR green RT-PCR Master Mix, containing HotStarTaq DNA polymerase, 1 µL of 10 µmol/L of each oligonucleotide primer, 0.2 µL of 100 × QuantiTect RT Mix (containing Omniscript and Sensiscript reverse transcriptases) and 2 µL of RNA extracted from samples or from ten-folds serial diluted virus RNA standard (from 10⁷ to 10 copies). The target fragment amplification was carried out as follows: reverse transcription at 50 °C for 30 min; initial activation of HotStar Taq DNA Polymerase at 95 °C for 15 min; 45 cycles in four steps: 94 °C for 10 s, 56 °C for 30 s, and 72 °C for 30 s. At the end of the amplification cycles, melting temperature analysis was carried out by a slow increase in temperature (0.1 °C/s) up to 95 °C.

Western blotting

To prepare total cellular protein extracts, the cells were lysed with RIPA buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.1% SDS, 1 × Roche protease inhibitor cocktail) with occasional vortexing. Lysates were collected by centrifugation at 14000 *g* for 10 min at 4 °C and protein concentrations were measured by the Bradford method (Bio-Rad, United States). Equal amounts (20 µg) of proteins from each sample were separated through 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). Membranes were blocked by 5% skim milk in Tris-Buffered Saline Tween-20 (TBST) (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.1% Tween 20) followed by incubation with specific antibodies against VP1 (PAB7631-

D01P, Abnova) or GAPDH (Santa Cruz Biotechnology). Target proteins were visualized with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and a chemiluminescence detection system (Amersham Biosciences). Each immunoblotting was carried out at least three times.

Statistical analysis

Data are depicted as mean ± SD. All statistical analyses were carried out with SPSS 14.0 software (SPSS Inc.). Two-tailed Student's *t* test was applied for two-group comparison. *P* < 0.05 was considered statistically significant.

RESULTS

Cytopathic effects and the kinetic of cell viability

Morphological changes were observed as early as 6 h p.i. when RD cells were infected with EV71 at either MOI 1 or 10 (Figure 1A, panels f and j). Initially, the cells rounded up and became more refractile. As the culture progressed, some infected cells detached from the culture plate and floated into the medium (Figure 1A, panels g, h, k and l). Compared with the cells infected at MOI 1, more cells were unhealthy at 6 h p.i. when infected at MOI 10 (Figure 1A, panel j *vs* f). Later on, the cells infected with EV71 both at MOI 1 and 10 underwent significant cell death and detached from the surface of culture dishes (Figure 1A, panels g and k). At 24 h p.i., most of the cells were detached from the surface of the plate in both infected groups (Figure 1A, panels h and l). The MTT assay showed that the viability of the cells infected with EV71 at MOI 1 did not significantly decrease at 12 h p.i., but slightly reduced in cells infected at MOI 10 (Figure 1B). At 18 and 24 h p.i., the viability of the cells infected with EV71 either at MOI 1 or 10 significantly decreased.

The kinetics of viral replication

To examine the kinetics of viral replication, the levels of intracellular viral RNA were measured by qRT-PCR at each time point. As shown in Figure 2, the intracellular viral RNA began to increase as early as 3 h p.i. and the exponential phase was from 3 to 6 h p.i. in both infected groups. In the case of infection at MOI 1, the intracellular viral RNA continually increased from 6 to 12 h p.i., and then gradually decreased until 24 h p.i. In the case of MOI 10, the intracellular viral RNA reached a peak between 6 and 9 h p.i., and then began to decrease.

The kinetics of viral protein synthesis

The intracellular viral protein VP1 was not detected until 6 h p.i. in the cells infected at MOI 1 and 10 (Figure 3). The VP1 protein level reached a peak at 9 h p.i. in the cells infected with EV71 at both MOI 1 and MOI 10. Obviously, the VP1 levels were much higher in the cells infected at MOI 10 than at MOI 1. In the case of infection at MOI 1, the VP1 level was maintained until 12 h p.i.; whereas the

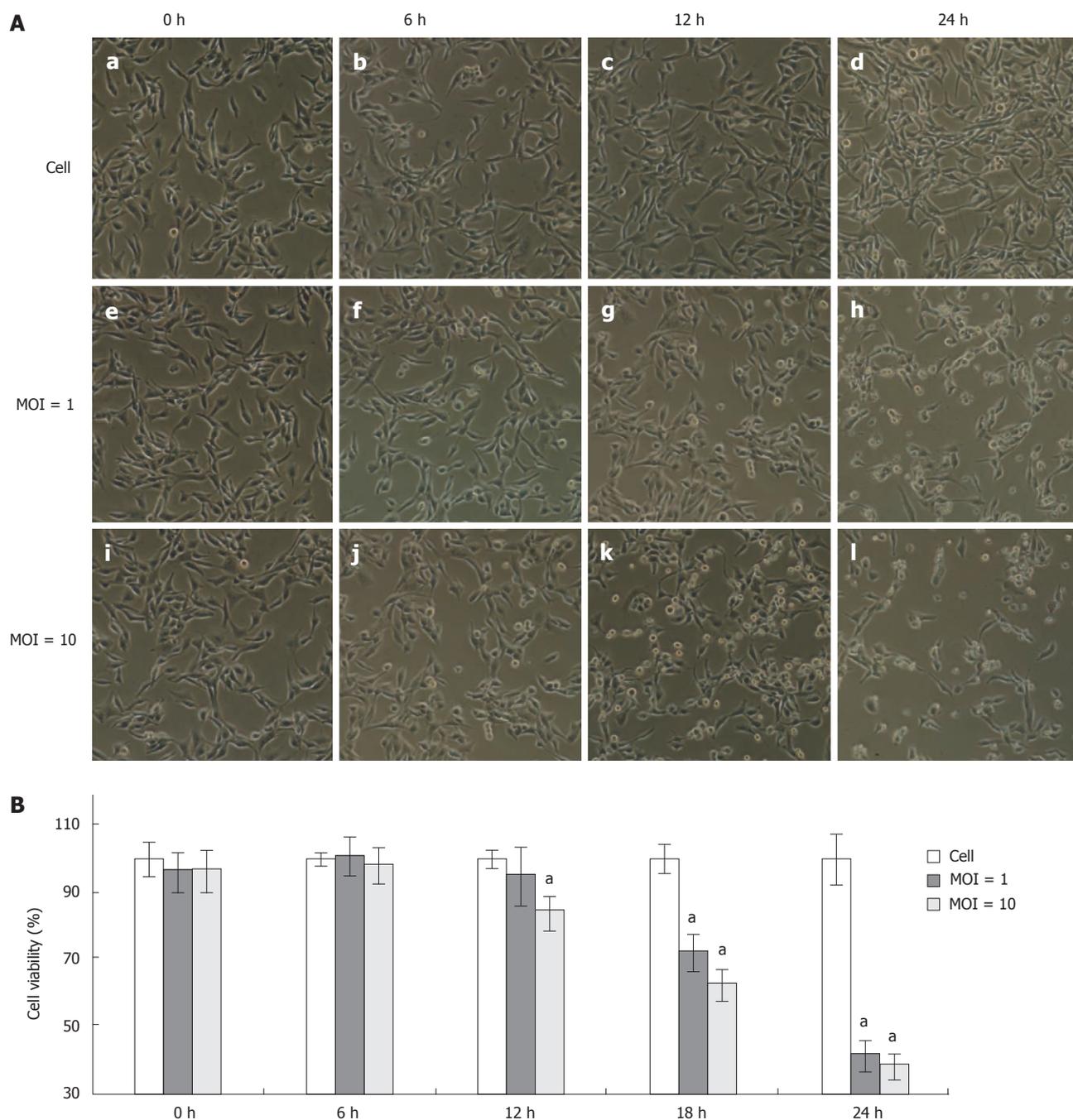


Figure 1 Cytopathic effects and kinetics of cell viability upon enterovirus 71 infection. Rhabdomyosarcoma cells were infected with enterovirus 71 (EV71) at multiplicity of infection (MOI) 1 or MOI 10. A: The cytopathic effects were shown by cell morphological changes (original magnification, $\times 100$); B: Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays at different time points after EV71 infection. Data are the mean \pm SD of three independent experiments, each carried out in triplicate. $^{\#}P < 0.05$.

VP1 protein level rapidly decreased after 9 h p.i. when the cells were infected with EV71 at MOI 10.

The kinetics of viral package

To determine the kinetics of virus package, the intracellular EV71 virions were quantified at different time points p.i. At 3 h p.i., the intracellular virions significantly decreased. Thereafter, the intracellular virions began to increase and enter into the exponential phase until 12 h p.i. when the amount of intracellular virions reached a peak.

The viral package kinetics were comparable for both the MOI 1 and MOI 10 infected groups (Figure 4). The total amounts of intracellular virions then decreased from 12 to 24 h p.i.

The kinetics of viral secretion

To determine the kinetics of virus secretion, the extracellular EV71 virions were quantitated. The EV71 virions began to be released from the cells infected either at MOI 1 or 10 3 h p.i. (Figure 5). The amounts of extra-

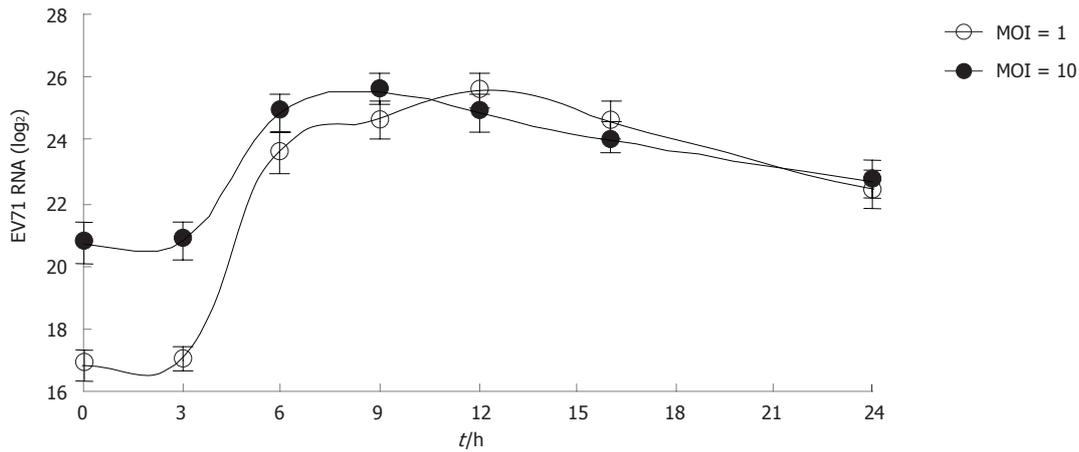


Figure 2 The kinetics of enterovirus 71 Replication. Rhabdomyosarcoma cells were infected with enterovirus 71 (EV71) virus at multiplicity of infection (MOI) = 1 or MOI = 10. At the indicated time points, the levels of total intracellular viral RNA were measured by quantitative real-time polymerase chain reaction. Data are the mean ± SD of three independent experiments; each carried out in triplicate.

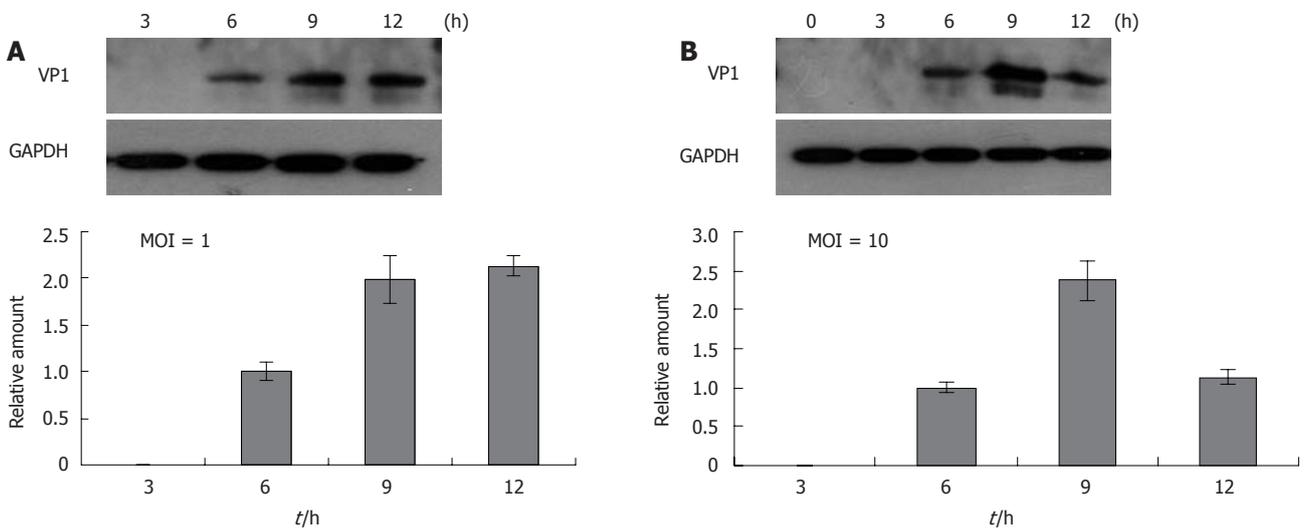


Figure 3 The kinetics of virus VP1 protein synthesis. Rhabdomyosarcoma cells were infected with enterovirus 71 (EV71) virus at multiplicity of infection (MOI) = 1 (A) and MOI = 10 (B). The intracellular viral protein VP1 was measured by Western blotting. The relative VP1 levels (the density of VP1/GAPDH) were calculated and are shown as solid bars.

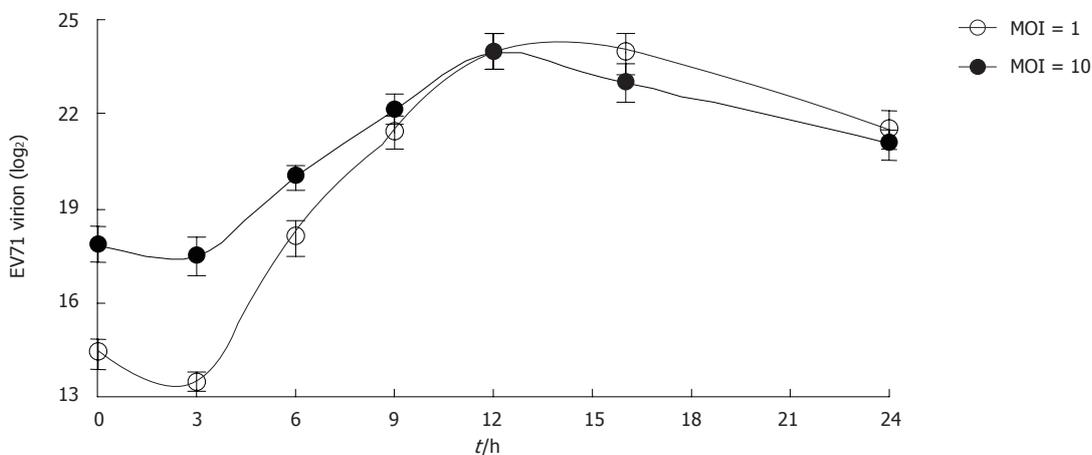


Figure 4 The kinetics of enterovirus 71 virus package. Rhabdomyosarcoma cells were infected with enterovirus 71 (EV71) virus at multiplicity of infection (MOI) = 1 or MOI = 10. The intracellular virus particles were isolated to measure the virion RNA by quantitative real-time polymerase chain reaction. Data are the mean ± SD of three independent experiments; each carried out in triplicate.

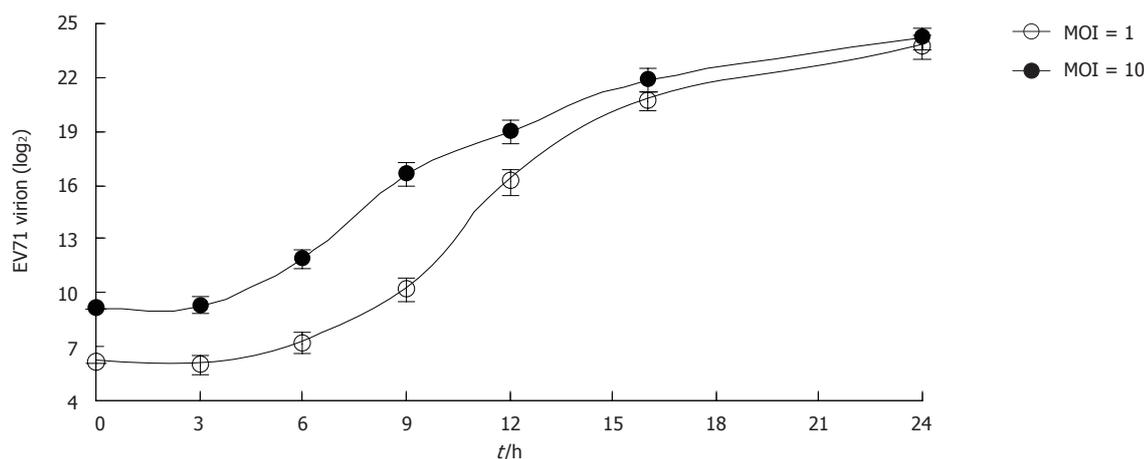


Figure 5 The kinetics of enterovirus 71 virus secretion. Rhabdomyosarcoma cells were infected with enterovirus 71 (EV71) virus at multiplicity of infection (MOI) = 1 or MOI = 10. Extracellular EV71 virions in the culture media were measured by quantitative real-time polymerase chain reaction at different time points post infection. Data are the mean \pm SD of three independent experiments; each carried out in triplicate.

cellular EV71 virions in the cultures of the two groups were constitutively increased. From 3 to 6 h p.i., the virions were slowly secreted into the culture media, and the virus secretion entered into the exponential phase from 6 to 12 h p.i. At 12 h p.i., the rate of increase declined and the total amount of extracellular virions reached a maximum at 24 h p.i. For cells infected at MOI 1 or 10, the virions in the culture media were similar at 24 h p.i.

DISCUSSION

Viral kinetics is an important parameter for demonstrating viral activities in the host cells and provides basic information on viral-host interactions and pathogenesis. The kinetics of some picornaviruses such as SVDV and FMDV have been described in several studies^[17,18]. However, little information on EV71 is available. Some studies provided brief descriptions on EV71 RNA replication and the growth kinetics of EV71 infected cells, however, the infection ratios used in these studies were too low (MOI \leq 0.01) to guarantee the synchronicity of infection^[21,22]. In this situation, some cells were undergoing cell death, whereas others just had a chance to be infected by new EV71 viruses secreted from the first round infected cells. Therefore, the viral life cycle could not be accurately examined. In addition, with the exception of RNA synthesis, no information was provided on viral protein expression, virus package and secretion. Our study, for the first time, comprehensively described the detailed viral kinetics in human RD cells. As RD cells infected by EV71 would develop cellular pathogenesis (CPE), these cells have been extensively used to investigate the viral activities of EV71 and host responses to EV71 infection^[23-26]. To obtain a synchronized infection, RD cells were pulse infected at high MOIs (MOI 1 and 10) to ensure that the majority of the cells were primarily infected in our study. Following infection, the unattached viruses were removed by washing the cells twice with PBS. This would minimize the interference of non-infectious virions. In this study, the kinetics of

viral replication, gene expression, package and secretion as well as the effects of viral activities on host cells were carefully examined at different time points.

We showed here that the intracellular virions significantly decreased by over 90% at 3 h p.i. (Figure 4), while the total intracellular RNA copies remained almost at the same levels (Figure 2). These results suggested that the virions were immediately uncoated after entry and virus replication was inactive within the first 3 h after infection. During this phase, the viral RNA could be translated to generate viral proteins essential for viral replication. From 3 to 6 h p.i., the virus underwent fast replication and the total intracellular viral RNA was rapidly accumulated (Figure 2). Similar results were also reported in other poliovirus infection^[27]. The total intracellular viral RNA increased by more than 64-fold within this period. In the meantime, viral gene expression was also initiated along with viral replication, as viral VP1 proteins in the host cells were clearly detected at 6 h (Figure 3). The viral package was also started (Figure 4) but very few virions were secreted (Figure 5). At 6 h p.i., about 1% (MOI 1) to 3% (MOI 10) of viral RNA was packaged into virions (Figures 2 and 4). Although the virus was rapidly replicating, the host cells were generally healthy during this period. From 6 to 9 h p.i., some cells became unhealthy (Figure 1), the viral replication entered a static stage as the total intracellular viral RNA only increased by about 2-fold in the MOI 1 and MOI 10 group. In the case of MOI 1, the total intracellular viral RNA increased a further 2-fold from 9 to 12 h p.i., but began to decrease 9 h p.i. in the cells infected at MOI 10. This suggested that viral RNA in cells infected with higher MOI reached maximal levels earlier. The viral gene expression and package were also actively processed in this period. The viral protein VP1 levels reached a peak at 9 h p.i. and rapidly decreased at 12 h p.i. in the MOI 10 group. In the case of MOI 1, the VP1 levels also reached a maximum at 9 h p.i. and maintained the same levels at 12 h p.i. Following viral protein synthesis, the intracellular virions also rapidly increased over 16-fold (MOI 10) or

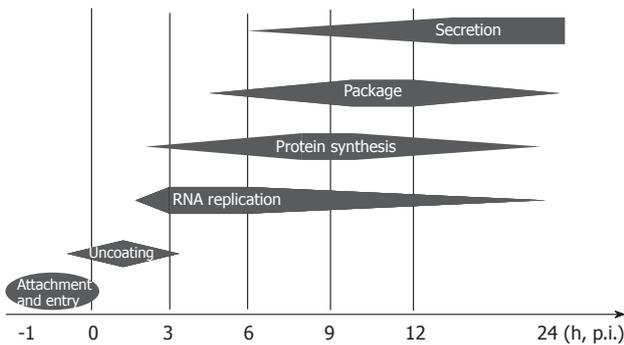


Figure 6 Schematic view of enterovirus 71 activities in rhabdomyosarcoma cells. Within one hour of inoculation, enterovirus 71 would first attach and enter into the host cell *via* its specific receptors. The virus was then uncoated in the first 3 h and started to synthesize the essential viral proteins for replication. From 3 to 6 h p.i., the virus rapidly replicated its genomic RNA and initiated structure protein synthesis. Fast viral package was observed from 6 to 12 h p.i. and virion secretion increased from 6 h p.i. to the end of the observation period.

64-fold (MOI 1). In both cases, the intracellular virions reached a peak and about 30% of viral RNA was packaged into the virions at 12 h p.i. (Figure 4 *vs* Figure 2). In the case of MOI 1, the extracellularly accumulated virions increased 8- and 64-fold from 6 to 9 h, and 9 to 12 h, respectively; while the extracellularly accumulated virions increased 30- and 5-fold during the same periods in the MOI 10 group. From 12 to 24 h p.i., as more and more infected cells became unhealthy and died, the intracellular viral RNA levels significantly decreased and viral replication became less active. These findings suggested that the cells could no longer sustain further viral replication and died^[28-30]. This was further supported by the data on intracellular and extracellular virion levels. We showed here that the intracellular virions maintained high levels at 16 h p.i. although more and more virions were secreted into the culture media. After that, the ratio of packaged viral RNA to total viral RNA was constant at about 50% in both the MOI 1 and MOI 10 group.

In summary, we have established a viral kinetics model of EV71 in human RD cells (Figure 6). We showed that upon infection, the virus uncoated within the first 3 h and started to synthesize the essential viral proteins for replication. From 3 to 6 h p.i., the virus rapidly replicated its genomic RNA and initiated viral package. The fast viral package displayed from 3 to 12 h p.i. and virion secretion from 6 h p.i. continued until death of the host cells. Host cells started to become unhealthy as early as 6 h p.i. but still supported viral replication, package and secretion until death. Thus, our study provides important information for further investigations into virus-host interactions and host pathogenesis.

COMMENTS

Background

Enterovirus 71 (EV71) infection causes hand-foot-and-mouth disease (HFMD) and neurological disease. Recently, EV71 infection has become a major health threat in China. However, the mechanisms of these diseases caused by EV71

infection are still largely unknown.

Innovations and breakthroughs

Studies on other human viruses such as human immunodeficiency virus, hepatitis C virus and hepatitis B virus have highlighted the importance of understanding viral kinetics. In this study, for the first time, the authors fully described the viral kinetics of EV71 in rhabdomyosarcoma (RD) cells and characterized the activities during each step of viral replication in detail.

Applications

Accurate information on viral kinetics will provide a valuable reference for investigating EV71-host interactions and the pathogenic mechanisms of diseases caused by EV71 infection.

Terminology

EV71 is a small positive RNA virus. During viral replication, the complementary minus-strand RNA is synthesised first and serves as a template for the next round of translation and replication. Thus, the viral RNA levels in cells, viral particles and culture supernatants represent the relative levels of viral replication, package and secretion.

Peer review

The authors provided detailed information on EV71 replication, package, secretion and viral protein expression in RD cells. The rapid increase of intracellular EV71 viral RNA and viral particles revealed that EV71 RNA was synthesized soon after infection and the viral particles could immediately package and released. These results will provide important information for further understanding the viral pathogenesis and EV71-host interactions.

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S- Editor Lv S L- Editor Webster JR E- Editor Zhang DN

Radiofrequency ablation vs hepatic resection for solitary colorectal liver metastasis: A meta-analysis

Yun-Zi Wu, Bin Li, Tao Wang, Shuang-Jia Wang, Yan-Ming Zhou

Yun-Zi Wu, Bin Li, Tao Wang, Shuang-Jia Wang, Yan-Ming Zhou, Department of Hepato-Biliary-Pancreato-Vascular Surgery, the First Affiliated Hospital of Xiamen University, Xiamen 361003, Fujian Province, China

Author contributions: Zhou YM participated in the design and coordination of the study, carried out the critical appraisal of studies and wrote the manuscript; Wu YZ and Li B developed the literature search, carried out the extraction of data, assisted in the critical appraisal of included studies and assisted in writing up; Wang T and Wang SJ carried out the statistical analysis of studies; Zhou YM interpreted data, corrected and approved the manuscript; All authors read and approved the final manuscript.

Correspondence to: Yan-Ming Zhou, MD, Department of Hepato-Biliary-Pancreato-Vascular Surgery, the First Affiliated Hospital of Xiamen University, 55 Zhenhai Road, Xiamen 361003, Fujian Province, China. zhouyms@yahoo.com.cn
Telephone: +86-592-2139708 Fax: +86-592-2137289

Received: December 22, 2010 Revised: January 19, 2011

Accepted: January 26, 2011

Published online: September 28, 2011

Abstract

AIM: To evaluate the comparative therapeutic efficacy of radiofrequency ablation (RFA) and hepatic resection (HR) for solitary colorectal liver metastases (CLM).

METHODS: A literature search was performed to identify comparative studies reporting outcomes for both RFA and HR for solitary CLM. Pooled odds ratios (OR) with 95% confidence intervals (95% CI) were calculated using either the fixed effects model or random effects model.

RESULTS: Seven nonrandomized controlled trials studies were included in this analysis. These studies included a total of 847 patients: 273 treated with RFA and 574 treated with HR. The 5 years overall survival rates in the HR group were significantly better than those in the RFA group (OR: 0.41, 95% CI: 0.22-0.90, $P = 0.008$). RFA had a higher rate of local intrahe-

patic recurrence compared to HR (OR: 4.89, 95% CI: 1.73-13.87, $P = 0.003$). No differences were found between the two groups with respect to postoperative morbidity and mortality.

CONCLUSION: HR was superior to RFA in the treatment of patients with solitary CLM. However, the findings have to be carefully interpreted due to the lower level of evidence.

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Key words: Hepatic resection; Colorectal liver metastases; Radiofrequency ablation; Efficacy; Meta-analysis

Peer reviewer: Selin Kapan, Dr., Associate Professor of General Surgery, Dr., Sadi Konuk Training and Research Hospital, Department of General Surgery, Kucukcekmece, Istanbul 34150, Turkey

Wu YZ, Li B, Wang T, Wang SJ, Zhou YM. Radiofrequency ablation vs hepatic resection for solitary colorectal liver metastasis: A meta-analysis. *World J Gastroenterol* 2011; 17(36): 4143-4148 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4143.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4143>

INTRODUCTION

Colorectal cancer continues to be one of the most common human malignancies, afflicting nearly one million individuals worldwide every year^[1]. Approximately 50% of patients with colorectal cancer develop hepatic metastases during the course of their disease. Survival without treatment is very limited, with a median of 7.4 to 11 mo^[2]. Hepatic resection (HR) is the only chance of cure for patients with colorectal liver metastases (CLM) and 5 years survival rates after radical resection are about 27%-58%^[3]. However, the great majority of

patients with CLM present with unresectable disease, mainly due to the extent or distribution of their disease, or concurrent medical disability, so only up to 20% of patients are candidates for HR^[4,5]. So, many nonsurgical ablative methods have been developed. The most widely utilized modality is radiofrequency ablation (RFA), which includes generation of high-frequency alternating current which causes ionic agitation and conversion to heat, with subsequent evaporation of intracellular water which leads to irreversible cellular changes, including intracellular protein denaturation, melting of membrane lipid bilayers, and coagulative necrosis of individual tumor cells.

Although RFA has established its role in the treatment algorithm of patients with inoperable CLM as a safe, well tolerated, easily repeated and less invasive procedure^[2,6,7], the therapeutic efficacy of RFA for those with resectable CLM remains controversial, especially for solitary lesions. For example, Oshowo *et al*^[8] reported equivalent median (41 mo *vs* 37 mo) and 3 years overall survival rates (55.4% *vs* 52.6%) between HR and RFA groups, whereas White *et al*^[3] reported better 5 years (71% *vs* 27%) and overall median survival (56 mo *vs* 36 mo) for resection *vs* RFA.

Meta-analysis can be used to evaluate the existing literature in both a qualitative and quantitative way by comparing and integrating the results of different studies and taking into account variations in characteristics that can influence the overall estimate of the outcome of interest^[9]. Therefore, we evaluated the available evidence comparing the clinical efficacy and safety of RFA and HR for treatment of solitary CLM using meta-analysis.

MATERIALS AND METHODS

Study selection

A MEDLINE, EMBASE, OVID, and Cochrane database search was performed on all studies between 1996 and 2010 to compare RFA and HR for solitary CLM. The following MeSH search headings were used: “colorectal liver metastases”, “hepatic resection”, “radiofrequency ablation” and “comparative study”. Only studies on humans and in English language were considered for inclusion. Reference lists of all retrieved articles were manually searched for additional studies.

Data extraction

Two reviewers (BL and TW, respectively) independently extracted the following parameters from each study: (1) first author and year of publication; (2) number of patients, patients’ characteristics, study design; and lastly (3) treatment outcome. All relevant text, tables and figures were reviewed for data extraction. Discrepancies between the two reviewers were resolved by discussion and consensus.

Criteria for inclusion and exclusion

For inclusion in the meta-analysis, a study had to fulfill

the following criteria: (1) compare the initial therapy effects of RFA and HR for the treatment of solitary CLM; (2) report on at least one of the outcome measures mentioned below; (3) clearly document indications for RFA and HR; and (4) if dual (or multiple) studies were reported by the same institution and/or authors, the one of higher quality or the most recent publication was included in the analysis.

Abstracts, letters, editorials and expert opinions, reviews without original data, case reports and studies lacking control groups were excluded. The following studies were also excluded: (1) those dealing with multiple CLM; (2) those with no clearly reported outcomes of interest; and (3) those evaluating patients with primary liver cancer.

Study objectives

The primary outcome was efficacy, including 5 years overall survival, local intrahepatic recurrence or 5 years disease-free survival. The secondary outcome was safety, including the morbidity and mortality.

Statistical analysis

The meta-analysis was performed using the Review Manager (RevMan) software, version 4.2.7. We analysed dichotomous variables using estimation of odds ratios (OR) with a 95% confidence interval (95% CI). Pooled effect was calculated using either the fixed effects model or random effects model. Heterogeneity was evaluated by χ^2 and I^2 . We considered heterogeneity to be present if the I^2 statistic was $> 50\%$. $P < 0.05$ was considered significant.

RESULTS

Selection of trials

After initial screening, 13 potentially relevant clinical trials were identified^[3,8,10-20]. Of these, in three trials including patients with multiple metastases, it was impossible to extract or calculate the appropriate data regarding solitary CLM^[10-12], two trials included patients with non-colorectal cancer^[13,14], and one trial lacked information concerning 5 years overall survival^[15]; all 6 studies were excluded. Finally, a total of 7 nonrandomized studies published between 2003 and 2009 matched the inclusion criteria and were therefore included^[3,8,16-20].

The characteristics of these 7 studies are summarized in Table 1. The 7 studies included a total of 847 patients: 273 in the RFA group and 574 in the HR group. Four studies were conducted in United States^[3,16,17,20], two in Korea^[18,19], and one in United Kingdom^[3]. The sample size of each study varied from 45 to 192 patients. The proportion of men ranged from 46.6% to 66.6%. Median duration of follow-up ranged from 17 to 68 mo.

Efficacy

The pooled analysis of the 7 studies furnishing data demonstrated a significant improvement in 5 years overall survival favoring HR over RFA (OR: 0.41, 95% CI:

Table 1 Baseline characteristics of studies included in the meta-analysis

Author/(yr)	Country	Group	n	M/F	Mean age (yr)	Mean tumor size (cm)	Median follow-up (mo)
Oshowo ^[8]	United Kingdom	RFA	25	11/14	57 (34-80)	3 (1-10) ¹	37 (9-67)
2003		HR	20	10/10	63 (52-77)	4 (2-7)	41 (0-97)
Aloia ^[16]	United States	RFA	30	23/7	-	3.0 (1.0-7.0) ¹	31.3 (4-138)
2006		HR	150	85/65	-	3.5 (0.5-17.0)	31.3 (4-138)
White ^[3]	United States	RFA	22	8/14	62 ± 7.5	2.4 ± 1.0	17
2007		HR	30	20/10	63 ± 9.6	2.7 ± 1.1	68
Berber ^[17]	United States	RFA	68	43/25	67 ± 1.4	3.7 ± 0.2	23 (2-86)
2008		HR	90	57/33	63.7 ± 1.3	3.8 ± 0.2	33 (2-132)
Lee ^[18]	Korea	RFA	37	26/11	59.0 (28-75) ¹	2.25 (0.8-5.0)	48.2 (0.9-133.9)
2008		HR	116	76/40	58.0 (26-79)	3.29 (0.5-18.0)	48.2 (0.9-133.9)
Hur ^[19]	Korea	RFA	25	15/10	62.6 (33-82)	2.5 (0.8-3.6)	42 (13-120)
2009		HR	42	27/15	58 (42-75)	2.8 (0.6-8)	42 (13-120)
Reuter ^[20]	United States	RFA	66	46/20	63.5	3.2	20
2009		HR	126	69/57	61.9	5.3	20

RFA: Radiofrequency ablation; HR: Hepatic resection; M: Male; F: Female. ¹Median.

Review: Meta-analysis of radio frequency ablation *vs* hepatic resection for solitary colorectal liver metastasis

Comparison: 01 Efficacy

Outcome: 01 5 years overall survival

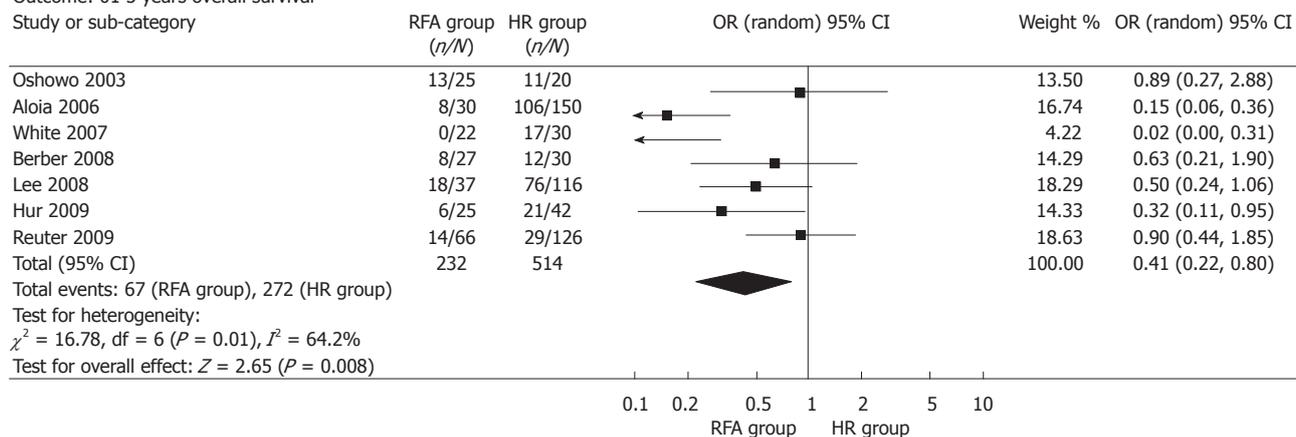


Figure 1 Results of the meta-analysis on 5 years overall survival. RFA: Radiofrequency ablation; HR: Hepatic resection; OR: Odds ratios; CI: Confidence intervals.

0.22-0.90, $P = 0.008$, $I^2 = 64.2\%$) (Figure 1).

Six trials investigated local intrahepatic recurrence^[3,16-20]. Local recurrence was more frequently observed after RFA than after HR (OR: 4.89, 95% CI: 1.73-13.87, $P = 0.003$, $I^2 = 77.3\%$) (Figure 2).

Only two studies reported on 5 years disease-free survival. Aloia *et al*^[16] reported that 5 years disease-free survival rates were higher after HR compared with RFA (50% *vs* 0%), whereas Lee *et al*^[18] reported equivalent results between two groups (25.7% *vs* 30.1%). We did not perform an analysis because of the small number of trials included in the review.

Safety

There was no statistically significant difference in the post-operative morbidity (five trials reported this data^[3,8,17,19,20], OR: 0.32, 95% CI: 0.07-1.52, $P = 0.15$, $I^2 = 75.6\%$) and mortality (all trials reported this data, OR: 0.58, 95% CI: 0.06-5.66, $P = 0.64$, $I^2 = 0\%$) between the two groups (Figures 3 and 4). There were no deaths reported in the

RFA group, and 2 in the HR group.

DISCUSSION

This meta-analysis shows that the HR treatment group had better 5 years survival outcomes than the RFA treatment group for solitary CLM. The major contributing factor for this finding may be the higher local recurrence rate after RFA. In addition to being more likely to have a recurrence, RFA patients also recurred earlier than resection patients^[3,20]. This could be due to incomplete ablation secondary to lesion size, heat sink effect, or the limitations of the modality^[20]. Resection of the entire area of preexisting tumor is more oncologically sound than attempting thermal destruction of a frequently ill defined region in the liver^[21]. This may explain the better outcomes following HR.

In a mouse xenograft model of CLM, von Breitenbuch *et al*^[22] revealed that RFA led to an increased survival of residual neoplastic cells and significantly promoted the

Review: Meta-analysis of radio frequency ablation vs hepatic resection for solitary colorectal liver metastasis

Comparison: 01 Efficacy

Outcome: 02 Local recurrence

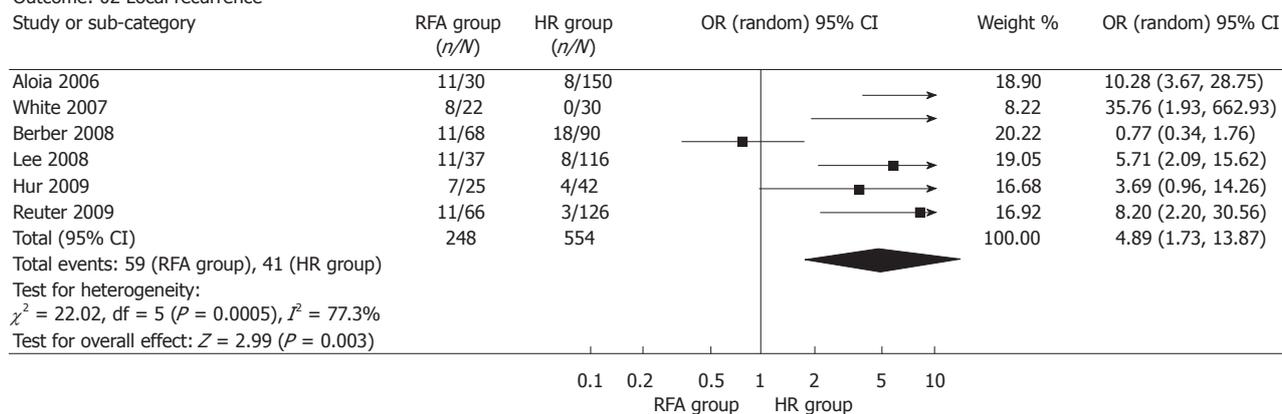


Figure 2 Results of the meta-analysis on local recurrence rate. RFA: Radiofrequency ablation; HR: Hepatic resection; OR: Odds ratios; CI: Confidence intervals.

Review: Meta-analysis of radio frequency ablation vs hepatic resection for solitary colorectal liver metastasis

Comparison: 02 Safety

Outcome: 01 Morbidity

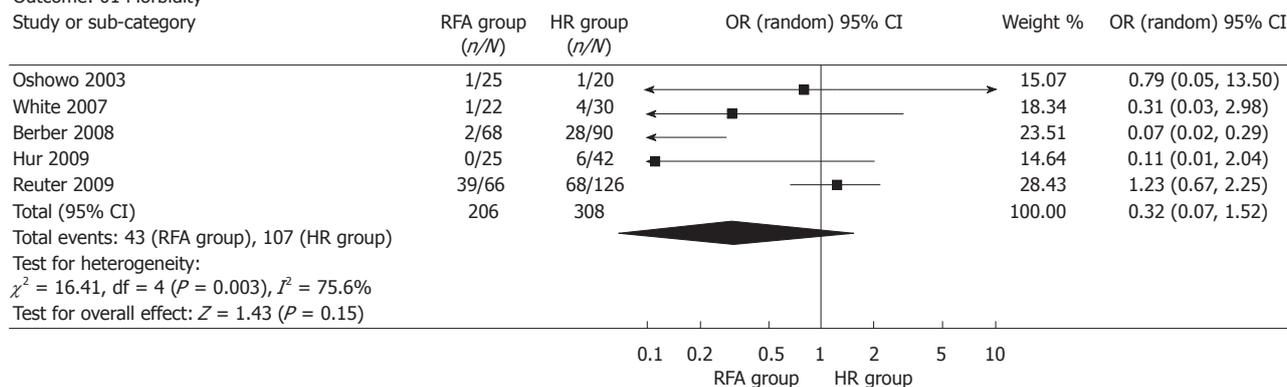


Figure 3 Results of the meta-analysis on postoperative morbidity. RFA: Radiofrequency ablation; HR: Hepatic resection; OR: Odds ratios; CI: Confidence intervals.

Review: Meta-analysis of radio frequency ablation vs hepatic resection for solitary colorectal liver metastasis

Comparison: 02 Safety

Outcome: 02 Mortality

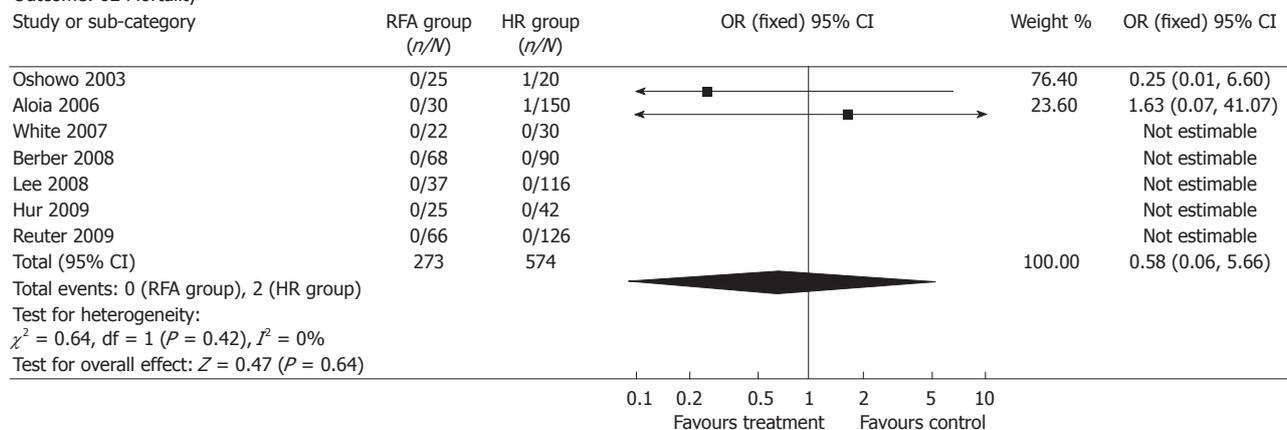


Figure 4 Results of the meta-analysis on postoperative mortality. RFA: Radiofrequency ablation; HR: Hepatic resection; OR: Odds ratios; CI: Confidence intervals.

proliferation of neoplastic cells. Recently, Nijkamp *et al*^[23] found that RFA treatment resulted in a highly localized

hypoxia-driven acceleration of tumor growth occurring in the transition zone between necrosis induced by RFA and the normal liver tissue, and that the stimulated outgrowth of perilesional micrometastases is associated with profound and chronic microvascular disturbances, chronic tissue and tumor hypoxia, and stabilization of hypoxia-inducible factor (HIF)-1 α and HIF-2 α . These experimental findings may further explain the better outcome after RFA compared with HR in current study.

For liver metastases ≤ 3 cm, Mulier and colleagues found that local recurrence after RFA is extremely low in a recent review, and the authors proposed a randomized trial comparing resection and RFA for resectable CLM ≤ 3 cm is warranted^[24]. However, in a study of 79 patients with solitary CLM ≤ 3 cm, RFA treatment resulted in a higher local recurrence rate than HR treatment (31% *vs* 3%, respectively). RFA was also associated with a marked decrease in the 5 years survival rate and the 5 years local recurrence-free rate compared with those of HR (18% *vs* 72% and 66% *vs* 97%, respectively)^[16]. Similarly, another study of 60 patients showed that both time to recurrence after treatment of liver metastases and overall survival were significantly shorter, and marginal recurrence significantly more frequent, in the RFA group^[15]. Although Hur *et al.*^[19] reported equivalent 5 years survival rates (56.1% *vs* 55.4%) and local recurrence-free survival rates (95.7% *vs* 85.6%) between HR and RFA groups in patients with tumors ≤ 3 cm, it must be noted that the limited number of patients ($n = 38$) in their study might have insufficient power to detect any differences.

In that review, Mulier *et al.*^[24] stated that the two randomized clinical trials^[25,26] showed equivalent survival after percutaneous RFA and surgical resection for small HCC will encourage the use of RFA for resectable CLM. However, in one of the two studies, 19 of 90 patients (21%) who were randomized for RFA converted to HR^[25]. More importantly, a recently published meta-analysis and a randomized clinical trial both found that HR was superior to RFA in the treatment of patients with small HCC with respect to survival and local control of the disease^[27,28]. Thus, we agree with the idea proposed by Curley that “it is not yet time for a randomized clinical trial comparing resection with RFA for resectable CLM.”

The results of this meta-analysis should be interpreted with caution for several reasons. First, all of data in the present study comes from nonrandomized studies, and the overall level of clinical evidence is low. Second, there is important heterogeneity between two groups, because it was not possible to match patients characteristics in all studies. We applied a random effect model to take between study variation into consideration. This does not necessarily rule out the effect of heterogeneity between studies, but one may expect a very limited influence. Finally, potential publication bias might be present due to the small number of trials included in the current study.

In summary, HR was superior to RFA in the treatment of patients with solitary CLM. RFA should be reserved for patients who are not optimal candidates for

resection, rather than being used as a first-line therapeutic option. However, the findings have to be carefully interpreted due to the lower level of evidence.

COMMENTS

Background

Hepatic metastases are the commonest cause of morbidity and death of patients with colorectal cancer. Survival without treatment is very limited, with a median of 7.4 to 11 mo. Hepatic resection (HR) is the only chance of cure for patients with colorectal liver metastases (CLM) and 5 years survival rates after radical resection are about 27%-58%. Unfortunately, only up to 20% of patients are candidates for HR.

Research frontiers

Radiofrequency ablation (RFA) is an established effective nonsurgical ablative method for treatment of inoperable CLM, but its therapeutic efficacy for resectable CLM remains controversial, especially for solitary lesions.

Innovations and breakthroughs

This meta-analysis shows for the first time that HR was superior to RFA in the treatment of patients with solitary CLM with respect to survival and local control of the disease.

Applications

The results suggest that RFA should be reserved for patients with solitary CLM who are not optimal candidates for resection, rather than being used as a first-line therapeutic option.

Peer review

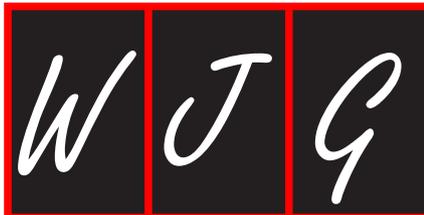
The article is a well written, well analysed one that is worth publishing.

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S- Editor Tian L L- Editor O'Neill M E- Editor Zhang DN



CD133 and membrane microdomains: Old facets for future hypotheses

Christine A Fargeas, Jana Karbanová, József Jászai, Denis Corbeil

Christine A Fargeas, Jana Karbanová, József Jászai, Denis Corbeil, Tissue Engineering Laboratories (BIOTEC), Technische Universität Dresden, D-01307 Dresden, Germany
Author contributions: Fargeas CA, Karbanová J, Jászai J and Corbeil D contributed equally to this letter to the editor.

Supported by Deutsche Forschungsgemeinschaft (TRR83 No. 6; SFB655 B3; CO298/5-1)

Correspondence to: Denis Corbeil, PhD, Tissue Engineering Laboratories (BIOTEC), Technische Universität Dresden, Tatzberg 47-49, D-01307 Dresden,

Germany. corbeil@biotec.tu-dresden.de

Telephone: +49-351-46340118 Fax: +49-351-46340244

Received: April 20, 2011 Revised: June 16, 2011

Accepted: June 23, 2011

Published online: September 28, 2011

Abstract

Understanding all facets of membrane microdomains in normal and cancerous cells within the digestive tract is highly important, not only from a clinical point of view, but also in terms of our basic knowledge of cellular transformation. By studying the normal and cancer stem cell-associated molecule CD133 (prominin-1), novel aspects of the organization and dynamics of polarized epithelial cells have been revealed during the last decade. Its association with particular membrane microdomains is highly relevant in these contexts and might also offer new avenues in diagnosis and/or targeting of cancer stem cells.

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Key words: AC133; Cancer; CD133; Membrane microdomains; Membrane vesicles; Prominin-1; Stem cell

Peer reviewer: Zoran Krivokapic, Professor, Dr., MD, FRCS, Institute for Digestive Disease, First Surgical Clinic, Clinical Center of Serbia, 6, Dr Koste Todorovica, Belgrade 11000, Serbia

Fargeas CA, Karbanová J, Jászai J, Corbeil D. CD133 and membrane microdomains: Old facets for future hypotheses. *World J Gastroenterol* 2011; 17(36): 4149-4152 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i36/4149.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i36.4149>

TO THE EDITOR

We read with great interest a recent Editorial entitled “Multifaceted nature of membrane microdomains in colorectal cancer” by Jahn *et al*^[1] published in issue 17 of the *World Journal of Gastroenterology* 2011 which proposes to describe the pioneering and recent studies on membrane microdomains (the so-called lipid rafts) and their potential roles in cancers. An important section dealing with prominin-1 (alias CD133), a cholesterol-binding glycoprotein often described as a stem and cancer stem cell marker, is unfortunately entirely based on a single publication released in 2009^[2], thus leaving out valuable biochemical and morphological information concerning CD133 and membrane microdomains from earlier works. We fear that as such, it might lead to underestimation of the importance and complexity of such a molecular association and contribute to certain confusion, particularly with regard to the debated AC133 epitope of CD133 and its association with cancer. We propose to expose here earlier overlooked data regarding its expression in epithelial cells and summarize the current knowledge on its cell biology and association with distinctive membrane microdomains. We hope that this might enlighten current issues regarding the implication of CD133 in colorectal cancer, whether it is in metastases, or as a prognostic marker or as a cancer stem cell marker.

Actually, the demonstration of the presence of CD133 in Caco-2 cells and its association with membrane microdomains is much less recent than 2009, since it was more than a decade ago that we reported its presence

in this widely used human colon carcinoma-derived cell line^[3]. The detection of CD133 by immunolabeling was originally documented by its particular epitope AC133 that appeared to be restricted to stem/progenitor cell populations but was also thought to be dependent on conformation and/or sensitive to changes in glycosylation^[4]. This antigen was attractive in the context of stem/progenitor and cancer stem cells and has often been used to define them in numerous organ systems including the digestive tract, but at the same time controversy was generated on the implication of CD133 as a specific marker^[5-10].

We have previously demonstrated in a key publication of 2000 using the Caco-2 cells as a model of enterocytic epithelial differentiation^[3] together with a later study^[11], that the AC133 epitope, but neither the CD133 transcript nor the CD133 protein, is down-regulated upon differentiation, with the result that only a minute sub-fraction of CD133 molecules will carry it^[11]. We have therefore stressed several times in the literature that it is important to consider that AC133 antibody detects only a subpopulation of human prominin-1/CD133 glycoproteins carrying the AC133 epitope, and that consequently, AC133 antigen is not fully synonymous with CD133^[11-13]. The importance of CD133 glycosylation states for the definition of cancer stem cells has been analyzed by Bindlingmaier and colleagues^[14]. In the meantime, the *prominin-1* (*PROM1*) gene was shown to be transcriptionally active all along the gastrointestinal tract as CD133 mRNA is detectable by Northern blot^[15], and several studies have demonstrated that in humans, as in mice, its protein is physiologically expressed in several differentiated epithelia^[11,16-20]. Thus, the AC133 epitope might be simply down- or up-regulated during the process of differentiation or transformation, respectively^[11]. The alteration of the general glycosylation pattern of intestinal cells might explain such a phenomenon^[21]. Importantly, the lack of AC133 detection might additionally reflect its instability^[22] or its differential accessibility^[19] (see below). Of note, the proportion of CD133 molecules carrying (or not) the AC133 epitope in a given differentiated cell remains, however, unknown.

As proposed earlier^[19] and pointed out in the Editorial of Jahn and colleagues, the molecular environment surrounding CD133 within the plasma membrane might influence the detection of certain epitopes (e.g., AC133 or those within putative ganglioside-binding sites^[2]). To fully appreciate the importance of CD133, one should bear in mind that, at the subcellular level, CD133 selectively marks plasma membrane protrusions, e.g., microvilli and primary cilia, that are located in the apical domain of polarized epithelial cells including Caco-2 cells, and was therefore originally named prominin (from Latin, *prominere*)^[3,16,23,24]. Within these protrusions, CD133 binds directly to plasma membrane cholesterol^[25,26] and is incorporated into membrane microdomains that differ from those found in non-protruding areas of the plasma membrane, as demonstrated biochemically using mild

detergents^[25], and morphologically by co-localization with the ganglioside GM1^[27]. Such protein-lipid interactions appear essential to maintain the proper localization of CD133 in microvilli^[25], and potentially its physiological function which yet remains elusive^[28,29]. Thus, the direct binding of certain gangliosides to CD133^[2,27] within the densely packed lipid microdomain might mask some CD133 epitope(s), particularly those in the vicinity of the membrane. Technically, they might be revealed, at least in part, using sensitive methods including harsh conditions for antigen retrieval as in the case of native tissues^[8,19,30,31], upon cell-detachment as in the case of cell lines (e.g., Caco-2 cells)^[32], or by chemical interference with membrane microdomain integrity^[2].

Although tightly associated with plasma membrane, CD133 is nonetheless released into numerous physiological body fluids including urine, saliva, seminal fluids and cerebrospinal fluids in association with small membrane vesicles^[33]. It is important to point out that such vesicles are budding from the tip of a microvillus or primary cilium by a molecular mechanism involving cholesterol-dependent membrane microdomains^[26,34]. In other words, their release might be modulated by the cholesterol level (and possibly that of other lipids) within the plasma membrane. Interestingly, such release occurs solely during and after the differentiation of Caco-2 cells or, *in vivo*, of neural progenitor cells^[33]. Based on the latter observation and the expression of CD133 (AC133 epitope in the case of humans) by numerous somatic stem cells, the concept of “stem cell-specific membrane microdomains” was postulated^[33]. Given that membrane microdomains are implicated in several signaling cascades by allowing the formation of active transduction complexes^[35], CD133-containing membrane microdomains might carry and/or functionally organize molecular determinants essential to maintain the stem cell and undifferentiated cell properties and their loss or disposal, e.g., *via* membrane vesicles, and could modify the status or even the fate of the cells^[33,36]. Yet, these microdomains, given their dependence on cholesterol, seem to differ from those defined by Hakomori and co-workers in the glycosynapse concept, and which have been implicated in several biological phenomena related to tumorigenesis^[37,38]. However, the coalescence of small CD133-lipid entities into the largest platform within the microvillar membranes might be dragged by carbohydrate moieties, as proposed earlier^[13,25,28]. Thus, a certain interdependence of lipid rafts and glycosynapses *per se* might exist. Whether CD133 molecules carrying AC133 epitope are preferentially released upon differentiation remains to be determined. Collectively, numerous physiological and technical parameters might interfere with immunodetection of certain CD133 epitopes, and importantly, the lack of their detection needs to be evaluated with some caution, and maybe alternative methods such as *in situ* hybridization should complement the investigation^[18,39].

Clinically, in addition to its potential value as a biomarker in tissue diagnosis, the association of CD133/

lipid complexes with extracellular membrane vesicles might offer an alternative screening method for the detection of cancers associated with the digestive tract as demonstrated for central nervous system diseases^[40]. Moreover, CD133 expression by cancer stem cells might contribute to outlining new prospects for more effective cancer therapy by targeting tumor-initiating cells.

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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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Dario Conte, Professor, GI Unit - IRCCS Osp. Maggiore, Chair of Gastroenterology, Via F. Sforza, 35, Milano 20122, Italy

Bijan Eghtesad, Dr, Associate Professor, Department of General Surgery, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland OH 44195, United States

Dr. Alessandro Ferrero, MD, Department of Surgery, Mauritian Hospital, Largo Turati 62, 10128 Torino, Italy

Alfred Gangl, Professor, Department of Medicine 4, Medical University of Vienna, Allgemeines Krankenhaus, Waehringer Guertel 18-20, Vienna A-1090, Austria

Satoru Kakizaki, MD, PhD, Assistant Professor, Department of Medicine and Molecular Science, Gunma University, Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan

Yuyuan Li, Professor, Department of Gastroenterology, First Municipal People's Hospital of Guangzhou, 1 Panfu Road, Guangzhou 510180, Guangdong Province, China

Fabrizio Montecucco, MD, Assistant, Division of Cardiology, Department of Internal Medicine, University of Geneva, Avenue de la Roseaie 64, 1211 Geneva, Switzerland

Dr. Vance Matthews, PhD, BS, Cellular and Molecular Metabolism Laboratory, Baker University of Texas Medical

Branch, IDI, PO Box 6492, St Kilda Road Central, VIC 8008, Melbourne, Australia

Noriko Nakajima, MD, PhD, Associate Professor, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Nihon University School of Medicine, 1-8-13 Kandasurugadai Chiyoda-ku, Tokyo 101-8309, Japan

Christoph Reichel, Priv.-Doz., Dr., Head of the Gastroenterological Rehabilitation Center Bad Brückenau, Clinic Hartwald, German Pension Insurance Federal Office, Schlüchtern Str. 4, 97769 Bad Brückenau, Germany

Francisco Rodriguez-Frias, PhD, Proteins Hepatitis Molecular Genetics Unitat, Biochemistry Department, Vall d'Hebron Unicersitary Hospital, Barcelona 08035, Spain

Chanjuan Shi, MD, PhD, Assistant Professor, Department of Pathology, Vanderbilt University, 1161 21st Ave. So, MCN C-2318A, Nashville, TN 37232-2561, United States

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

Phil Sutton, Associate Professor, Centre for Animal Biotechnology, School of Veterinary Science, University of Melbourne, Melbourne, VIC 3010, Australia

Masahiro Tajika, MD, PhD, Department of Endoscopy, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan

Yuk Him Tam, Dr., Department of Surgery, Prince of Wales Hospital, Shatin, Hong Kong, China

Tamara Vorobjova, Senior Researcher in Immunology, Department of Immunology, Institute of General and Molecular Pathology, University of Tartu, Ravila, 19, Tartu 51014, Estonia

Chunqing Zhang, Professor, Department of Gastroenterology, Shandong Provincial Hospital, Jinan 250021, Shandong Province, China

Events Calendar 2011

January 14-15, 2011

AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011

Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011

Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011

9. Gastro Forum München, Munich,
Germany

February 4-5, 2011

13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011

Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011

APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011

Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011

2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011

International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011

Canadian Digestive Diseases Week,
Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 1, 2011

Childhood & Adolescent Obesity:

A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011

42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011

Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011

British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011

41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011

Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011

UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011

MedicRes IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011

26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011

IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011

International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011

Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011

Pediatric Emergency Medicine:
Detection, Diagnosis and Developing

Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011

9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011

The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011

Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011

4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011

Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011

2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011

1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011

22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011

4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011

The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011

Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011

International Scientific Conference
on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011

ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011

XI Congreso Interamericano
de Pediatría "Monterrey 2011",
Monterrey, Mexico

September 2-3, 2011 Falk Symposium

178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne,
Martinstr. 29-37, 50667 Cologne,
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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,
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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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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books*Personal author(s)*

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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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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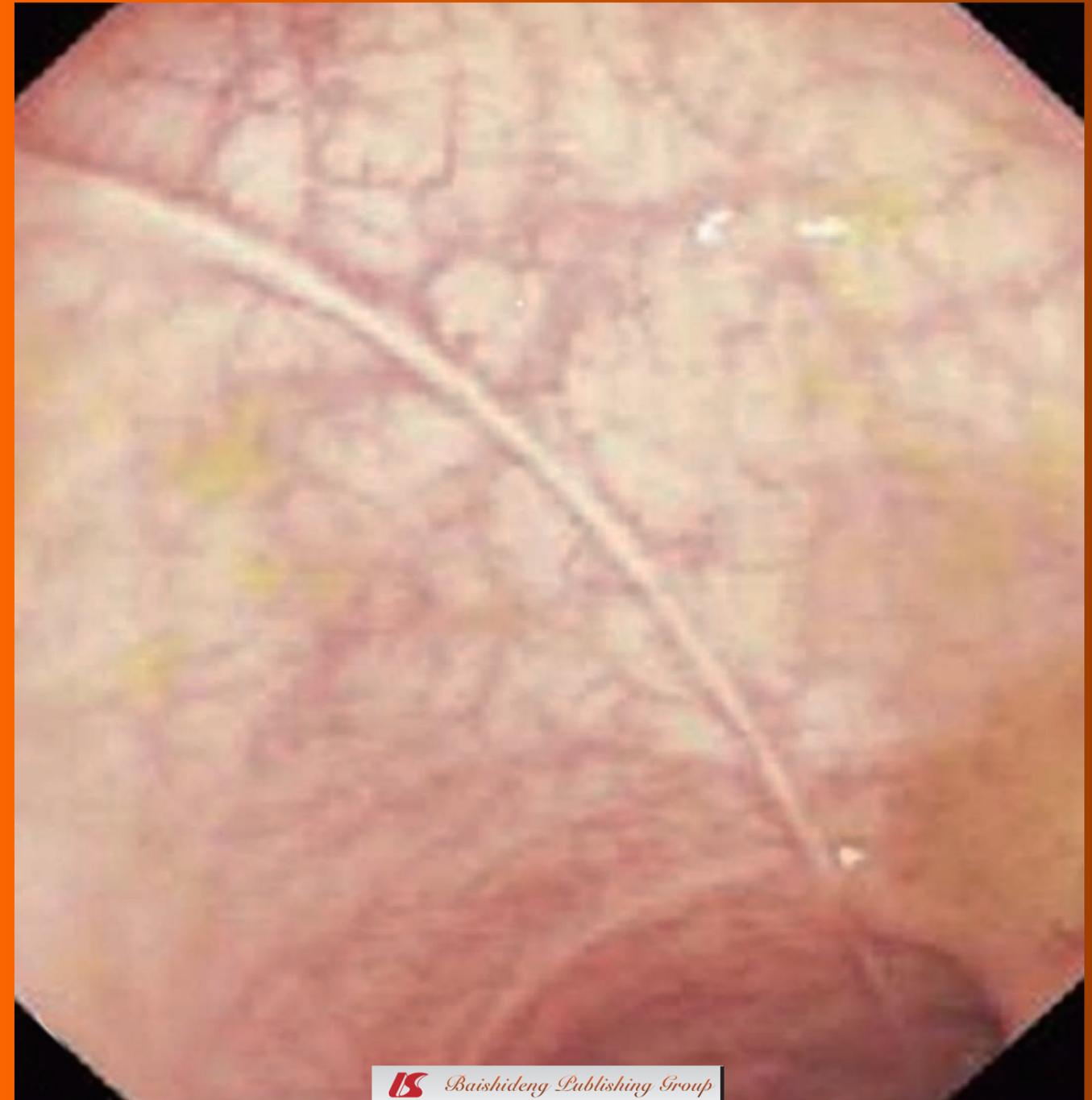
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ISSN 1007-9327



World Journal of *Gastroenterology*

World J Gastroenterol 2011 October 7; 17(37): 4153-4250



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Weekly Volume 17 Number 37 October 7, 2011

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<http://www.wjgnet.com/1007-9327/full/v17/i37/4157.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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Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
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PUBLISHING
Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building, No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
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SUBSCRIPTION
Beijing Baishideng BioMed Scientific Co., Ltd.
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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
October 7, 2011

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

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Selection criteria for cytoreductive surgery and hyperthermic intraperitoneal chemotherapy in gastric cancer

Ingmar Königsrainer

Ingmar Königsrainer, Department of General, Visceral and Transplant Surgery, Tübingen University Hospital, 72076 Tübingen, Germany

Author contributions: Königsrainer I was the sole contributor to this paper.

Correspondence to: Ingmar Königsrainer, MD, Department of General, Visceral and Transplant Surgery, Tübingen University Hospital, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany. ingmar.koenigsrainer@med.uni-tuebingen.de

Telephone: +49-7071-2985073 Fax: +49-7071-295588

Received: January 24, 2011 Revised: June 9, 2011

Accepted: June 16, 2011

Published online: October 7, 2011

Abstract

Peritoneal carcinomatosis in gastric cancer is associated with a dismal prognosis. Systemic chemotherapy is not effective because of the existence of a blood-peritoneal barrier. Cytoreductive surgery and intraperitoneal chemotherapy can improve survival and quality of life in selected patients. Patient selection for this multimodal approach is one of the most critical issues, and calls for interdisciplinary evaluation by radiologists, medical and surgical oncologists, and anaesthetists. This article sets forth criteria for selection of gastric cancer patients suffering from peritoneal carcinomatosis.

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Key words: Peritoneal carcinomatosis; Gastric cancer; Hyperthermic intraperitoneal chemotherapy; Cytoreductive surgery; Selection criteria

Peer reviewer: Kevin Michael Reavis, MD, Assistant Clinic Professor, Department of Surgery, Division of Gastrointestinal Surgery, University of California, Irvine Medical Center, 333 City Boulevard West, Suite 850, Orange, CA, United States

Königsrainer I. Selection criteria for cytoreductive surgery and hyperthermic intraperitoneal chemotherapy in gastric cancer. *World*

J Gastroenterol 2011; 17(37): 4153-4156 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4153.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4153>

INTRODUCTION

Gastric cancer is one of the most frequent causes of cancer-related mortality worldwide^[1,2]. Surgical resection after neoadjuvant chemotherapy in primary locally extended cases remains the mainstay for treating patients suffering from this disease. Surgery is limited by various factors: impaired general status, severe concomitant diseases, and distant metastases. One form is peritoneal dissemination of cancer cells within the abdominal cavity. Peritoneal carcinomatosis (PC) is detected in more than 30% of patients with advanced gastric cancer, and almost 60% of deaths are caused by peritoneal dissemination. In contrast to lymphatic and hematogenous metastasis, peritoneal carcinomatosis can be considered a local disease limited to the peritoneal cavity. Based on this rationale, cytoreductive surgery and intraabdominal chemotherapy have become a relevant treatment option for patients.

Various intraabdominal chemotherapy protocols have been established, varying from hyperthermic intraperitoneal chemotherapy (HIPEC), to early postoperative intraperitoneal chemotherapy, normothermic intraperitoneal chemotherapy, and delayed postoperative intraperitoneal chemotherapy^[3]. They differ in the heat of the administered agent, the chemotherapy dosage, and time of administration of the chemotherapy. HIPEC seems to have the most beneficial impact on overall survival^[3]. Retrospective analyses of patients treated with cytoreductive surgery plus HIPEC show a clear survival benefit when complete cytoreduction was possible. The randomized trial by Fujimoto *et al*^[4] in 141 gastric cancer patients, who were curatively resected, showed a significantly reduced peritoneal recurrence rate and improved long-term survival when HIPEC was part of the treatment as

compared to the “surgery alone” group. This observation was confirmed by Kim *et al*^[5], who showed a significantly lower peritoneal recurrence rate and an improvement in the five-year survival rate in the multimodally treated patients as compared to the “surgery alone” group.

Systemic chemotherapy is not as effective as surgery plus HIPEC because of the blood-peritoneal barrier^[6].

For metachronous peritoneal carcinomatosis from gastric cancer, there is no evidence to show which patient should be treated with the presented multimodal strategy. The dilemma arises when patients are very young and systemic chemotherapy is ineffective. In such cases, an individual approach with maximal tumor debulking may be an option and is justified in highly selected patients. However, in metachronous peritoneal carcinomatosis, the tumor often extensively involves the abdominal cavity with infiltration of the retroperitoneum, liver hilus, etc., which makes surgery impossible.

The aim of this article is to summarize the recent knowledge on patient selection for cytoreductive surgery and perioperative intraperitoneal chemotherapy for primary gastric cancer with peritoneal carcinomatosis or positive cytology.

GENERAL STATUS

Patients with limited peritoneal carcinomatosis from gastric cancer do not suffer from symptoms such as dysphagia or dysmotility, and PC is frequently found even in low T and negative N stages. Therefore, if the general status is acceptable, the option of radical surgical treatment should always be considered.

A detailed preoperative anesthesiological check-up is of importance and all patients should undergo a preoperative lung and cardiac function test. Concomitant diseases, that may influence surgical and anesthesiological risks, should be identified. As for most general elective operations, a low ASA score is mandatory. Age is still a matter of concern, because biological age does not always correlate with numerical age. However, most groups dealing with cytoreductive surgery and HIPEC restrict patient selection to an age below 65. Most importantly, informed consent with discussion of all alternative therapies must be obtained from the patients, and patients should be offered psychooncological support.

PREOPERATIVE DIAGNOSTICS

A high-end computed tomography is actually the standard and should be performed also to exclude extraabdominal spread and liver metastases. Recently, we demonstrated that positron emission tomography computed tomography (PET-CT) correlates well with intraoperative tumor load in peritoneal carcinomatosis^[7]. However, gastric cancer with PC is frequently of mucinous character; therefore, PET-CT is not helpful in selecting candidates for radical resection. Nodules smaller than 5-8 mm cannot be consistently detected^[8,9]. In particular, nodules on the small bowel and its mesentery are difficult to diagnose, but relevant for indication.

To date, there is no imaging method that can sufficiently predict intraoperative tumor load. Therefore, explorative laparoscopy is an invasive alternative for candidates in whom radiological work-up was not sufficient to determine operability.

Laparoscopy permits determination of the peritoneal carcinomatosis index (PCI) and cytology in locally advanced cases. Laparoscopy is highly accurate for the diagnosis of peritoneal carcinomatosis, with good correlation to the open surgical exploration found by Yonemura *et al*^[9].

Therefore, every patient should undergo explorative laparoscopy before neoadjuvant therapy or primary gastrectomy. As the first step of treatment, some groups even administer HIPEC *via* laparoscopy in patients with synchronous PC or positive cytology^[10,11].

PCI

PCI describes the tumor load in the abdomen and varies from 0 to 39, depending on the compartments involved^[12].

In contrast to colorectal cancer, where the PCI should be lower than 20 so that patients potentially profit in terms of overall survival^[13], in gastric cancer, the PCI should be much lower, because the biological behaviour of the tumor is more aggressive.

In a recent work by Yonemura *et al*^[14], complete cytoreduction was achieved in 91% of the patients when the PCI was lower than 6, but in only 42% of the patients with a PCI \leq 7. Overall survival was also better in the PCI \leq 6 group. In gastric cancer with peritoneal carcinomatosis, lymph nodes should be removed only if they are infiltrated; however, prophylactic D2 lymphadenectomy is unnecessary.

Patients with liver metastasis, involving para-aortic lymph nodes and extraabdominal metastases, are not candidates for cytoreductive surgery and HIPEC. The treatment of metachronous metastases remains controversial, and cancer masses tend to infiltrate the retroperitoneum and liver hilus with vascular structures, which makes surgery impossible.

COMPLETENESS OF CYTOREDUCTION SCORE

The completeness of cytoreduction score CC score describes the completeness of cytoreduction after operation. Ideally, all tumor nodules can be removed macroscopically (CC0). Otherwise, a CC1, CC2 or CC3 score describes non-resectable tumor nodules that vary in size and influence on prognosis. Non-resectability is caused either by diffuse peritoneal carcinomatosis with a high PCI, where surgical resection is oncologically not justified, or by diffuse infiltration of the small bowel or the mesenteric axis, and infiltration of the retroperitoneum.

NEW PROTOCOLS

Neoadjuvant intraperitoneal systemic chemotherapy pro-

tocol (NIPS) is a newly developed neoadjuvant intraperitoneal treatment modality developed by Yonemura *et al*^[15]. A good predictor of the possibility of achieving a CC0 status is preoperative cytology. Yonemura *et al*^[16] performed NIPS and achieved CC0 status in 27 of 52 patients with negative cytology, but only in four of 27 with positive cytology. Peritoneal wash cytology may, therefore, be a good predictor of the potential for CC0 status.

SUMMARY AND FUTURE PERSPECTIVES

Gastric cancer with peritoneal carcinomatosis is a biologically aggressive tumor, and surgery is still the gold standard of treatment if abdominal spread is limited and PCI is low, ideally < 10. HIPEC may have a potential impact on remaining free cancer cells, although it has not been proven in randomized trials. In metachronous peritoneal carcinomatosis, the surgical approach is often limited by the extensive intraabdominal tumor load and by the aggressive biological behaviour of the tumor itself. NIPS is a promising therapy, and may improve resectability and survival. Intense research is currently being done in experimental peritoneal carcinomatosis, which will eventually modulate current indications.

Concerning promising biomarkers, Phosphoglycerate-kinase 1 (PGK1), an adenosine-triphosphate (ATP)-generating enzyme of the glycolytic pathway, which also affects DNA replication and repair, seems to be an interesting enzyme that is significantly involved in the pathogenesis of gastric cancer and PC^[17-19]. Concerning tumorigenesis, it is assumed that genes involved in the glycolytic pathway are responsible for providing solid tumor cells with ATP. A newly discovered link between metabolic changes, including PGK1, and differentiation, has intriguing connections to an old hypothesis advocated by Otto Warburg for tumor metabolism. Further, recent *in vitro* and *in vivo* studies showed that PGK1 overexpression is associated with an elevated tumor invasion and metastatic rate in gastric cancer^[17-19]. Those results demonstrate that PGK1 might be a crucial enzyme enabling cancer cells to metastasize, and, therefore, may serve as a target molecule for therapy in gastric cancer in the near future.

CONCLUSION

Nowadays, a radical combined treatment should be considered for a motivated patient with good performance status and low-grade peritoneal carcinomatosis. In addition, the patient should be sent to a peritoneal surface malignancy center.

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Anastasios Koulaouzidis, MD, MRCP, Series Editor

Distinct colonoscopy findings of microscopic colitis: Not so microscopic after all?

Anastasios Koulaouzidis, Athar A Saeed

Anastasios Koulaouzidis, Endoscopy Unit, Centre for Liver and Digestive Disorders, The Royal Infirmary of Edinburgh, EH164SA Edinburgh, Scotland, United Kingdom

Athar A Saeed, Gastroenterology Department, Queen Elizabeth Hospital, NE96SX Gateshead, England, United Kingdom

Author contributions: Koulaouzidis A and Saeed AA contributed equally to this work; Koulaouzidis A designed the paper and performed the research; Koulaouzidis A and Saeed AA analyzed the data; and Koulaouzidis A and Saeed AA wrote the paper.

Correspondence to: Anastasios Koulaouzidis, MD, MRCP, FEBG, Endoscopy Unit, Centre for Liver and Digestive Disorders, The Royal Infirmary of Edinburgh, 51 Little France Crescent, Edinburgh, EH164SA, Scotland,

United Kingdom. akoulaouzidis@hotmail.com

Telephone: +44-131-2421126 Fax: +44-131-2421618

Received: February 3, 2011 Revised: May 20, 2011

Accepted: May 27, 2011

Published online: October 7, 2011

mucosal defects with the use of lansoprazole seems to exist. Adoption of the proposed lesion description herein is recommended in order to improve homogeneity of future reports.

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Key words: Collagenous colitis; Microscopic colitis; Endoscopy; Mucosa; Lesion

Peer reviewer: Dr. Stefan Riss, Department of Surgery, Medical University of Vienna, Währingergürtel 18-20, 1090 Vienna, Austria

Koulaouzidis A, Saeed AA. Distinct colonoscopy findings of microscopic colitis: Not so microscopic after all? *World J Gastroenterol* 2011; 17(37): 4157-4165 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4157.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4157>

Abstract

Microscopic colitis (MC) is considered an “umbrella term”, comprising two subtypes, i.e., collagenous colitis (CC) and lymphocytic colitis (LC). They are classically associated with normal or unremarkable colonoscopy. In the last few years, reports have been published revealing findings that are thought to be characteristic or pathognomonic of MC, especially CC. A systematic electronic and manual search of PubMed and EMBASE (to December 2010), for publications on distinct endoscopic findings in MC, resulted in 42 relevant reports for inclusion in this review. Eighty eight patients with collagenous colitis were presented. Only one publication describing a distinct endoscopic pattern in LC was found. Typical findings in CC are alteration of the vascular mucosal pattern, mucosal nodularity, a sequence of change from mucosal defects to mucosal cicatricial lesions, and perhaps (although of doubtful relevance) mucosal pseudomembranes. A causal connection of

INTRODUCTION

Microscopic colitis (MC), regarded as a rare entity in the early 80s (and certainly overlooked), has now emerged as an increasingly common cause of chronic, non-bloody/watery diarrhea^[1].

MC is an “umbrella term”, comprising two entities/subtypes, i.e., collagenous colitis (CC) and lymphocytic colitis (LC)^[2]. The two entities are characterized by a variable, yet apparently benign, clinical course of protracted, non-bloody diarrhea and classically normal or unremarkable colonic mucosa on endoscopy^[3]. In 1984, Gledhill^[4] established that thickening of the colonic acellular basement membrane by > 15 µm is invariably associated with diarrhea.

The histological abnormalities in MC are discontinuous, subtle and often unequally located in the colon, making it necessary to take multiple biopsies from various

colonic regions for identification of the pathognomonic microscopy, i.e., thickened sub-epithelial collagen band and increased intraepithelial lymphocytes^[5] (Figure 1).

However, there are occasions where endoscopy reveals findings that are thought to be characteristic or pathognomonic of MC, and especially CC. Although the estimated prevalence of MC is up to 10% in patients with chronic diarrhea^[5], there are few reports of macroscopic findings in MC. This review attempts to describe the known characteristic endoscopy findings in MC and to categorize them in different types.

PATHOPHYSIOLOGICAL BACKGROUND

CC was first described in 1976, independently in Sweden by Lindström^[6] and in Canada by Freeman^[7], while LC was first described by Lazenby *et al*^[8] in 1989. An increase in their incidence has been recently reported, but this is most likely an artifact secondary to increased awareness and prompt diagnosis^[9]. In the absence of persistent endoscopic findings, diagnosis is based mainly on specific histological criteria^[9].

It is not clear whether CC and LC are separate entities or part of the spectrum of a single disease^[2]. With regard to pathogenesis, several hypotheses have been suggested, including inflammation secondary to medication, smoking, immune dysfunction, autoimmunity, and/or infection.

Studies of collagen typing in patients with CC have produced conflicting results. Electron microscopy findings have suggested that the collagen in CC appears similar to that found in granulation tissue, supporting the hypothesis that its presence would suggest a reparative response to injury^[10]. In fact, it is plausible to assume that overproduced, multiple, and different collagen types may deposit in the sub-epithelial layer of the colon and manifest clinically as CC^[11]. Günther *et al*^[12] showed that increased connective tissue growth factor expression might be the final mediator of local fibrosis in CC.

Non-steroidal anti-inflammatory drugs (NSAIDs) have been implicated as causative factors, through their ability to inhibit prostaglandin synthesis from the colonic mucosa. More recently, several reports have been published incriminating proton pump inhibitors (PPIs), especially lansoprazole, in the induction of CC. Most of the findings to support this came from the temporal relationship of resolution of symptoms with cessation of NSAID or PPI therapy. PPI-induced conformational changes in the cytoskeleton of epithelial cells may result in alterations in the function of the tight junction, leading to increased paracellular permeability. Keszthelyi *et al*^[13] postulated that this could allow the luminal contents to easily penetrate the lamina propria causing an immune and/or inflammatory reaction. On this basis, and in light of some recent reports^[14], which incriminate lansoprazole as the main cause of linear mucosal defects in CC, it may be plausible to suggest that CC is a syndrome with various causes and perhaps graded histopathology.

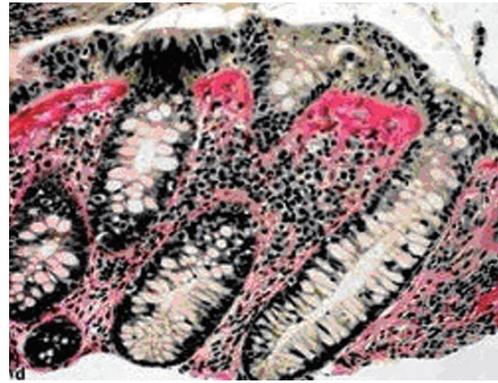


Figure 1 Van Gieson Stain, sub-epithelial collagen table.

The purpose of this review is to present the published experience on distinct endoscopic findings in MC and suggest a unifying lexicon for the reported lesions.

SEARCH STRATEGY

We conducted a PubMed and EMBASE computer search (to December 2010) in order to identify articles on microscopic colitis and endoscopic findings. Our search strategy for PubMed was [“Colitis, Microscopic” (MeSH) or “Colitis, Collagenous” (MeSH) or “Colitis, Lymphocytic” (MeSH)] and [“Endoscopy” (MeSH) or “colonoscopy” (MeSH) or “intestinal mucosa” (MeSH)]. We confined our search to articles in humans but we did not apply any language restriction. In order to search EMBASE we used the following key words: “collagenous colitis”, “microscopic colitis” or “lymphocytic colitis”, “endoscopy” or “colonoscopy”. A further search of electronic journals was undertaken.

Duplicate articles identified in PubMed or EMBASE were manually deleted. The first selection, based on the title and/or abstract was carried out by one of the authors (AK). From the outset, we agreed not to include for further review reports or studies on endoscopic technology, e.g., confocal laser endomicroscopy, which is not yet widely available or restricted to a small number of tertiary institutions. The full paper of each potentially relevant report was then obtained. Thereafter, the two authors independently assessed publications for inclusion in the review. In addition, the reference lists of relevant reports and review papers were cross-searched, in order to identify papers that our initial computer search may have missed.

The following data were extracted from each included publication: year of publication and first author, country of origin, number of cases reported, gender and age of the cases, described endoscopic findings, histopathological diagnosis, post-endoscopy/clinical complications and any important clinical associations (Table 1).

SEARCH FINDINGS

Our initial computational search returned 89 articles in

Table 1 Overview of reports of endoscopic findings/appearance of collagenous colitis

Year	Ref.	No. of cases, gender, age	Endoscopic findings	Lesion location collagen table thickness	Clinical associations	Complications
1990	Giardiello <i>et al</i> ^[15]	1, M, 60	Pseudomembranes	S colon 50-70 μ m	Watery diarrhea, received NSAIDs/antibiotics	None
1993	Richieri <i>et al</i> ^[16]	1, F, 43	Linear mucosal tears/lacerations Absent vascular mucosal pattern	R colon 30-40 μ m	Watery diarrhea, abdominal pain Successful therapy with steroids, some bloody stools	None
1993	Smiley <i>et al</i> ^[17]	1, F, 53	6 mo later; many linear cicatricial lesions Carpet-like patch with nodularity (5 cm)	R colon	Watery diarrhea	None
1995	Katanuma <i>et al</i> ^[18]	1, F, 72	Similar to sessile villous adenoma Diminished vascular pattern	20-40 μ m Pancolonial	Therapy with bulking agents RA on sulindac, diarrhea and wt loss	None
1997	Katsinelos <i>et al</i> ^[19]	1, M, 65	Edematous/red mucosa Multiple red mucosal spots Diminished vascular pattern	n/s R colon n/s	Treated by discontinuation of NSAID Watery diarrhea, Successful therapy with steroids	None
1997	Yabe <i>et al</i> ^[20]	1, F, 47	Multiple red mucosal spots Diminished vascular pattern	Pancolonial n/s	6 F/U colonoscopies showed no improvement	None
1998	Sato <i>et al</i> ^[21]	1, F, 78	Crowded/tortuous vascular pattern I/C spray: coarse/nodular surface	R + T colon n/s	Watery diarrhea	None
1999	Bermejo <i>et al</i> ^[22]	1, F, n/s	Pseudomembranes and aphthae	n/s	Watery diarrhea, received NSAIDs/antibiotics	None
2001	Freeman <i>et al</i> ^[23]	1, F, 37	Deep, elliptical mucosal defect/ulcer	S colon n/s	Watery diarrhea, acute abdomen Diagnostic laparotomy + IV antibiotics	Perforation
2001	Yagi <i>et al</i> ^[24]	1, F, 77	Mucous-covered lesions in R colon Ulcer in descending colon 2nd look: rectal pseudomembranes	R + T colon 30-60 μ m	Watery diarrhea, 4 colonoscopy linear lesions in rectum, ASA-associated	None
2002	Cruz-Correa <i>et al</i> ^[25]	2, F, (73/61) 1, M, 62	Deep lacerations/tears 0.5-4 cm long (to muscularis mucosa)	R and T colon n/s	All had hypothyroidism Therapy with tetracycline/5-ASA	None
2003	Kakar <i>et al</i> ^[26]	8, F, (a. r: 37-91) 1, M, 27	Linear ulcers or lacerations (5) Diminished vascular pattern (2)	R colon (5) S colon (3)	Aspirin and NSAID-associated CC Treated with discontinuation, bismuth	None
2003	Sato <i>et al</i> ^[27]	1, F, 78	Aphthae (2), pseudomembranes (1) 1st colonoscopy: 3 mm nodule 2nd look: crowded/tortuous vascular pattern I/C spray: coarse and nodular, uneven surface	n/s Pancolonial R: 40-70 μ m L: 20 μ m	Mesalamine or azathioprine/6-MP Watery diarrhea, wt loss ASA-associated	None
2003	Byrne <i>et al</i> ^[28]	1, F, 27	Erythematous mucosa Multiple pseudomembranes	S colon n/s	Watery diarrhea, common variable immunodeficiency (CVID)	None
2003	Yuan <i>et al</i> ^[29]	6, F, (a. r: 54-81)	Linear ulcers (1), R colon ulcers (2), inflamed rectum (1)	T colon n/s	Pseudomembranes in CC, only endoscopic cases included	None
2004	Buchman <i>et al</i> ^[30]	1, F, 58 1, F, 46	Hemorrhagic mucosal spots and erythema, granularity/ pseudomembranes	R colon	Prednisolone, antibiotics, TPN, PPI, hypoalbuminemia	None
2004	Sherman <i>et al</i> ^[31]	3, F, (a. r: 66-73) 1, M, 69	Mucosal tears and fractures Granularity of mucosa at places	R + T colon 40-50 μ m	Watery diarrhea, wt loss, hypoalbuminemia	Perforation in 3/4 cases
2006	Wickbom <i>et al</i> ^[32]	3, F, (a. r: 73-86)	Mucosal tears and fractures (4-5 cm long) Mucosal scars on repeat colonoscopy	R + T colon 14-40 μ m	All on aspirin ACE/lansoprazole-induced (1 case)	None
2006	Koulaouzidis <i>et al</i> ^[33]	1, F, 83	Mucosal tears	Cecum n/s	Iron deficiency anemia	None
2007	Poupardin-Moulin <i>et al</i> ^[34]	1, F, 80	Longitudinal mucosal fractures	R + T colon n/s	No significant clinical associations, diagnosis missed	None

Koulaouzidis A *et al.* Distinct colonoscopy findings in microscopic colitis

2007	Smith <i>et al</i> ^[35]	1, F, 43	Long, linear mucosal fractures	R colon n/s	Treated with sulfasalazine	Perforation Hemicolectomy
2007	McDonnell <i>et al</i> ^[36]	3, n/s, n/s	Bright linear marks/parallel corkscrew lesions: "cat scratch" colon	R colon n/s	n/s	none
2008	Allende <i>et al</i> ^[37]	9, F, (a. r: 44-80)	Mucosal fractures to muscularis propria (7)	R colon (6)	2/12 underwent barium enema	Perforation all cases 2 during colonoscopy
		1, M, 71	Ragged mucosal defect (1)	T colon (3)		
2008	Umeno <i>et al</i> ^[38]	7, n/s, (a. r: 37-92)	Wall induration (1), constriction (1) Longitudinal mucosal defects (ulcers/tears)	L colon (1) L colon	Only in the lansoprazole treated group	None
2008	Hashimoto <i>et al</i> ^[39]	1, F, 66	Whirling/circling mucosal vessel network Linear (20 cm) ulcer/scar in the descending	Pancolonic n/s	SLE, treated with mesalamine 2nd look: normal vessels, smaller scar	None
2009	Watanabe <i>et al</i> ^[40]	1, F, 68	Multiple, longitudinal thin ulcers	L colon 30 µm	Lansoprazole, discontinued and healed	None
2009	Yusuke <i>et al</i> ^[41]	1, F, 78	Ragged and linear, long mucosal tear	S colon	Abrupt abdominal pain, PR blood	None
2009	Cuoco <i>et al</i> ^[42]	1, F, 68	Hypertrophic scar Deep linear ulcer-type defects	n/s R + L colon	Lansoprazole, discontinued Watery diarrhea, abdominal pain	None
2009	Dunzendorfer <i>et al</i> ^[43]	1, F, 60	7 cm long in ascending 3 cm hypertrophic mucosal scar	n/s S colon	4 L PEG for cleansing Long history of constipation	None
2009	Chiba <i>et al</i> ^[44]	1, F, 70	Distinct diffuse mucosal cloudiness	Pancolonic	Wt loss, combination therapy On lansoprazole and loxoprofen, treated with sulfasalazine	Reoccurred on a further Lansoprazole course
2009	Sekioka <i>et al</i> ^[45]	1, F, 82	Indistinct vascular pattern (UC-like pattern) 2 longitudinal mucosal fractures	n/s T colon	Lansoprazole-associated (6 mo) Treated by discontinuation	Peritonitis, pre-endoscopy
2010	Couto <i>et al</i> ^[46]	1, F, 48	2nd look: A ridge-type cicatricial lesion Hemorrhagic mucosal tears Longitudinal white ridges/lines	n/s T + L colon n/s	OA on nimesulide and lansoprazole, abdominal pain, wt loss (10%)	None Colonoscopy halted at T colon
2010	Sawada <i>et al</i> ^[47]	1, M, 77	Disappearance of vascular network, Red (numerous) mucosal spots	L colon 25 µm	Lansoprazole-associated (6 years) Wt loss, treated by discontinuation collagen table reduced on 2nd look	None
2010	Koulaouzidis <i>et al</i> ^[48]	1, M, 83	Fine cicatricial line	L colon n/s	n/s	None
2010	van Velden <i>et al</i> ^[49]	1, F, 45 1, F, 63	Hypertrophic mucosal scar Linear tears Diminished vascular pattern and edema 2nd look colonoscopy: multiple linear scars	R + S colon 20 µm	Instrumentation-induced and insufflation-induced mucosal tears	Perforation Treated conservatively
2010	Nomura <i>et al</i> ^[50]	1, F, 67	Linear mucosal defect x 2, Linear scar in sigmoid, I/C spray	L colon n/s	Lansoprazole-associated Improved on discontinuation	None Painful left abdomen
2010	Miyagawa <i>et al</i> ^[51]	1, M, 81	Longitudinal mucosal defect	L colon n/s	Lansoprazole and hemodialysis	None
2010	Milestone <i>et al</i> ^[52]	3, F; 1, M (a. r: 57-75)	Long (5-20 cm) linear ulcers, non-hemorrhagic with evidence of healing	S colon n/s	Treated with budesonide and/or bismuth subsalicylate	None
2010	Kawamura <i>et al</i> ^[53]	3, n/s, n/s	Longitudinal mucosal ulcers	L + S colon n/s	Lansoprazole induced	None
2010	Fasoulas <i>et al</i> ^[54]	1, F, 68	"Cat scratch" colon	R colon n/s	n/s	None
2010	Cimmino <i>et al</i> ^[55]	4, F, (a. r: 24-77)	Mosaic pattern (honeycomb image), I/C spray: for delineation of pattern	Rectum+ S colon n/s	Case control study Mosaic pattern had high LR+/spec	None

n/s: Not stated; M: Male; F: Female; a. r: Age range; I/C spray: Indigo carmine spray; R colon: Right colon; T colon: Transverse colon; L colon: Left colon; S colon: Sigmoid colon; LR: Likelihood ratio; spec: Specificity; UC: Ulcerative colitis; TPN: Total parenteral nutrition; OA: Osteoarthritis; ASA: Acetyl salicylic acid; wt: Weight; PPI: Proton pump inhibitor; 6-MP: 6-mercaptopurine; PEG: Polyethylene glycol; ACE: Angiotensin converting enzyme; NSAIDs: Nonsteroidal antiinflammatory drugs; CC: Collagenous colitis; SLE: Systemic lupus erythematosus; RA: Rheumatoid arthritis.

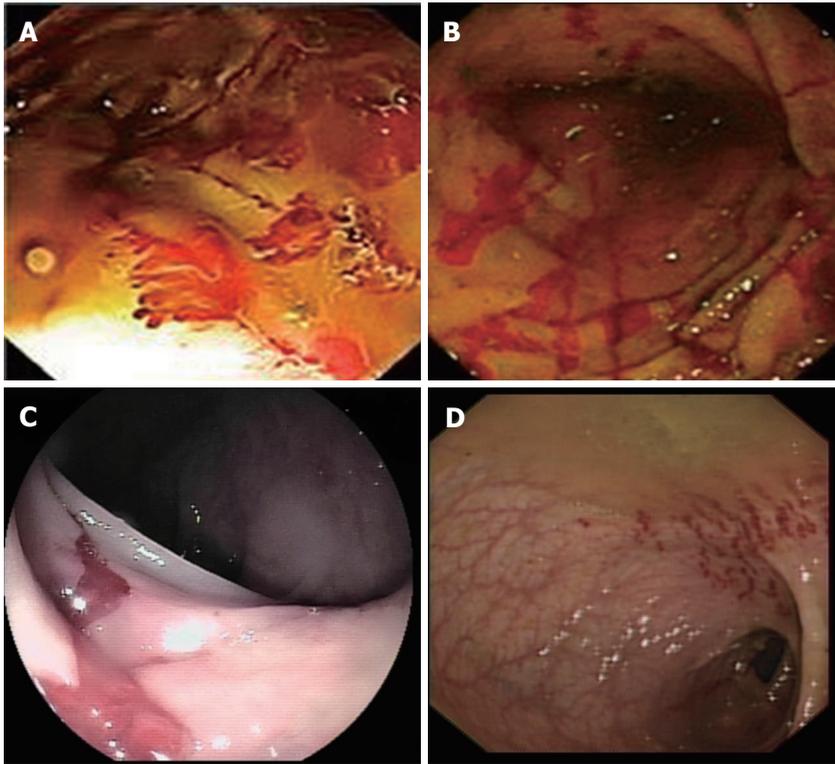


Figure 2 Colon lacerations/mucosal breaks in collagenous colitis. A, B and C: Colon lacerations/mucosal breaks; D: Cat-scratch colon.

PubMed and 499 in EMBASE. Nineteen and 50 articles, from PubMed and EMBASE respectively, were included for further review. After obtaining the full papers, 35 papers were selected. Another seven publications were identified from references lists and included in the final analysis.

The terms mucosal break, defect, tear, fracture or laceration were used indiscriminately. For the purpose of this review and in order to standardize the terminology, we agreed to use the term “mucosal defect” as a collective one, under which there are two subtypes of lesions: (1) mucosal lacerations/tears which are the longitudinal (superficial or deep) and mainly fresh/hemorrhagic in appearance mucosal breaks (Figure 2); and (2) mucosal fractures describing the deeper (with occasional exposure of the muscularis mucosa) and white-based or more chronic looking mucosal defects (Figure 3).

Although to an extent arbitrary, we believe that this terminology will aid the introduction of a universal lexicon for future reports of similar lesions. It is obvious that in accordance with the above, the “cat scratch colon” belongs to the first category, i.e., mucosal lacerations or tears.

Eighty eight cases [65 females, 10 males, 13 not stated (n/s); median age: 67 years] were reported in 41 publications. Of these, 14 publications were from Japan^[18,20,24,27,37-41,44,45,47,50,51], 12 from the United States^[15,17,25,26,28-31,35-37,43], three from the United Kingdom^[33,48,52], two each from France^[16,34], Sweden^[21,32], and Greece^[19,54], and one each from Argentina^[55], Canada^[23], Italy^[42], the Netherlands^[49], Portugal^[46], and Spain^[22]. Where reported, the

submucosal collagen table thickness ranged from 14–70 μm . The only publication reporting endoscopic findings in LC described the presence of a subtle mucosal change in an 85-year-old female^[56].

Gardiello *et al*^[15] were the first to report distinct endoscopic findings in CC (i.e., pseudomembranes), but in fact it was Richieri *et al*^[16] who first described the presence of multiple linear mucosal lacerations with sharp edges in the right colon of a 43-year-old female, with sub-epithelial collagen table thickness of 30–40 μm . Eventually, on repeat colonoscopy 6 mo later the lesions had healed, resulting in fine cicatricial lines on an otherwise unremarkable colonic mucosa. Therefore, Richieri *et al*^[16] had effectively pointed to a pattern seen in some of the reports that followed, i.e., the continuum of laceration to cicatricial healing of the mucosa.

Since this report, 53 cases (34 females/6 males/13 n/s; median age: 69 years) of linear, long or shorter and finer (cat-scratch type) mucosal tears, fractures and ulcers have been reported^[25,26,29,31-42,45,46,49-54]. Sixteen patients with mucosal defects were on lansoprazole, and in the majority, discontinuation of the medication resulted in symptomatic, endoscopic and histopathological improvement.

On the other hand, only 11 (10 females/1 male) cases of mucosal cicatricial lesions have been reported to date, identified either during the index colonoscopy that revealed the mucosal defects, or at follow-up colonoscopic examinations^[16,32,38,41,43,45,46,48-50]. The lesions ranged from hypertrophic (celoid-type mucosal scars)^[32,38,41,43,45,46,48-50] to fine, cicatricial lines^[16,48] (Figure 4).

We did not manage to establish an association of any

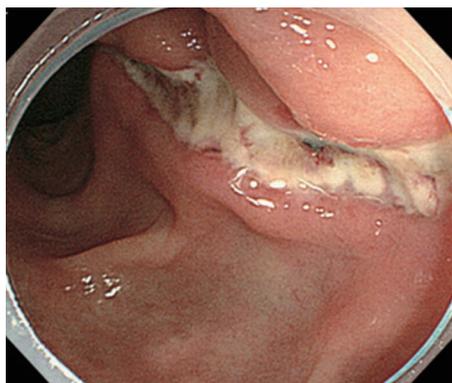


Figure 3 Colon mucosal fractures in collagenous colitis.

of these lesions either with the collagen table thickness or with symptom severity in the review cohort.

The right colon (for the purpose of this review defined as the area from the cecum to the hepatic flexure), irrespective of the type of findings, was affected in 32 cases, the transverse colon in 16 and the left (descending, sigmoid and rectum) colon in 32. Five reports presented cases with pancolonic mucosal involvement^[18,20,27,39,44].

Although the sign of a mosaic pattern or mucosa nodularity (“honeycomb mucosa”) was noted first by Smiley *et al*^[17] in 1993 in the ascending colon of a 53-year-old woman, a retrospective case-control study was only published in 2010^[55]. In the appropriate clinical context of watery diarrhea, the “honeycomb pattern” had an odds ratio of 19.4 with a specificity of > 99% for diagnosis of CC. The authors though pointed out that, due to both the retrospective nature of the study and the high possibility of under-reporting, this may be an over-estimation.

Dye spray (indigocarmine), for improved delineation of the identified lesions, was utilized in four reports^[21,27,50,55], and seems helpful in the context of subtle mucosal changes and/or disturbed vascular architecture. However, this should be balanced against the greater resource implications and procedure time.

With regard to complications, there were 17 recorded perforations/peritonitis in the review cohort^[23,31,35,37,45,49]. As expected, these were all associated with cases where mucosal defects (tears or fractures) were evident on colonoscopy^[52,57].

WHAT IS CURRENTLY KNOWN

We found four broad categories of distinct endoscopic findings in CC: (1) pseudomembranes^[15,22,24,26,28,30], (2) mucosal vascular pattern alteration which includes an indistinct appearance of the blood vessels and a variable degree of pruning of the mucosal vasculature, or a crowded, dilated and tortuous capillary network^[16,18-21,26,27,39,44,47]; (3) mucosal abnormalities such as red spots and some mucosal nodularity or textural alteration, evident with or without chromoendoscopy^[17,19-21,27,30,31,55]; and (4) a continuum of mucosal breaks/defects, i.e., mucosal lacera-

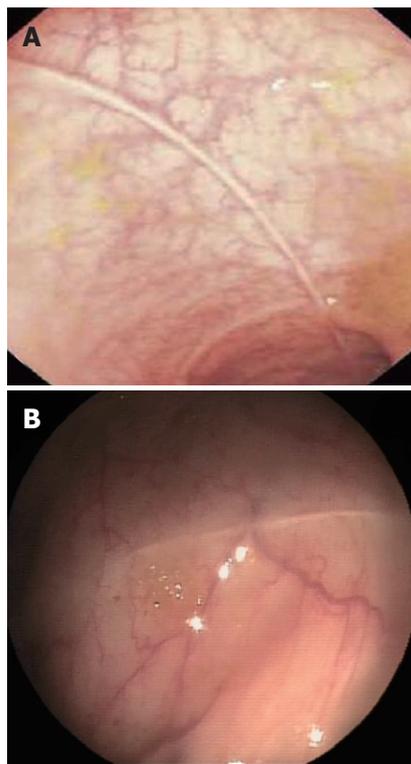


Figure 4 Cicatricial mucosal lesions in collagenous colitis (A and B).

tions/tears, including the so-called “cat scratch colon” pattern, or fractures usually along the long axis of the colonic wall^[16,25,26,29,31-42,45,46,49-54] to the fine linear cicatricial lines or thick scar-like ridges of the mucosal surface (effects of the mucosal healing process of mucosal defects)^[16,32,38,41,43,45,46,48-50]. There was only one publication describing a characteristic endoscopic pattern in LC^[56].

Hemorrhagic mucosal breaks have an appearance that could be liberally described as “colon craquelé”^[16]. The term mucosal fracture was introduced by Sherman *et al*^[31] in 2004 and it is admittedly a successful descriptive one. Thickened and abnormal sub-epithelial collagen table leads, at some areas, to loss of attachment with the epithelial component, and this in turn causes stretching of the mucosa over the deeper wall layers, and eventually tearing of the detached mucosal surface (in a “zip” fashion, hence the longitudinal lesions). The sharply demarcated margin of these mucosal defects, as if the mucosa has been slashed with a sharp knife, helps to differentiate them from ischemic colitis^[50].

Mucosal defects are more likely to be found in the right colon as a result of a colonic insult, i.e., instrumentation or air insufflation due to the abundant presence of a thicker and denser (hence dysfunctional) collagen type III table, in association with increased colon diameter on that side^[25,37,58]. The right colon thinner wall and its expansion to a greater diameter during fecal storage and transit, produce greater relative wall tension (Laplace’s law, i.e., tension on the wall of a cylinder is proportional to the radius). Therefore, a competent ileocecal valve and a deformed sigmoid are sufficient to cause colonic air

entrapment in a closed space^[59], and eventually “cracking” of the brittle colonic mucosa^[11,25,31,32,48,58]. Although the colon can not be seen as a simple cylinder^[37], we suggest that these breaks can occur spontaneously, and postulate that increased intra-colonic pressure during peristalsis and defecation leads to mucosal stretching and defects that will heal with time leaving behind various types of cicatricial lesions^[33,48].

McDonnell *et al*^[36] coined the term “cat scratch colon” to describe the red linear marks in the cecum or ascending colon seen in 21 of 8277 patients undergoing colonoscopy. They reported a 14% prevalence of CC of in their cohort. They also postulated that these marks were due to barotrauma from insufflation^[36,59-61]. However, it is unclear whether biopsies were taken in all patients undergoing the test for diarrhea, other than in those that had the “cat scratch” appearance. Furthermore, endoscopic findings are non-specific for CC and have been described in the normal colon (attributed to barotrauma from excessive insufflation during colonoscopy), in diversion colitis, and even in chronic cholestasis^[54,61,62].

The true prevalence of mucosal tears is unknown due to the rarity of reported cases, but it is estimated to be around 1%. Under the assumption that not all of the relevant cases have been reported, the true prevalence may be much higher. However, based on the type of publications included in this review, i.e., case reports or series, it is not possible to estimate prevalence. In addition, practices vary worldwide and up until recently flexible sigmoidoscopy was considered sufficient to diagnose MC (it is believed that left-sided biopsies probably miss less than 5% of MC cases, due to its patchy nature), and as lesion awareness rises, the incidence of macroscopic findings will increase^[63]. On the other hand, the increased frequency of reports published during the last decade show that there is an increased awareness of the distinct endoscopic appearances in MC, and perhaps endoscopist enthusiasm may result in over-diagnosis (as mucosal tears/scratches have been described in the normal colon, diversion colitis and in lansoprazole colitis^[36,64,65]) of an entity whose main hallmark remains histological confirmation.

It is also now known that mucosal defects in CC represent a marker of increased risk of colonic perforation^[52,54]. A recent review found 21 cases of perforation in CC. The majority of these were either colonoscopy-associated (15 cases) or barium enema-associated (four cases), while the rest seem to have occurred spontaneously^[57].

There are several reports of remission, including disappearance of the collagen layer on follow-up. This would indicate that an environmental factor such as medication may be responsible in susceptible individuals. NSAIDs or PPIs have been implicated. It has also been suggested that collagen plate thickness is greater with lansoprazole^[38]. The pathophysiologic mechanism by which lansoprazole induces microscopic colitis and mucosal defects is not well understood. Although a clear temporal correlation exists, it should be remembered

that, due to the fluctuating nature of CC^[66], it might simply represent a coincidence, as PPIs are one of the most commonly prescribed drug categories worldwide.

It has been postulated that this may be due to higher concentrations of drugs such as NSAIDs in the right colon^[26]. However, it is possible that more right sided biopsies are taken because of endoscopic abnormalities, more likely to be observed in the right colon, as mentioned above. More case control studies and multivariate analysis may provide the answer^[14].

In conclusion, the endoscopic appearances of CC are becoming more familiar amongst the endoscopic community. We recommend adoption of the proposed lesion description herein in order to improve homogeneity of future reports.

ACKNOWLEDGMENTS

We feel indebted to Ms Sarah Douglas, Senior Scientist and Dr. JN Plevris, Consultant and Reader in Gastroenterology, The Royal Infirmary of Edinburgh for their invaluable suggestions in proofreading. We are grateful to Dr. T Sekioka for allowing us to reproduce image (Figure 3) from Ref. 45. Figures 1, 2A and B from Koulaouzidis *et al*^[33], Endoscopy 2006, with permission. Figure 4A and B from Koulaouzidis^[48], GIE 2010, with permission.

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S- Editor Sun H L- Editor Cant MR E- Editor Zhang DN

Optimizing 6-mercaptopurine and azathioprine therapy in the management of inflammatory bowel disease

Kara Bradford, David Q Shih

Kara Bradford, Department of Medicine, Cedars Sinai Medical Center, Los Angeles, CA 90048, United States

David Q Shih, Inflammatory Bowel and Immunobiology Research Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, United States

Author contributions: Bradford K and Shih DQ solely contributed to this paper.

Supported by Grant from Inflammatory Bowel and Immunobiology Research Institute, Cedars-Sinai Medical Center

Correspondence to: David Q Shih, MD, PhD, Inflammatory Bowel and Immunobiology Research Institute, Cedars-Sinai Medical Center, 8700 Beverly Blvd, Suite 4066, Los Angeles, CA 90048, United States. david.shih@csmc.edu

Telephone: +1-310-4237722 Fax: +1-310-4230224

Received: March 22, 2011 Revised: June 20, 2011

Accepted: June 27, 2011

Published online: October 7, 2011

dosing of 6-MP.

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Key words: Azathioprine; Drug levels; Inflammatory bowel disease; 6-Mercaptopurine; Thiopurine

Peer reviewers: Ferenc Sipos, MD, PhD, Cell Analysis Laboratory, 2nd Department of Internal Medicine, Semmelweis University, Szentkirályi u 46, Budapest 1088, Hungary; Wojciech Blonski, MD, PhD, University of Pennsylvania, GI Research-Ground Centrex, 3400 Spruce St, Philadelphia, PA 19104, United States

Bradford K, Shih DQ. Optimizing 6-mercaptopurine and azathioprine therapy in the management of inflammatory bowel disease. *World J Gastroenterol* 2011; 17(37): 4166-4173 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4166.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4166>

Abstract

The thiopurine drugs, 6-mercaptopurine (6-MP) and azathioprine, are efficacious in the arsenal of inflammatory bowel disease (IBD) therapy. Previous reports indicate that 6-thioguanine nucleotide (6-TGN) levels correlate with therapeutic efficacy, whereas high 6-methylmercaptopurine (6-MMP) levels are associated with hepatotoxicity and myelotoxicity. Due to their complex metabolism, there is wide individual variation in patient response therein, both in achieving therapeutic drug levels as well as in developing adverse reactions. Several strategies to optimize 6-TGN while minimizing 6-MMP levels have been adopted to administer the thiopurine class of drugs to patients who otherwise would not tolerate these drugs due to side-effects. In this report, we will review different approaches to administer the thiopurine medications, including the administration of 6-mercaptopurine in those unsuccessfully treated with azathioprine; co-administration of thiopurine with allopurinol; co-administration of thiopurine with anti-tumor necrosis factor α ; 6-TGN administration; desensitization trials; and split

INTRODUCTION

Inflammatory bowel disease (IBD) encompassing Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory disorder caused by dysregulated immune responses in a genetically predisposed individual. Given the role that the immune system plays in IBD, the hallmark of therapy is immune modulation. The thiopurine drugs, 6-mercaptopurine (6-MP) and its prodrug azathioprine (AZA), remain the mainstay of immunomodulator therapy for IBD and are indicated in steroid-dependent and -refractory patients, as prophylaxis in CD^[1-3]. Chebli found that AZA maintained steroid-free clinical remission for three years in UC patients, previously steroid-dependent^[2]. Of note, however, AZA has not been shown to be effective in treating active UC flare^[4]. Rather, thiopurines have also been noted to induce and maintain remission in UC and CD patients, more effectively than 5-aminosalicylic acid^[1,5-10]. However efficacious, their use

is often limited, as an estimated 30% to 50% of patients discontinue these drugs due to either side-effects or lack of clinical efficacy^[11-13]. The lack of response to these immunomodulators has been attributed to differences in individual variations in drug metabolism^[14,15]. The 6-thioguanine nucleotide (6-TGN) metabolite of 6-MP and AZA appears to be the predominant active metabolite responsible for therapeutic efficacy, whereas 6-methylmercaptapurine (6-MMP) levels correlate with the risk of hepatotoxicity and possibly myelotoxicity^[15,16]. Theoretically, if the thiopurine metabolite profile can be shifted to 6-TGN, a greater percentage of IBD patients would benefit from immunomodulator therapy. A meta-analysis has confirmed that higher 6-TGN levels are associated with remission among IBD patients^[17]. In this review, we will discuss the thiopurine metabolic pathway, monitor the drug metabolite levels, and evaluate the different approaches that have been developed to enhance clinical efficacy and minimize the side-effects of AZA and 6-MP.

THIOPURINE METABOLIC PATHWAY

To achieve the active cytotoxic form, AZA is metabolized *via* a series of biochemical pathways summarized in Figure 1. Initially, approximately 90% is non-enzymatically cleaved to 6-MP in the liver^[18,19]. There are three competitive metabolic pathways in 6-MP metabolism. It can be inactivated to 6-thiouric acid (6-TU) *via* xanthine oxidase (XO), activated to 6-MMP *via* thiopurine methyltransferase (TPMT), or to the therapeutic 6-TGN *via* enzymes hypoxanthine phosphoribosyl transferase (HPRT), inosine monophosphate dehydrogenase (IMPDH), and guanosine monophosphate synthetase (GMPS)^[11,20,21]. A complete understanding of its mode of action is unknown^[19]; however, based on its structural similarity to the purine guanine, 6-TGN is a purine antagonist that inserts within the DNA of leukocytes^[22]. Intracellular build up of 6-TGN is thought to be the cytotoxic form that inhibits DNA synthesis and downstream T cell proliferation for its immunosuppressive activity^[20,23,24]. Using a genome-wide expression profiling approach, 6-TGN was found to inhibit several immune and inflammation-related genes including tumor necrosis factor-related apoptosis-inducing ligand, tumor necrosis factor receptor superfamily member 7, and $\alpha 4$ -integrin in activated but not resting T lymphocytes^[25]. Thus, 6-TGN may additionally exert its immunosuppressive effect by down-regulating the expression of pro-inflammatory and gut-homing factors. Another report found that the immunosuppressive role of thiopurine medications may in part be due to its metabolite 6-thioguanine triphosphate (6-TGTP) suppression of the Rac1 protein, which participates in T cell maturation and proliferation, thus inducing T lymphocyte apoptosis^[19,26].

MONITORING THIOPURINE METABOLITE LEVELS

Because AZA is 55% of 6-MP by molecular weight and

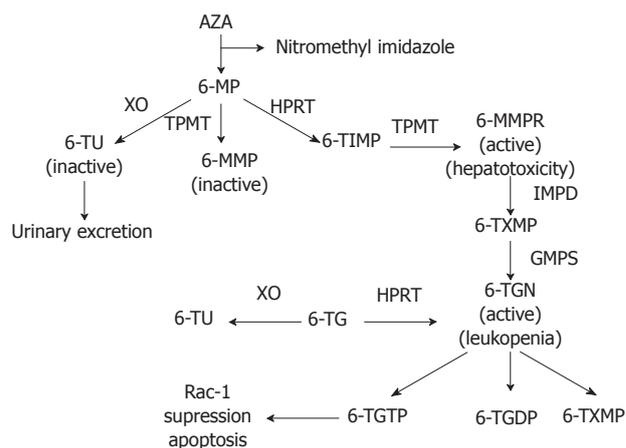


Figure 1 Thiopurine metabolic pathway. Metabolic pathway for AZA and 6MP is shown in the diagram. AZA: Azathioprine; 6-MP: 6-mercaptopurine; 6-TU: Thiouric acid; 6-MMP: 6-methylmercaptapurine; TIMT: Thiopurine methyltransferase; 6-MMPR: Methyl-mercaptapurine ribonucleotide; TXMP: 6-thioxanthosine monophosphate; 6-TGN: Thioguanine nucleotide; 6-TG: Thioguanine; 6-TGDP: 6-thioguanine diphosphate; 6-TGTP: 6-thioguanine triphosphate; XO: Xanthine oxidase; TPMT: Thiopurine methyltransferase; HPRT: Hypoxanthine phosphoribosyl transferase.

88% of AZA is converted to 6-MP, historically, thiopurines are dosed by the patient's weight; the maintenance dose of AZA is 2-2.5 mg/kg per day and 6-MP is dosed at half that of AZA, or 1-1.5 mg/kg per day in IBD patients^[1,22,7-29]. Individual variation in drug metabolism account for the differences in therapeutic efficacy and development of adverse reactions^[30,31]. Fortunately, advances in thiopurine metabolite monitoring can help predict which patients are more at risk of developing side effects, allowing for adjustments in drug dosages^[11,15].

TPMT is a key enzyme whose activity determines the level of 6-MMP as well as 6-TGN metabolite levels^[15,20,24,30]. TPMT methylates 6-MP to 6-MMP and 6-TIMP to 6-methylmercaptapurine ribonucleotide (MMPR) (Figure 1)^[15]. Elevated levels of 6-MMP (> 5700 pmol/ 8×10^8 erythrocytes) are associated with hepatotoxicity, whereas 6-TGN is the metabolite responsible for the therapeutic activity of thiopurines. Monitoring the thiopurine metabolite levels can help to optimize immunomodulator therapy and minimize adverse events. A retrospective study showed that patients who did not respond to AZA or 6-MP either had a high 6-MMP concentration or 6-MMP/6-TGN ratio^[12]. Furthermore, a subset of IBD patients preferentially metabolize thiopurines to the hepatotoxic 6-MMPR which explains why some patients develop toxic metabolite accumulation, side effects and ultimately cannot be maintained on thiopurine therapy^[30]. Even though 6-TGN is associated with therapeutic immunosuppressive activity, an excess amount of this metabolite poses an increased risk for myelosuppression^[32]. The therapeutic efficacy of 6-MP or AZA are correlated with 6-TGN levels between 235-450 pmol/ 8×10^8 erythrocytes^[15,22,33,34]. Because several studies have shown that weight-based dosing is poorly correlated with 6-TGN levels^[16,35,36], monitoring thiopurine metabolite levels can help optimize immunomodulatory therapy while mini-

mizing adverse effects^[15,30,34].

Differences in patient response to thiopurines may in part be due to patient-specific metabolism and genetic variation^[30]. TPMT activity is inversely related to clinical response to AZA^[11]. Different genetic polymorphisms code for the level of TPMT activity^[37]. 0.3% of the Caucasian population are homozygous for low enzyme activity; 11% are heterozygous and 89% are homozygous for high enzyme activity^[38]. Allelic frequency patterns vary among different ethnic groups. In Caucasian populations, intermediate or low TPMT activity is most frequently associated with TPMT*2, TPMT*3A or TPMT*3C alleles^[38], while in African-Americans, TPMT*3C is the most prevalent variant allele^[39]. Wild-type or heterozygous TPMT deficient patients have high TPMT activity > 14 units/mL RBC, which were associated with higher levels of 6-MMP and lower levels of 6-TGN, and thus a decreased likelihood of achieving complete remission (termed 6-MP resistance) and increased risk for hepatotoxicity^[11,13,30]. Dose escalation of thiopurine level may optimize 6-TGN levels, but must be done under caution given that TPMT also catalyzes the formation of the toxic metabolite 6-MMP^[40]. A meta-analysis found that TPMT polymorphisms are related to adverse drug reactions and myelotoxicity, but not hepatotoxicity or pancreatitis^[37]. It is thought that the higher level of TPMT activity may cause higher 6-MP catabolism resulting in higher 6-MMP and decreased 6-TGN levels^[11]. Low TPMT and thus high 6-TGN is associated with a higher risk for leukopenia^[13,15].

Studies have found that checking TPMT activity may be cost-effective as compared to standard therapeutic dose administration^[33,41]. Traditionally, AZA or 6-MP was started at a low dose and progressively titrated up because of safety concerns (bone marrow suppression, hepatotoxicity, etc.). Using this strategy, time to initial response is delayed and can take up to 6 mo to reach therapeutic response^[6,33,42,43]. Compared to traditional thiopurine dosing, monitoring TPMT can allow faster achievement of initial response (22.4 wk *vs* 18.9 wk) and lower costs at 1 year (\$7142 *vs* \$3861)^[33]. Thus, patients found to have normal TPMT could have dose escalation sooner therefore avoiding delay in achieving response^[33]. The cost-effectiveness of measuring TPMT activity was independently shown in a separate study^[41]. Furthermore, awareness of TPMT activity can help to avoid potential deleterious consequences of thiopurine therapy. For example, in patients with low TPMT activity, a lower initiation dose or avoidance of either 6-MP or AZA is recommended due to risks of leukopenia^[16,44]. Albeit, TPMT activity monitoring is not universally available to all practitioners; in these cases, thiopurine may be started at a low-dose (50 mg daily) and titrated up with weekly monitoring of CBC and liver function tests during the first 2 mo, and once every 3 mo thereafter^[20].

APPROACHES TO OPTIMIZING THIOPURINE METABOLITES

Use of 6-MP in patients who are intolerant of AZA

In addition to bone marrow suppression and hepatotoxicity,

early hypersensitivity reactions including fever and gastrointestinal side effects including diarrhea, nausea, and emesis can occur in as many as 10% of patients^[45]. These adverse reactions often cause IBD patients to discontinue thiopurine therapy. Several studies have shown that among patients intolerant of AZA, 6-MP may be a safe and effective alternative^[29,45,46]. One study showed that 20 of 29 (69%) IBD patients with a history of AZA hypersensitivity tolerated 6-MP^[46]. An AZA to 6-MP change appears to be more effective in UC compared to CD patients as by the end of the first year, none of the CD patients were maintained on 6-MP^[46]. In addition to hypersensitivity reactions, up to 60% of IBD patients with AZA intolerance due to nausea, emesis, and flu-like illness tolerated switching to 6-MP^[45]. In contrast, patients who discontinued AZA due to hepatotoxicity or pancreatitis were less likely to tolerate 6-MP^[45]. Another study found that 48% of patients previously intolerant to AZA due to myalgia and arthralgia were able to tolerate 6-MP^[46]. Another report showed that 11 of 15 (11 CD, 4 UC) patients (73.3%) who discontinued AZA due to epigastric pain, nausea and vomiting tolerated 6-MP and reached therapeutic goals^[47]. A retrospective study showed that 19 out of 140 patients discontinued AZA therapy (4 patients for clinical inefficacy, 13 due to side-effects, 2 due to leucopenia)^[48]. Of these 19 patients, 11 (58%) tolerated the switch to 6-MP^[48]. Consistent with the above findings, another report showed that 6 of 11 patients who initially could not tolerate AZA, were able to tolerate 6-MP and achieve response^[18]. The reasons behind the observation that 6-MP bypasses the adverse reactions caused by AZA are unclear, but may in part be due to the nitro-imidazole structure that is released as AZA is cleaved to 6-MP^[49].

Based upon the above studies, we propose that 6-MP should be considered in IBD patients who require continuing immunosuppressive therapy but are intolerant of AZA. We caution that there has been variable success among those who are switched to 6-MP (Table 1), and unfortunately many of the same reactions to AZA develop with 6-MP over time.

Use of AZA in 6-MP intolerant patients

The converse treatment strategy of administering AZA to patients who did not tolerate initial 6-MP therapy has not proved as effective^[48,50,51]. In a trial of AZA after 6-MP adverse reactions, similar side-effect profiles were seen^[18,48,50,51]. This is likely due to the fact that AZA is converted in the liver to 6-MP (Figure 1), thereby, yielding similar adverse reactions (Table 1). Based upon the lack of clinical efficacy, we do not recommend using AZA in patients who were previously intolerant of 6-MP.

Desensitization

Some investigators propose desensitization in the subset of patients who experience hypersensitivity reactions to AZA or 6-MP within the first month of treatment. Korrelitz *et al*^[51,52] retrospectively reviewed 591 charts of IBD

Table 1 Summary of strategies to optimize thiopurine metabolite levels

Method	Effectiveness (%)	Side effect
AZA to 6-MP	48-73 ^[45-48]	Nausea, vomiting, hepatotoxicity, neutropenia, pancreatitis
6-MP to AZA	Not effective ^[48,50,51]	Nausea, vomiting, hepatotoxicity, neutropenia, pancreatitis
Desensitization	25 ^[51]	Hypersensitivity reaction
Combination infliximab/thiopurine	25 ^[72,74]	Lymphoma, infection
6-TG	46-82 ^[50,58]	NRH, veno-occlusive disease and possible tumor
Allopurinol supplementation	25-75 ^[68,69]	Skin rash, renal impairment, leukopenia
Split-dosing	60 ^[75]	Reduces 6-MP, AZA associated adverse effects

AZA: Azathioprine; 6-MP: 6-mercaptopurine; NRH: Nodular regenerative hyperplasia; 6-TG: 6-thioguanine.

patients treated with 6-MP. Four of 16 patients who had early hypersensitivity reactions were successfully desensitized to 6-MP or AZA and achieved long-term clinical remission. One patient tolerated the direct switch from 6-MP to AZA. Of the remaining 11 patients, 5 needed surgery, 2 were changed to methotrexate (MTX), and 4 had chronic symptoms. In this study, desensitization began at one-quarter tablet per day, with an increase in the dose every 3 d for several weeks until a full dose was reached^[51,52]. A similar case report describes a CD patient who developed skin rash after 4 wk of treatment with AZA^[53]. Upon drug withdrawal, the rash resolved. A skin test was positive for AZA allergy, suggesting an IgE mediated hypersensitivity; and the patient was desensitized with AZA, with the patient's CD successfully in remission^[53]. Another case report describes a CD patient who developed a macular, erythematous truncal rash and fever after treatment with 6-MP^[54]. This patient was able to tolerate 6-MP after desensitization^[54]. The process of desensitization for patients with hypersensitivity reactions to AZA or 6-MP may be an empiric strategy for maintenance of immunomodulator therapy. However, we caution that more studies are needed to confirm the efficacy of this strategy.

6-thioguanine

As a possible alternative to those who cannot be maintained on 6-MP or AZA, treatment using 6-TG has been proposed. This drug has been used in children with acute lymphoblastic leukemia^[55]. Compared to AZA, 6-TG is directly converted to 6-TGN by HPRT (Figure 1)^[56,57]. Since 6-TG is a poor substrate for TPMT, hepatotoxic 6-MMP production would be low^[50,55]. Therefore, 6-TG bypasses several of the steps in thiopurine metabolism that are responsible for producing toxic metabolite

build-up and its association with the aforementioned potential adverse effects^[13,50]. One study found that of the 49 CD patients who were either resistant or intolerant to AZA or 6-MP, 46% of patients at 6 mo and 79% of patients at 12 mo were in remission and none of the patients developed pancreatitis or bone marrow toxicity^[58]. Although up to 82% of patients tolerated 6-TG^[50], this drug has been associated with several possible toxicities, notably nodular regenerative hyperplasia (NRH). One study found that the prevalence of NRH to be 16 out of 26 (62%) biopsies taken from patients treated with 6-TG^[59]. Several studies further found that the incidence of NRH associated with 6-TG use varied from 4%-27% among thiopurine naive patients (Table 1)^[60-62]. Other 6-TG associated adverse effects include secondary liver tumors, veno-occlusive disease, and other vascular liver pathologies^[50,58,63-65]. Formal dose-ranging studies for 6-TG are lacking and only limited data are available on the therapeutic efficacy and dosing regimens^[13]. As a general rule, dosage should not exceed 25 mg daily, as higher dosing has been associated with an increased risk of developing NRH^[13,66].

6-TG can be considered a rescue drug in IBD patients intolerant of or refractory to AZA or 6-MP. However, given the potential complications including NRH, and the small number of long-term safety monitoring and limited formal dose-range studies, we do not recommend 6-TG therapy at this time.

Allopurinol supplementation

In some patients, with AZA or 6-MP dose escalation, rather than achieving therapeutic levels of TGN, 6-MMP levels increase and resultant hepatotoxicity ensues^[67]. These patients are known as preferential 6-MMP metabolizers^[50]. Among IBD patients with high TPMT activity who favor 6-MMP production with reduced 6-TGN levels, adding the XO inhibitor allopurinol can favor the production of 6-TGN over 6-MMP^[68,69]. The addition of allopurinol to 6-MP or AZA resulted in improved disease activity as measured by the partial Harvey Bradshaw index in CD and Mayo scores in UC patients; decreased prednisone maintenance dose, and improved liver function laboratory values^[68,69]. However, at the 18 mo follow-up, 25% of these patients were escalated to anti-tumor necrosis factor (TNF) α therapy and 2 required surgery.

The exact mechanism of shifting thiopurine metabolites from 6-MMP to 6-TGN by the XO inhibitor is unknown, but may involve reduced production of the inactive thiouric acid (TU) metabolite in favor of the cytoactive metabolites 6-MMP and 6-TGN^[67,70,71]. However, if XO inhibition favors MMP and TGN production, then the toxic 6-MMP would also be expected to increase (Figure 1). Interestingly, allopurinol does not increase the level of 6-MMP or its associated hepatotoxicity, but mainly shifts the metabolite to 6-TGN.

Potential side effects of allopurinol include skin rash and renal impairment^[68]. In addition, the addition of allopurinol to thiopurine therapy may lead to supra-

therapeutic levels of 6-TGN, leading to leukopenia^[40,68]. Therefore, it is recommended that if allopurinol is used in combination with 6-MP or AZA, the dose of thiopurine medications should be reduced by at least 50% with close laboratory monitoring for leukopenia with weekly CBC monitoring during the first month, followed by every other week for the next month.

Combination therapy: Thiopurine and anti-TNF

Anti-TNF therapies are generally used for patients who are refractory to first-line medications^[72,73]. Colombel *et al.*^[72] conducted a randomized, double blind study of moderate-to-severe CD patients, comparing infliximab and AZA alone *vs* in combination and found that the primary endpoint, steroid-free remission at 26 wk, was achieved in a greater number among those treated in combination *vs* monotherapy. Mucosal healing, a secondary endpoint was also greater among patients who received combination therapy. These patients were immunosuppressant- and biologic therapy-naïve patients. Caution must be used, however, as studies have found an increased risk of non-Hodgkin's lymphoma in patients treated with anti-TNF with a history of thiopurine use^[74].

Split dose administration of thiopurines

As discussed above, patients who are preferential 6-MMP metabolizers exhibit high 6-MMP levels with subtherapeutic 6-TGN levels when thiopurines are dosed in the traditional weight-based, once-a-day fashion. 6-MP/AZA dose escalation in this subset of patients in an attempt to push the 6-TGN level into the "therapeutic range"-often results in dose-dependent leukopenia, transaminitis and/or flu-like symptoms (headache, nausea, myalgia, fatigue, general malaise). Overproduction of 6-MMP and side-effects resolve with dose reduction, but the lower dose often fails to adequately suppress IBD disease activity, resulting in suboptimal symptom control.

Anecdotally, we observed that simply splitting the daily dose of thiopurine (e.g., 50 mg *BID* rather than 100 mg once daily) can reduce the 6-MMP metabolites while maintaining 6-TGN levels. To confirm our observation, we performed a retrospective chart review of patients with baseline 6-MMP levels greater than $7000 \text{ pmol}/8 \times 10^8$ red blood cells (RBC) who underwent split dosing ($n = 20$). Dividing the daily thiopurine dose led to a significant reduction in 6-MMP levels ($11\,879 \text{ vs } 5955 \text{ pmol}/8 \times 10^8$ RBC; $P < 0.0001$) without adversely affecting clinical disease activity (HBI) or 6-TGN levels ($250 \text{ vs } 227 \text{ pmol}/8 \times 10^8$, $P = \text{NS}$)^[75]. Side-effects associated with 6-MMP, such as abnormal liver function test (LFT), leukopenia and flu-like symptoms, improved in seven of eight patients^[75]. After a mean follow-up of 42 mo, 12 of 20 patients were able to be maintained on a split dose of 6-MP with control of their IBD activity^[75]. To our knowledge, this is the first study to demonstrate the effectiveness of dose splitting on preferential metabolism. This approach has several advantages over other strategies. Dose splitting does not sacrifice potential efficacy associated with

dose reduction, and may even allow for further upward titration of thiopurine to efficacy if needed. It avoids the introduction of possible additional medication side effects as can be seen with co-administration of allopurinol and the potential cost burden of designer biologic inventions. This maneuver is relatively simple for both patients and practitioners alike.

Independent studies are needed to confirm that split-dose administration of thiopurine is an effective approach to manage 6-MMP preferential metabolizers. However, in an IBD patient who might otherwise not tolerate immunomodulator therapy and require ongoing steroid exposure and/or escalation of therapy to biologics, splitting the daily dose of 6MP or AZA may be attempted.

POTENTIAL RISK OF LYMPHOPROLIFERATIVE DISEASE

Thiopurines have been linked with chromosomal abnormalities and an increased risk of lymphoma among rheumatoid arthritis patients^[76]. Whether the same risk exists in IBD patients is controversial. A meta-analysis of six studies showed a four-fold increased risk of developing lymphoma among IBD patients treated with immunomodulators^[77]. Similarly, in the CESAME prospective cohort study by Beaugerie, a five-fold increased risk of lymphoproliferative diseases was shown among IBD patients on thiopurines^[78]. Whether this risk stems from underlying disease or iatrogenic medication is unclear. Studies have shown that the risk is greater in IBD patients who are male and between 15 to 40 years old^[79,80]. One study focused on hepatosplenic T-Cell lymphoma and noted cases among men younger than 35 years who had been treated with either anti-TNF and thiopurines or thiopurine monotherapy^[79]. In contrast, a study by Vos *et al.*^[81] found no increased risk of lymphoma among a nationwide study of 17 834 IBD patients. Interestingly, however, they did find an association between Epstein-Barr Virus-positive lymphoma and thiopurine use. EBV-positive lymphoma implies the effect of immunosuppression as a factor^[77,80,81]. Nevertheless, the consensus has been that the benefits of thiopurines outweigh the risk^[77,82]. Lewis *et al.*^[82] conducted a decision analysis and found that a 9.8-fold increase in lymphoma is needed to favor an alternative therapy over AZA. Further studies are needed, as it has yet to be determined how this risk changes with discontinuation of thiopurines^[77].

CONCLUSION

Given the complex metabolism of thiopurines and the individual variability among patients in response to this medication, various dosing strategies have been adopted. Several approaches have been promising among patients who develop toxicities to the initial strategy. However, no single strategy has proven completely effective in all patients. Further studies will inevitably provide more in-

formation to assist in optimizing administration of these vital IBD medications. This is significant given that there are a limited number of medications available in the IBD arsenal. In all dosing strategies, however, close monitoring of metabolites as well as determination of pharmacogenetics are pivotal for patient safety and medication efficacy.

ACKNOWLEDGMENTS

We thank Cindy Ting, PharmD for critical reading of this manuscript.

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S- Editor Tian L L- Editor Webster JR E- Editor Zhang DN

Barrett's esophagus with high-grade dysplasia: Focus on current treatment options

Leonidas Lekakos, Nikolaos P Karidis, Dimitrios Dimitroulis, Christos Tsigris, Gregory Kouraklis, Nikolaos Nikiteas

Leonidas Lekakos, Nikolaos P Karidis, Dimitrios Dimitroulis, Christos Tsigris, Gregory Kouraklis, Nikolaos Nikiteas, Second Propedeutic Department of Surgery, Medical School, University of Athens, General Hospital Laiko, Athens 11527, Greece
Christos Tsigris, First Department of Surgery, Medical School, University of Athens, General Hospital Laiko, Athens 11527, Greece

Author contributions: Lekakos L and Karidis NP organized and prepared the draft of the present review; Karidis NP and Dimitroulis D contributed to reference collection and final preparation of the manuscript; Tsigris C, Kouraklis G and Nikiteas N coordinated and reviewed the manuscript.

Correspondence to: Nikolaos P Karidis, MD, General Surgeon, Second Propedeutic Department of Surgery, University of Athens, General Hospital Laiko, Athens 11527, Greece. npkaridis@gmail.com

Telephone: +30-210-9350100 Fax: +30-210-7791456

Received: December 12, 2010 Revised: April 21, 2011

Accepted: April 28, 2011

Published online: October 7, 2011

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Key words: Barrett's esophagus; High-grade dysplasia; Endoscopic ablation; Endoscopic excision; Surgical treatment

Peer reviewers: Marco Giuseppe Patti, MD, Professor of Surgery, Director, Center for Esophageal Diseases, University of Chicago Pritzker School of Medicine, 5841 S. Maryland Avenue, MC 5095, Room G 201, Chicago, IL 60637, United States; Lesley A Anderson, PhD, MPHe, BSc (Hons), Academic Fellow in Cancer Prevention, Cancer Epidemiology and Prevention Research Group, Centre for Clinical and Population Sciences, Mulhouse Building Grosvenor Road, Belfast, BT12 6BJ, United Kingdom

Lekakos L, Karidis NP, Dimitroulis D, Tsigris C, Kouraklis G, Nikiteas N. Barrett's esophagus with high-grade dysplasia: Focus on current treatment options. *World J Gastroenterol* 2011; 17(37): 4174-4183 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4174.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4174>

Abstract

High-grade dysplasia (HGD) in Barrett's esophagus (BE) is the critical step before invasive esophageal adenocarcinoma. Although its natural history remains unclear, an aggressive therapeutic approach is usually indicated. Esophagectomy represents the only treatment able to reliably eradicate the neoplastic epithelium. In healthy patients with reasonable life expectancy, vagal-sparing esophagectomy, with associated low mortality and low early and late postoperative morbidity, is considered the treatment of choice for BE with HGD. Patients unfit for surgery should be managed in a less aggressive manner, using endoscopic ablation or endoscopic mucosal resection of the entire BE segment, followed by lifelong surveillance. Patients eligible for surgery who present with a long BE segment, multifocal dysplastic lesions, severe reflux symptoms, a large fixed hiatal hernia or dysphagia comprise a challenging group with regard to the appropriate treatment, either surgical or endoscopic.

INTRODUCTION

In the era of minimally invasive therapies, numerous treatment options for Barrett's esophagus (BE) with high-grade dysplasia (HGD) are available. Nevertheless, since therapy is individualized, the standard of care remains debatable for a large number of patients without clear-cut guidelines. The aim of this review is to briefly present and compare current therapeutic modalities with an emphasis on endoscopic approach, outline factors that can aid in the choice of the appropriate treatment (medical, endoscopic or surgical) and underline the lack of a properly designed study so far that compares the outcomes of these therapies.

BE is the result of chronic gastroesophageal reflux disease (GERD) and represents the end stage of the natural course of this disease. It has been estimated that 20% of the population in the United States suffers from gastroesophageal reflux^[1] and that about 10% of

these patients are diagnosed with BE^[2]. Commonly, BE is discovered during endoscopy for the evaluation of GERD symptoms. The severity of GERD symptoms is not considered an indicator of BE presence, whereas the chronicity of GERD symptoms may be related to the possibility of BE transformation^[3]. It is documented that longstanding exposure of esophageal mucosa to gastric acidity results in cellular damage of the stratified squamous epithelium and creates an abnormal environment, which stimulates repair in the form of intestinal epithelial metaplasia^[4,5]. Moreover, BE is related to a serious mechanical insufficiency of the lower esophageal sphincter, a functional derangement of the esophageal body, as well as an insufficient esophageal clearance^[6-8].

In BE it is possible to encounter three histologic types of columnar epithelium: (1) the specialized intestinal metaplasia type, in which the epithelium exhibits a villous surface and intestinal-type crypts lined by columnar cells that secrete mucous and goblet cells containing mucin; (2) the gastric fundus epithelial type; and (3) the junctional type. Among these three histological types, only the intestinal type represents an important premalignant state.

In BE, the stratified squamous epithelium, which physiologically lines the esophageal mucosa, is replaced by a pathological, specialized columnar epithelium which is neither of cardiac nor of stomach type, but exhibits features of the intestinal type of epithelium^[4]. This pathological type of epithelium usually demonstrates DNA alterations that predispose to malignancy^[2,9,10]. The alterations in BE are histologically classified into three categories, depending on whether or not they exhibit dysplasia: (1) BE without dysplasia; (2) BE with low-grade dysplasia; and (3) BE with HGD^[11-13]. In BE with HGD, dysplasia is confined to the mucosa without crossing the basement membrane. If dysplasia extends beyond the basement membrane into the lamina propria through the in-coming lymphatic network, it is defined as intramucosal (superficial) adenocarcinoma, whereas if it invades the muscularis mucosa layer it becomes invasive adenocarcinoma. Thus, **BE with HGD is considered a precursor of invasive adenocarcinoma**. Six to twenty percent of patients with BE and HGD are at greatest risk of developing adenocarcinoma within a short period of time, ranging from 17 to 35 mo at follow-up^[14]. Esophagectomy specimens from patients with BE and HGD revealed invasive adenocarcinoma in 30%-40% of cases^[15]. A recent meta-analysis demonstrated that patients with BE and HGD developed esophageal adenocarcinoma with an average incidence of 6 every 100 patients per year, during the first 1.5 to 7 years of endoscopic surveillance^[16]. Furthermore, the majority of esophageal adenocarcinoma is thought to have evolved from cells that have undergone Barrett's metaplasia^[17].

BE is also classified into two categories according to the extent of intestinal metaplasia above the gastroesophageal junction: (1) **long segment BE, if the extent of the intestinal epithelium is greater than 3 cm**; and (2)

short segment BE, if it is less than 3 cm^[18]. Among patients who undergo endoscopy for symptoms of GERD, the incidence of long segment BE is 3%-5%, whereas short segment BE occurs in 10%-15%^[4]. Whether long and short segment BE share the same pathogenetic alterations or the same predisposition to malignancy still remains unclear; however, both conditions are currently treated in the same manner^[19].

ENDOSCOPIC SURVEILLANCE IN PATIENTS WITH BE

Concerning the therapeutic management of BE, endoscopic follow-up of the patient at regular intervals, so-called endoscopic surveillance, plays a pivotal role. There is great difference of opinion when dealing with the problems of therapeutic management of BE. The value of endoscopic surveillance in patients with BE represents one of the many controversies that exist amongst gastroenterologists.

As aforementioned, BE represents a strong risk factor for developing adenocarcinoma, which is a particularly lethal malignancy^[19]. In order to diminish the risk of cancer development, the American College of Gastroenterology introduced the application of a surveillance protocol that is, in summary, as follows^[20]: (1) **patients who are diagnosed with BE at two consecutive endoscopies should undergo endoscopy every 3 years**; (2) **if Barrett's dysplasia is diagnosed, it should be confirmed by a second specialized pathologist**; (3) **patients who are definitely diagnosed with low-grade dysplasia after examination of sufficient biopsy specimen should undergo endoscopic surveillance every year**; (4) **patients diagnosed with HGD should undergo a new endoscopy with a second adequate biopsy specimen, to check the possible presence of invasive cancer**; (5) **if the results are positive, the biopsy specimen should be examined by a second specialized pathologist**; and (6) **if multiple HGD foci are confirmed, then the patient should undergo either surgical therapy (esophagectomy) or endoscopic surveillance every 3 mo**.

This protocol, concerning high-risk patients, is considered strict in various aspects. Many authors believe that surveillance is not justified in a cost-effectiveness analysis^[21-23]. Others compare endoscopic surveillance of patients with BE with the endoscopic follow-up of patients with ulcerative colitis for early detection of malignancy or mammography for early diagnosis of breast cancer and conclude that the former is lacking, in terms of cost-effectiveness, compared to the other two^[24,25].

Furthermore, many studies have shown that the survival of patients with BE is not different from that of the general population^[26]. This observation, as paradoxical as it may appear, can be explained by the low absolute number of adenocarcinoma cases in patients with BE^[19]. Current data demonstrate that patients with BE develop adenocarcinoma at a low rate of 0.5% which is, nevertheless, 30-40 times higher than that of the general population^[27,28]. The subgroup of patients with BE and HGD

develop esophageal adenocarcinoma at a higher rate of 6.58 per 100 patient-years, as shown in a recent meta-analysis^[16]. Moreover, survival studies in patients with BE primarily include elderly individuals, for whom the risk of death from other lethal co-morbid conditions is much higher than the annual 0.5% risk of death from esophageal adenocarcinoma^[29]. Apart from the above, a long-term prospective study involving young patients with BE demonstrating a decreased life span in these patients has not yet been published^[19].

Concerning the endoscopic surveillance of patients with BE, this is not a risk-free procedure. It is estimated that the risk of development of adenocarcinoma is one in every 200 or 300 patients with BE, whereas the risk of a major complication from an endoscopic procedure is one in 1000 esophagogastrosopies^[28-30]. The program of the American College of Gastroenterology also differs from that of the British Society of Gastroenterology^[31] and the NHS Technology Review^[32] in the value of endoscopic surveillance as a screening tool. Despite the previously mentioned contradictory views, many authors indicate a benefit of endoscopic surveillance in cost-effectiveness analyses for the early diagnosis of cancer^[33-36].

CURRENT MANAGEMENT OF BE WITH HGD

Controversy is also perpetuating between surgeons and gastroenterologists. BE with HGD carries a high risk of developing esophageal adenocarcinoma, at a rate of 6%-20%, within a short period of time (17-35 mo)^[14,16]. Therefore, in the presence of such risk, the traditional standard therapy was en bloc esophagectomy with regional lymph node dissection. This approach has been supported by the fact that invasive adenocarcinoma was previously diagnosed in patients with HGD at a rate of 30%-40%^[15], although more recent data have revealed a significantly lower incidence (12%)^[27]. Nevertheless, en bloc esophagectomy carries a high mortality (4%-19%)^[37], high postoperative morbidity (20%-47%)^[38] and unacceptable late postoperative quality of life^[39].

During the last few years, while surgeons try to improve their surgical technique and the results of esophageal resection (esophagectomy without lymph node dissection and/or without thoracotomy, esophagectomy with vagal preservation or laparoscopic esophagectomy), endoscopists have been developing minimally invasive therapeutic methods for the management of BE with HGD. It should be noted that the problem of GERD persists with these endoscopic methods and endoscopic surveillance is necessary for all endoscopic treatment options.

Management of gastroesophageal reflux disease

The therapeutic goal in patients with BE is similar to that of patients with GERD, i.e., relief of symptoms and reversal of the epithelial damage caused by increasing gastric reflux. In cases of BE with HGD, the question is whether medical or surgical management of GERD can

have beneficial effects on the dysplastic lesions. Therefore, the following questions come into play:

Can either surgical or medical antireflux therapy achieve regression of the epithelium in BE?

Evidence clearly indicates that medical therapy of GERD does not lead to acceptable results, with regard to the regression of dysplastic epithelial lesions^[40]. Surgical therapy may have better results than conservative therapy in terms of regression, but is far from being considered adequate. In a series of five publications that included 151 patients submitted to surgical management of gastroesophageal reflux (fundoplication), surgical therapy achieved full regression of these lesions in 6 patients only, whereas in 31 patients only a decrease in the length of BE lesions was observed and 6 patients developed invasive adenocarcinoma. Furthermore, other published data corroborate that antireflux surgery does not decrease the rate of adenocarcinoma in patients with gastroesophageal reflux^[41-43]. Data from the subgroup of patients with BE are also conflicting and pose an unsettled issue^[44].

Does antireflux surgery prevent the metaplastic evolution of the mucosa in BE?

Evidence suggests that surgery is superior to conservative therapy as it can abolish, at high rates, the progression of metaplastic mucosal lesions in BE^[45,46] and therefore protect from dysplasia and malignancy. On the other hand, systematic review indicates that antireflux surgery in patients with BE is associated with regression of BE lesions and/or dysplasia, but evidence supporting the assertion that surgery decreases the rate of adenocarcinoma comes from non-controlled studies^[47]. In a study from the Mayo Clinic in 118 patients who underwent antireflux surgery and follow-up for 18.5 years, it was stated that only 3 patients developed adenocarcinoma within the first three years postoperatively^[48]. This outcome suggested that the lesion probably existed during the operation^[49,50]. Encouraging data come from patients with low-grade dysplastic mucosa and antireflux surgery who, at endoscopic surveillance, showed conversion from a dysplastic to a non-dysplastic mucosa at a rate of about 70%^[51]. Concerning the endoscopic antireflux interventions (Stretta procedure, Bard EndoCinch, Wilson-Cook Endoscopic Suturing Device, NDO Plicator, Enteryx, Gatekeeper Reflux Repair System and Plexiglas), these are currently under evaluation and evidence is lacking to support their role in the therapy of BE with HGD^[52].

Should antireflux therapy accompany other treatment modalities when confronting metaplasia or dysplasia of BE epithelium?

The combination of medical or surgical antireflux therapy with endoscopic mucosal ablation has yielded promising results^[53,54]. These early observations concluded that the resected mucosa undergoes re-epithelialization by normal squamous epithelium and is preserved with the aid of antireflux therapy; usually proton pump inhibitors. Further research is nevertheless needed in this field.

Endoscopic treatment of BE with HGD

It is documented that BE with HGD or intramucosal adenocarcinoma constitute diseases amenable to cure in most cases. Data from high volume centers of esophageal surgery have indicated rare lymph node metastasis, ranging in incidence between 2%-6%^[36,49,55,56]. Newer, less invasive treatment modalities such as endoscopic therapies or less aggressive surgical operations are currently being evaluated in an effort to achieve the least postoperative morbidity and the best quality of life.

Current endoscopic methods include two major therapeutic categories: (1) endoscopic ablation of Barrett's mucosa that can be achieved by thermal, photodynamic and/or radiofrequency energy; and (2) endoscopic mucosal resection.

Thermal therapy

In methods implementing thermal energy, the endoscopic elimination or destruction of the diseased superficial esophageal mucosa is achieved by the administration of heat with one of the following specialized devices: (1) electrocoagulation; (2) argon plasma coagulation (APC); (3) heat probe; and (4) Nd: neodymium-doped yttrium aluminium garnet laser. Another version of thermal therapy consists of cryospray ablation, but experience with this method is limited^[57].

The more widely used first two methods of thermal therapy, probably due to greater availability in endoscopy units, provoke a superficial mucosal injury with a low rate of serious complications. APC has been evaluated at twelve independent centers in 444 patients with BE, making this technique by far the most commonly applied method^[55]. However, the significant variation in the regression of intestinal metaplasia and the formation of new squamous epithelium, together with the complications of this method, resulted in dismissal of APC as the method of choice^[55]. In published series, full regression of BE has ranged from 36%^[58] up to 98%^[59] in an average time frame of 36 and 12 mo, respectively.

Two studies have focused on the effect of APC on intestinal metaplasia in association with the amount of administered energy. In one, no recurrence of BE was noted, while in the other, recurrent disease occurred in 30% of cases. It is noted that in the patients of the first study ($n = 70$) a higher energy device (90 W) was utilized and higher doses of omeprazole (40 mg three times a day) were administered. In 69 patients (98.6%) complete BE eradication with associated squamous regeneration was achieved after a median of two APC sessions (range 1-5). During a median follow-up of 12 mo (range 2-51 mo) with continuous acid suppression, no case of dysplasia relapse was noted. Of these patients, only 3 developed stenosis (4.3%), for whom dilatation was advocated for therapy^[59]. In the second study, where low energy was administered in 27 patients, 70% showed regeneration of squamous epithelium with no persistent intestinal metaplasia and in 30%, areas of intestinal metaplasia were present under the new squamous epithelium, after a

median follow-up of 9 mo (range 6-18 mo). Overall, two cases of perforation were reported, one of which was fatal^[60]. In a third study of 33 patients treated with APC energy between 65 W and 70 W and 60 mg omeprazole daily, complete restoration of the normal squamous epithelium was noted in all cases after 1.96 sessions (range 1-4). Esophageal stenosis occurred in 3 patients, for whom dilatation was deemed necessary, 5 patients developed mediastinal syndrome (high fever and pleural effusion) and one patient pneumomediastinum. After a follow-up period of 10.6 mo, only one recurrence of BE was observed^[61]. Accordingly, the amount of energy administered with APC seems to be directly related to the recurrence rate of BE, favoring the use of high energy devices for a median follow-up of 9 to 12 mo, although data for long-term effectiveness are still lacking. It should be stated that the emergence of APC-related complications depends not only on the amount of energy, but also on other parameters such as mucosal contact at different pressures and repetitive therapy in the same area^[55].

Photodynamic therapy

Photodynamic therapy requires previous administration of a photosensitizer and selection of a specific wavelength of light that stimulates a specific target area or the whole of BE. As a result, singlet oxygen is formed that causes damage to the esophageal mucosa. 5-aminolevulinic acid (5-ALA) is an oral photosensitizing agent that incites severe superficial injury in the patients with HGD and superficial cancer. In the United States, intravenous porfimer sodium, which causes deeper injury, is used. Overholt *et al.*^[62] applied a technique of introducing a cylindrical inflatable balloon through which light was administered in 101 patients with HGD. After a follow-up of at least 4 years, the analysis of the therapeutic effect showed that in 54% of cases there were no residual BE lesions. Successful eradication of low- or high-grade dysplasia or cancer reached 93%, 78% and 48%, respectively. It is thus suggested that HGD and cancer exhibit the greatest resistance to therapy. The total rate of stenosis reached 30%, reflecting the effect of this therapy in deeper esophageal layers.

Great value to this type of therapy is attributed by a large multicentric, semi-blinded, randomized study by Overholt *et al.*^[62] in 208 patients with HGD. Patients were randomly divided, in a 2:1 ratio, into a study group treated with photodynamic therapy and omeprazole and a control group receiving only omeprazole. A statistically significant difference ($P < 0.0001$) regarding the complete eradication of HGD was noted in favor of photodynamic therapy (106/158, 77%), compared to the control group (27/70, 39%). The therapeutic response persisted even after 5 years of follow-up. It should be noted that endoscopic ablation was combined with a long-term follow-up and was, thus, more costly. Nevertheless, this approach has proved to be a better treatment option in terms of cost-effectiveness, compared to the standard follow-up and radical surgery for the treatment

of dysplasia, although clinical trials directly comparing these strategies are warranted^[63]. Additionally, esophagectomy provided 11.82 quality adjusted life years (QUALYs) compared to photodynamic therapy with 12.31 QUALYs and long-term follow-up^[63]. Furthermore, anecdotal time-life analysis of several cases has revealed that many patients with HGD and even early cancer could be controlled with ablative techniques and careful follow-up for 5-10 years^[39].

Radiofrequency energy ablation

This method is a novel therapeutic approach employing (1) energy emitted from a controlled radiofrequency (RF) source [Halo360 or Halo90 RFA (where A stands for ablation), BARRX Medical Inc, Sunnydale, CA]; (2) a sizer balloon catheter, that is introduced into the esophagus and measures esophageal width; and (3) an EFA balloon catheter. The controller of the RFA source is preset to deliver energy of 12 J/cm² which causes complete destruction beyond the lamina propria^[64]. The RFA balloon is 3 cm long and consists of 60 narrowly spaced electrode rings in a bipolar fashion. After the esophageal diameter is measured by the sizer balloon, the RFA balloon catheter is introduced in the esophagus and placed in its position. The balloon is then inflated and the RFA source releases energy circumferentially on the esophageal surface for 300 ms. The whole procedure is performed under general anesthesia^[65].

The use of radiofrequency for the ablation of the dysplastic epithelium in BE is more effective, posing less risk for damage beyond the desired limits, while also controlling the depth of the damage^[66]. In contrast to photodynamic therapy, radiofrequency mucosal ablation is not associated either with the development of esophageal strictures or with recurrent disease resulting from buried Barrett's glands. According to current opinion, the development of strictures after photodynamic or thermal therapy has been attributed to the circumferential destruction of the mucosa. Despite the fact that during RFA therapy destruction is also circumferential, no strictures are observed, as a result of better control of the depth of ablation attained by this method^[65,66]. In order to safely evaluate this method and its long-term effects, studies with larger series, longer duration of follow-up and endoscopic surveillance are expected, so as to document the recurrent dysplasia-free interval.

Recently, endoscopic radiofrequency ablation was evaluated in a study as the definitive treatment of 25 patients with ultralong-segment (≥ 8 cm) BE, using balloon- and/or plate-based devices (BARRX Medical Inc., Sunnydale, CA). Complications for all 25 patients included hemorrhage in one, stricture in two, and nausea and vomiting in two cases. The time from the initial procedure was such that 15 patients underwent at least one post-ablation biopsy. One patient was elected to undergo esophagectomy based on biopsies. Of these patients, 78.5% (11/14) had a complete response. The number of ablations in this group was 2-3 (median 2.5). The authors

concluded that the method is safe and feasible in patients with ultralong-segment BE and can be applied to the entire length of intestinal metaplasia during one session^[67]. Radiofrequency ablation has also been recommended as a single-modality therapy for flat type mucosa, or as a supplementary therapy after endoscopic resection of visible lesions. The treatment protocol consists of initial circumferential ablation, using a balloon-based electrode, followed by focal ablation of residual Barrett's epithelium. The authors believe that radiofrequency is less frequently associated with stenosis and buried glandular mucosa, in contrast to other ablation techniques. This method has been shown to be safe and effective in the treatment of patients with BE and early cancer^[68].

Endoscopic mucosal resection

Endoscopic mucosal resection (EMR) with a curative intent, beyond the scope of mucosal resection for biopsy, is being investigated more than any other endoscopic method for the treatment of HGD in BE. Since the first publication in 2000^[69], several other similar reports have emerged in the United States as well as in Europe^[56,70-76].

The landmark study by Ell *et al*^[69] included 35 low-risk patients with superficial cancer and well or moderately differentiated BE less than 2 cm in diameter who underwent EMR. With an average of 1.3 interventions and an average follow-up time of 1 year, complete regression was observed at a rate of 97% and local recurrence or metachronous cancer at a rate of 17%, with only one case of hemorrhage that was controlled endoscopically. In another study, a group of 70 patients with HGD or early cancer similarly underwent EMR, with an average follow-up interval of 34 mo, and demonstrated regression of lesions in 98% of cases, with a complication rate of 9.5%. Metachronous or recurrent disease occurred in 30% of cases^[77].

EMR has demonstrated satisfactory rates of complete regression; up to 82.5% in 550 patients with HGD or Barrett carcinoma, at an average follow-up interval of 12 mo. The best results were documented in patients with HGD and small (< 20 mm), well or moderately differentiated Barrett carcinomas, at a rate of 97%^[78]. Recently, in a retrospective, single center study from the University of Chicago, 49 patients, 33 with high-grade dysplasia and 16 with early carcinoma, underwent complete Barrett's eradication with the aid of EMR. The rate of stenosis was significant, but it resolved easily with endoscopic dilatation. The authors noticed the presence of Barrett's epithelium underneath the squamous resection margin (Z line) in 13 of 47 patients (28%) at initial mucosectomy. Based on their findings and surveillance biopsies, they concluded that ablative therapy should extend to 1 cm proximal to the endoscopically determined squamocolumnar junction. They also concluded that EMR, with close endoscopic surveillance, is an effective treatment modality for BE with HGD and intramucosal carcinoma^[79]. Another recent study, originating from two Australian academic hospitals, involved 75 patients; 89% with Barrett's HGD

and 11% with early esophageal cancer, who were treated by EMR over a 7-year period. The treatment resulted in complete Barrett's excision in 94% of cases with short segment BE. During the mean follow-up of 31 mo (range, 3-89) there was no recurrence although 11% developed metachronous lesions. Five patients underwent esophagectomy because the endoscopic resection specimen demonstrated submucosal invasion. The complications were one aspiration and six strictures, which were managed with endoscopic dilatation. This study concluded that EMR alters histological grade or local T stage in 48% of patients and dramatically reduces esophagectomy rate, thus providing a safe and effective therapy^[80].

The development of EMR allows full eradication of the neoplastic mucosal lesions and simultaneous accurate staging. Nevertheless, the greatest value of this method is focused on the ability to detect a metachronous lesion, in 50% of cases, in the residual portion of BE. From a therapeutic approach, EMR is promising but has been associated with persistent HGD, persistent Barrett's epithelium and serious recurrence rates of dysplasia or neoplasia in the residual Barrett's epithelium, thus necessitating endoscopic surveillance after resection. It is, therefore, obligatory to completely extirpate intestinal metaplasia at its whole extent, a target that can be accomplished with the combination of EMR with other therapeutic modalities. Combinations of EMR and photodynamic therapy with porfimer sodium, 5-ALA, or meta-tetrahydroxyphenylchlorine^[73,76,77] have been applied in selected patients and have yielded successful results with regard to the eradication of dysplastic lesions^[55].

Another novel therapeutic approach with the intent to eliminate local recurrence involves the use of circumferential mucosal resection with complete excision of the visible Barrett's epithelium^[81]. High success rates in eradication of Barrett's epithelium with a low rate of complications have been demonstrated^[82,83]. These findings suggest that this method could be beneficial for all patients with BE and HGD or intramucosal cancer. Larghi *et al*^[84] have used the technique of cylindrical mucosal excision in 26 patients with BE and HGD as a way to achieve complete excision of Barrett's mucosa. The technique utilized either endoscopic cap suction or endoscopic snare mucosectomy or a combination of both methods. The method of endoscopic cap suction was applied as previously described^[80,84], with the aid of commercially available kits (K001 and K002, Olympus America Inc.). The method of endoscopic snare mucosectomy was performed in the way described by Soehendra *et al*^[85], in which a single-channel therapeutic endoscope (type GIF-IT, Olympus America Inc.) and a single-channel mucosectomy snare (type D3422161 M-C, Endo-Flex GmbH, Voerde, Germany) were used. From the follow-up of 23 patients over an average period of 28 mo, complete eradication of lesions occurred in 21 patients (87.5%), whereas in one patient Barrett's epithelium developed underneath the neo-squamous epithelium three mo after excision, and in another an HGD nodule was detected and excised at

twelve mo during follow-up. Finally, many authors appreciate this method owing to its high therapeutic yield, but also stress the need for additional larger cohort studies, with longer duration of follow-up and endoscopic surveillance, in order to deduce definitive conclusions. It is also suggested that new equipment will aid in the en bloc resection and possibly prove more effective in completely excising the mucosa along with eliminating the possibility of residual Barrett's epithelium. Improvement in the skills needed to perform such techniques with optimal results is expected to accompany technological advances^[84,86,87].

CHOICE OF THERAPEUTIC APPROACH

The classical therapy of BE with HGD has been based on the well renowned, *en bloc* esophagectomy with thoracotomy, vagotomy and lymph node dissection; an operation that, as already mentioned, carries high perioperative mortality, morbidity and a poor quality of life. Considering these disadvantages, many patients are considered unfit for such an operation, whereas others fail to accept it as an option. Additionally, studies from high volume centers in esophageal surgery have demonstrated rare lymph node metastasis, in the region of about 5%, rendering lymph node dissection unnecessary^[36,88,89]. The above data have led surgeons as well as gastroenterologists to in-depth research regarding less invasive endoscopic procedures and operations with decreased mortality, morbidity and an acceptable quality of life, such as laparoscopic vagal-sparing esophagectomy. However, the decision for the appropriate therapeutic approach is often difficult and currently there is considerable controversy over which method is better, i.e., surgery or endotherapy (techniques involving endoscopy). Nevertheless, it must be noticed that a number of other parameters may affect the choice of the therapeutic method.

The histopathological diagnosis of HGD, or even the distinction between low- and high-grade dysplasia, remains alarmingly subjective. The kappa values for intra-observer and inter-observer variability are 0.64 and 0.45^[90] and the accordance for the diagnosis of dysplasia attains a rate of 94% and 88%, respectively. Furthermore, agreement between specialized and non-specialized pathologists as to the definition and the histopathological characteristics of HGD exists in only 50% of cases^[88]. When the need to distinguish HGD from intramucosal carcinoma arises, agreement is even poorer. It is also common knowledge that the natural course of dysplasia differs from patient to patient. Thus, some researchers announce cancer development in 60% of patients at 8 mo, while others report a cumulative cancer rate of 9% at 5 years and only 16% over a 15.9-year period, as documented by endoscopic surveillance^[91].

The presence of esophageal cancer in BE represents yet another diagnostic problem. With meticulous examination of esophagectomy specimens in an effort to detect invasion through the submucosal layer, the kappa values for intra-observer and inter-observer variability

are 0.56 and 0.42, respectively^[92]. There is also significant discrepancy between the prevalence of carcinoma in esophagectomy specimens of patients who are operated for HGD (0% up to 75%)^[93-95] and that of invasive adenocarcinoma in patients with HGD who are under endoscopic surveillance (16% up to 60%)^[88,93,95]. Further disagreement exists as to the presence of occult cancer in patients with BE and HGD. Cameron *et al*^[94] histologically mapped esophagectomy specimens from patients operated for early adenocarcinoma and depicted areas with occult cancer that are extremely small and can easily evade attention.

Obviously, current data are not sufficient to dictate clear-cut therapeutic indications for this specific patient population. The question in doubt is whether to choose endoscopic therapy, particularly EMR, over esophagectomy. Nevertheless, this does not apply to patients who reject surgical intervention or are considered unfit to undergo a major operation. The therapeutic indication for these patients is limited to the choice of an appropriate endoscopic method. Additionally, it must be pointed out that endoscopic therapy should probably be precluded for a group of patients with BE and HGD who are young (about 55 years old), otherwise healthy without significant co-morbidities, with a high risk of developing invasive adenocarcinoma^[95]. Therefore, the selection of the appropriate treatment is questionable for older patients who are eligible for esophagectomy.

Proponents of endoscopic treatment, even in the absence of comparative studies between surgical units and endoscopic departments, advocate that endoscopic therapy carries lower morbidity and mortality than esophagectomy. They also raise the argument that the FDA has already approved porfimer sodium for the photodynamic eradication of premalignant lesions in patients with BE who do not undergo esophageal resection. It seems that even technology works in favor of the endoscopic therapy argument. Recently, in the field of optical spectroscopy, a technique that allows detection of molecular degeneration and minute dysplastic alterations in real time was developed. This technique is expected to allow simultaneous detection and destruction in a single endoscopic session^[79,80].

On the other hand, surgeons argue that the patient is subject to the risk of being lost during the follow-up with endoscopic surveillance and may reappear later with inoperable disease. Moreover, the techniques of endoscopic destruction of the lesion may not provide adequate samples for histological examination. At EMR, residual foci of dysplastic cells remain deeper in the regenerated squamous epithelium; however, HGD is often multifocal and early reports of endoscopic excision have documented an unacceptably high rate of positive excision margins. The majority of studies evaluating endoscopic treatment of BE with HGD were neither randomized nor controlled, included small numbers of patients and the duration of follow-up was relatively short, thus unreliable for extraction of safe conclusions. From a surgeon's point of

view, before choosing a therapeutic approach, the severity of GERD as well as the gravity of symptoms should be taken into account. Thus, avoiding esophagectomy and implementing an endoscopic therapy should be considered for patients with few symptoms, normal esophageal function and short segment BE, with associated low risk of intramucosal cancer. Accordingly, esophagectomy is reserved for patients with BE and HGD or intramucosal cancer who present with severe symptoms of GERD or dysphagia, long segment BE, a large hiatal hernia and poor function of esophageal body^[96].

Currently, the optimal therapy of BE with HGD is, at best, controversial, despite the vast number of emerging new techniques in the fields of both surgery and endoscopy. No properly designed prospective randomized controlled trial, comparing the various therapeutic modalities, has yet been conducted, rendering the undertaking of such a study mandatory in order to elucidate the ideal therapy^[97].

CONCLUSION

The modern era surgeon is confronted with multiple dilemmas concerning the best therapeutic management of patients with BE and HGD, which represents an area of dispute between esophagogastric surgeons and gastroenterologists. The ideal therapy for BE with HGD is further perplexed by the unclear natural history of the disease, the discordance of histopathologic diagnosis and its relation to malignancy, i.e., coexistent disease or subsequent development of esophageal adenocarcinoma. When considering the best therapeutic approach for these patients, multifocality, extent and pretreatment staging of the disease, as well as patient's preference and performance status, should all be taken into account. Therefore, the ideal therapy should be individualized. Many advocate esophagectomy as the gold standard therapy for BE with HGD. Nevertheless, new and emerging minimally invasive, endoscopic and ablative techniques have more recently yielded significant results and gained popularity. Randomized controlled trials are still required to properly define their optimal role in the armamentarium against BE with HGD and current research is expected to lead to the incorporation of these techniques in standard clinical practice.

ACKNOWLEDGMENTS

We would like to thank Professor Gregory Kouraklis for his constant support throughout the design and preparation of this review.

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S- Editor Tian L L- Editor Logan S E- Editor Xiong L

Hepatic steatosis prevents heme oxygenase-1 induction by isoflurane in the rat liver

Patrick Stoll, Christian I Schwer, Ulrich Goebel, Hartmut Buerkle, Alexander Hoetzel, Rene Schmidt

Patrick Stoll, Christian I Schwer, Ulrich Goebel, Hartmut Buerkle, Alexander Hoetzel, Rene Schmidt, Department of Anesthesiology and Critical Care Medicine, Freiburg University Medical Center, D-79106 Freiburg, Germany

Author contributions: Stoll P performed all animal experiments and most of the molecular analyzes and wrote the first draft of the manuscript; Schwer CI participated in the research; Goebel U participated in data analysis; Buerkle H wrote the paper; Hoetzel A participated in research design and data analysis; Schmidt R designed the study and wrote the paper.

Supported by Departmental funding

Correspondence to: Rene Schmidt, MD, DESA, Department of Anesthesiology and Critical Care Medicine, Freiburg University Medical Center, Hugstetter Strasse 55, D-79106 Freiburg, Germany. rene.schmidt@uniklinik-freiburg.de

Telephone: +49-761-27023060 Fax: +49-761-27023960

Received: December 30, 2010 Revised: April 7, 2011

Accepted: April 14, 2011

Published online: October 7, 2011

Abstract

AIM: To characterize the inductive effects of isoflurane (ISO) on hepatic heme oxygenase-1 (HO-1) in an animal model of hepatic steatosis.

METHODS: Lean (LEAN) and obese (FAT) Zucker rats were randomized into 4 groups: 1: LEAN + pentobarbital sodium (PEN); 2: LEAN + ISO; 3: FAT + PEN; 4: FAT + ISO. The animals were mechanically ventilated for 6 h. *In vitro* analyses of liver tissue included determination of HO-1 mRNA and protein expression as well as measurement of HO enzyme activity and immunohistochemical analyses.

RESULTS: Compared to PEN treatment, ISO administration profoundly induced hepatic HO-1 mRNA and protein expression and significantly increased HO enzyme activity in lean Zucker rats. In contrast, no difference in *HO-1* gene expression was observed after ISO or PEN anesthesia in obese Zucker rats.

CONCLUSION: The present study demonstrates that ISO is an inducer of hepatic *HO-1* gene expression in non-steatotic organs but failed to upregulate HO-1 in steatotic livers.

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Key words: Isoflurane; Heme oxygenase; Hepatic steatosis; Heme oxygenase-1; Volatile anesthetics

Peer reviewers: Kostas Pantopoulos, Associate Professor, Department of Medicine, McGill University, Lady Davis Institute for Medical Research, 3755 Cote Ste-Catherine Road, Montreal, Quebec, H3T 1E2, Canada; Valentina Medici, MD, Assistant Professor, Division of Gastroenterology and Hepatology, Department of Internal Medicine, University of California Davis, 4150 V Street, Suite 3500, Sacramento, CA 95817, United States

Stoll P, Schwer CI, Goebel U, Buerkle H, Hoetzel A, Schmidt R. Hepatic steatosis prevents heme oxygenase-1 induction by isoflurane in the rat liver. *World J Gastroenterol* 2011; 17(37): 4184-4190 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4184.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4184>

INTRODUCTION

The development of hepatic ischemia/reperfusion (I/R) injury is a fundamental problem in major hepatic surgery including liver transplantation, causing a higher rate of morbidity and mortality^[1,2]. Surgical interventions such as warm hepatic inflow occlusion (Pringle maneuver) or cold ischemia in the transplant setting followed by reperfusion are important and often unavoidable techniques used to reduce blood loss or preserve organs for subsequent transplantation. I/R injury frequently results in apoptosis and necrosis of hepatocytes, which could consequently lead to organ failure or graft dysfunction^[3,4]. In recent years liver surgery has become safer due to improvements in surgical techniques, anesthetic procedures

and postoperative care. However, due to the epidemic increase in obesity, the prevalence of hepatic steatosis has significantly increased during the last few decades. The Dallas Heart Study reported a prevalence of hepatic steatosis in their population of about 38% indicating the high relevance for today's health care system^[5]. It has been repeatedly shown that steatotic livers are especially vulnerable to I/R injury. Liver surgery in patients with severe hepatic steatosis is associated with higher morbidity and mortality rates due to the underlying pathogenic features affecting important mechanisms during I/R, liver regeneration and recovery^[6-8]. Various strategies have been proposed to improve the postoperative outcome of these patients including pharmacological approaches aiming at upregulation of cytoprotective genes.

Heme oxygenase-1 (HO-1) and its catalytic products have been identified as major players in cell protection in different organs^[9-12]. Whereas HO-1 represents the inducible form of the HO family, HO-2 is expressed constitutively. HO catabolizes the first and rate-limiting step in heme degradation producing carbon monoxide (CO), free iron and biliverdin, which is converted into bilirubin by biliverdin reductase^[13]. A multitude of HO-1 inducers are well known, but most of them are toxic which limit their therapeutic application in humans^[14,15]. We have previously shown that volatile anesthetics are potent non-toxic inducers of *HO-1* gene expression in the rat liver^[16]. Isoflurane (ISO) pretreatment induces hepatic HO-1 mRNA and protein followed by an increase in HO activity, thereby reducing portal resistance^[17]. Experimental and clinical evidence support the hypothesis that administration of volatile anesthetics could be a promising approach to limit I/R injury and to improve the outcome of patients undergoing liver surgery^[18,19]. To date, no data are available regarding the effects of anesthetics on hepatic HO-1 induction in steatotic livers. Therefore, the present study was designed to characterize the effects of ISO administration on hepatic *HO-1* gene expression in an established animal model of hepatic steatosis using genetically modified Zucker rats.

MATERIALS AND METHODS

Reagents

Isoflurane was obtained from Abbott (Wiesbaden, Germany) and pentobarbital sodium from Alvetra (Neumuenster, Germany). Pancuronium was purchased from Organon (BH Oss, Netherlands). All other reagents used were purchased from Sigma Aldrich (Deisenhofen, Germany), if not specified otherwise.

Animals

All animal experiments were approved by the local animal care and use committee and were in accordance with the Guide for the Care and Use of Laboratory Animals. Homozygous obese (FAT) male Zucker rats and heterozygous lean (LEAN) male Zucker rats aged 12 wk were obtained from Charles River (Sulzfeld, Germany). Animals were fasted for 6 h before the beginning of the

experiments but were allowed free access to water.

Experimental protocol

The animals were assigned to 4 groups: group 1, LEAN + PEN (pentobarbital sodium, 40 mg/kg per hour i.v.); group 2, LEAN + ISO; group 3, FAT + PEN; group 4, FAT + ISO. Animals treated with PEN received one initial intraperitoneal injection of pentobarbital sodium (40 mg/kg) followed by an intravenous infusion of 40 mg/kg per hour. For compensation of evaporative losses 10 mL/kg per hour of saline solution 0.9% were continuously infused. Rats in the ISO groups were anesthetized by inhalation of ISO (2.8-3.1 Vol%). After induction of anesthesia, a tail vein was cannulated and a tracheostomy was performed. Relaxation was achieved by injection of pancuronium (1 mg/kg i.v.) and all animals were mechanically ventilated (Rodent Ventilator UB 7025-10, Harvard Apparatus, March-Hugstetten, Germany). Doses of 0.5 mg/kg pancuronium were repeated every 3 h to maintain muscle paralysis. Cannulation of the left carotid artery with polyethylene (PE-50, Smith Medical, Ashford, United Kingdom) tubing was performed for arterial blood pressure monitoring and blood gas analysis. The right jugular vein was cannulated for fluid administration. Blood gas analyses were performed using an autoanalyzer ABL 800 Flex (Radiometer, Willich, Germany). At the end of the experiment (6 h after onset), the animals were killed and blood and liver tissue were removed for subsequent analyses.

Enzyme determination

Blood samples were collected at the end of each experiment and immediately centrifuged at 4 °C. Rat α -glutathione s-transferase (α -GST) serum concentration was evaluated using an anti-rat α -GST enzyme immunoassay (Argutus Medical, Dublin, Ireland). The procedures were performed according to the manufacturer's instructions.

RNA isolation

Total RNA was extracted from liver tissues using the TRIzol method (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's recommendation. RNA amounts were normalized to a concentration of 50 ng/ μ L diluted with RNase-free Water (Qiagen, Hilden, Germany).

Semi-quantitative real-time reverse transcriptase-polymerase chain reaction

Total RNA was reverse transcribed to single-stranded cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc, Foster City, CA 94404 United States) according to the manufacturer's protocol. Briefly 250 ng of purified total RNA were subsequently used in 50 μ L reverse transcription reactions employing random hexamers. 50 ng of the resulting cDNA were used in semi-quantitative real-time polymerase chain reaction (PCR) analysis in a 50 μ L final volume. Reactions were performed on an ABI Prism 7000 (Applied Biosystems, Foster City, CA, United States) in duplicate for each animal using TaqMan Mastermix reagents (part number

4309169, Applied Biosystems, Foster City, CA, United States) with a specific TaqMan® Probe against HO-1 cDNA (Assay ID: Rn00561387_mL) as described in the manufacturer's protocol. Parameters for quantitative PCR were as follows: 10 min at 95 °C, followed by 40 cycles of amplification for 15 s at 95 °C, and 1 min at 60 °C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as endogenous control in all real time analyses using a VIC®/MGB labeled Probe (part number: 4352338E, Applied Biosystems, Foster City, CA, United States). The obtained data were analyzed by the $\Delta\Delta CT$ method.

Determination of heme oxygenase enzyme activity

The heme oxygenase (HO) enzyme activity assay was performed as previously described^[20]. Briefly, frozen liver tissue was homogenized and added to a reaction mixture containing NADPH, liver cytosol, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and hemin. The reaction was performed at 37 °C for 1 h in the dark and stopped by the addition of chloroform. The extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm.

Hematoxylin-eosin staining of liver sections

For histological analysis, liver sections were fixed with 4% buffered formalin (pH 6.9) and embedded in paraffin. Livers were sliced (5 μ m) and stained with hematoxylin-eosin according to a standardized protocol.

Immunohistochemical staining

Liver tissue samples were formalin-fixed, paraffin embedded and cut in a microtome to 4 microns. The slides were deparaffinized with xylene, rehydrated and then rinsed with tap water. Antigen retrieval was performed by microwave irradiation in a sodium citrate buffer (pH 6) and slides were blocked with a ready-to-use peroxidase blocking reagent (Dako North America, Inc., CA 93013 United States) for 10 min at room temperature. After subsequent treatment with normal goat serum, slides were incubated with the primary antibody (dilution 1:50) as used in Western blotting for 1 h at room temperature. Following three washing steps with phosphate buffered saline (PBS) the slides were incubated with the HRP-conjugated secondary antibody (Goat A-rabbit-HRP, Dako, Denmark) diluted 1:200. The slides were again washed 3 times with PBS and then incubated with liquid diaminobenzidine and substrate (Dako North America, Inc., CA, United States) as the chromogen for 6 min and rinsed with deionized water. Finally the sections were counterstained with Mayer's hematoxylin (Merck KG, Darmstadt, Germany), dehydrated and mounted in an organic mounting media. For assessment of the severity of hepatic steatosis, hematoxylin and eosin-stained sections were evaluated without immunohistochemical treatment.

Western blotting analysis

Western blotting analysis was performed with total cell lysates as described previously^[19]. Briefly, frozen liver tissue was homogenized on ice in activated RIPA buffer (Santa

Cruz, CA, United States). Total protein concentration was determined in the supernatant using the Bradford assay (Bio-Rad Laboratories, Munich, Germany). Each lane of a 10% sodium dodecyl sulfate gel contained 100 μ g of total protein. After separation and electroblotting, HO-1 was detected by a rabbit polyclonal anti-HO-1 antibody (1:1000 dilution, SPA 895; Stress Gen Biotechnologies, Victoria, British Columbia, Canada) using the enhanced chemiluminescence detection kit (Amersham Pharmacia) according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean \pm SE of the mean with $n = 5$ animals per group as indicated. Statistical differences within each group were determined using a one-way analysis of variance (ANOVA) for repeated measurements and between the different groups by one-way ANOVA followed by the post hoc Student-Newman-Keuls test for pairwise comparisons. When criteria for parametric tests were not met, Kruskal-Wallis ANOVA on ranks followed by Dunn's test was used. These data are presented as median (box: 25th and 75th percentiles; error bars: 5th and 95th percentiles) for $n = 5$ animals per group. Data were considered significant when $P < 0.05$. Statistical analysis was performed using the Sigma Stat and Sigma Plot 11 software package (Jandel Scientific, San Rafael, CA, United States).

RESULTS

Analysis of vital parameters and weight

The animals in the LEAN + PEN group had a significantly higher mean arterial pressure at the respective time points during the experiments (Table 1). There were no differences in heart rate between the different groups. Body temperature dropped during induction of anesthesia but returned to normal values after at least 2 h in all groups (Table 1). **Homozygous Zucker rats (FAT) had a higher body weight compared to the age-matched heterozygous controls (LEAN) (Table 2).** There were no significant differences in blood gas parameters.

Effect of ISO treatment on hepatic HO-1 mRNA expression

Semi-quantitative HO-1 mRNA real-time analysis of isolated liver extracts is shown in Figure 1A. ISO treatment over 6 h led to a significant increase in HO-1 mRNA in lean rats (4.81 ± 0.82) compared to all other groups. ISO inhalation in FAT Zucker rats (2.57 ± 0.22) did not lead to a significant induction of HO-1 compared to the respective PEN group (2.83 ± 0.39). A significant difference in HO-1 mRNA levels was detected between liver extracts of animals from the FAT+PEN and FAT + ISO group compared to rats assigned to the LEAN + PEN group (2.03 ± 0.38).

Effect of ISO administration on hepatic HO-1 protein levels and HO enzyme activity

In line with RT-analyses, representative Western blotting showed higher HO-1 protein levels in liver extracts

Table 1 Time course of mean arterial pressure, heart rate and temperature

	Time (h)	LEAN+PEN	LEAN+ISO	FAT+PEN	FAT+ISO
MAP (mmHg)	0	127 ^a ± 5	82 ± 11	106 ± 5	99 ± 5
	2	86 ^a ± 3	72 ± 2	77 ± 4	73 ± 2
	4	85 ^a ± 5	69 ± 2	74 ± 4	69 ± 2
	6	84 ^a ± 7	69 ± 2	72 ± 3	70 ± 2
	HR (bpm)	0	305 ± 3	302 ± 4	314 ± 2
Temp. (°C)	0	34.4 ^a ± 0.3	34.7 ^a ± 0.6	34.5 ^a ± 0.2	34.7 ^a ± 0.3
	2	36.6 ± 0.3	37.2 ± 0.3	36.6 ± 0.3	36.9 ± 0.1
	4	37.1 ± 0.2	37.5 ± 0.1	37.2 ± 0.1	37.0 ± 0.2
	6	37.1 ± 0.2	37.5 ± 0.0	37.2 ± 0.1	37.0 ± 0.1

Data are presented as mean ± SE of the mean for $n = 5$ animals per group. ^a $P < 0.05$ vs all other groups, ^c $P < 0.05$ vs all other time points. MAP: Mean arterial pressure; HR: Heart rate; Temp.: Temperature; LEAN: Lean Zucker rats; FAT: Obese Zucker rats; ISO: Isoflurane; PEN: Pentobarbital sodium.

Table 2 Body weight and baseline values of blood gas parameters

	LEAN+PEN	LEAN+ISO	FAT+PEN	FAT+ISO
Weight (g)	321 ± 19	294 ± 16	462 ^b ± 14	426 ^b ± 18
pH	7.46 ± 0.03	7.48 ± 0.03	7.48 ± 0.04	7.42 ± 0.01
pCO ₂ (mmHg)	40 ± 3	37 ± 3	32 ± 4	33 ± 4
pO ₂ (mmHg)	211 ± 52	279 ± 69	258 ± 33	308 ± 74

Data were obtained after induction of anesthesia. Data are presented as mean ± SE of the mean for $n = 5$ animals per group (^b $P < 0.001$ vs Lean + Pentobarbital sodium and Lean + Isoflurane). pCO₂: Arterial carbon dioxide partial pressure; pO₂: Arterial oxygen partial pressure; LEAN: Lean Zucker rats; FAT: Obese Zucker rats; ISO: Isoflurane; PEN: Pentobarbital sodium.

from animals in the LEAN + ISO group compared to the other groups (Figure 1B). Equal loading was verified by reprobing the membrane with a GAPDH antibody. In addition, HO enzyme activity was significantly higher in ISO treated lean Zucker rats (Figure 1C). Interestingly, we found no differences in HO activity between the FAT + PEN and FAT + ISO group.

Expression pattern of hepatic HO-1 protein after ISO treatment

HO-1 immunoreactive protein was restricted to spindle-shaped sinusoidal lining cells in PEN anesthetized control animals (Figure 2A). HO-1 protein was markedly upregulated in hepatocytes predominantly located in the perivenular area after ISO treatment in lean Zucker rats (Figure 2B). In sharp contrast, we did not detect any upregulation of HO-1 protein in hepatocytes of the perivenular area in obese Zucker rats after treatment with ISO (Figure 2C and D).

Hematoxylin-eosin staining

For confirmation of steatosis hepatis, hematoxylin-eosin staining was performed. Obese Zucker rats (FAT) showed

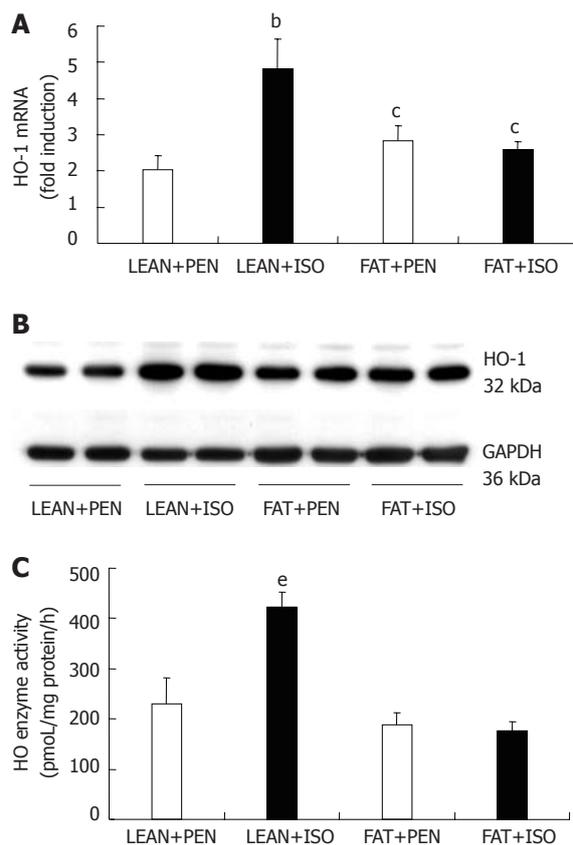


Figure 1 Hepatic heme oxygenase-1 gene expression and heme oxygenase enzyme activity in lean and obese Zucker rats after treatment with pentobarbital or isoflurane. A: Reverse transcriptase-polymerase chain reaction was performed for determination of HO-1 mRNA expression. Data are presented as mean ± SE of the mean for $n = 5$ per group. ^b $P < 0.01$ vs all other groups; ^c $P < 0.05$ vs LEAN + PEN; B: Western blotting analysis for determination of HO-1 protein expression in two representative animals from each group; C: Measurement of HO enzyme activity. Data are presented as mean ± SE for $n = 5$ per group. ^e $P < 0.001$ vs all other groups. HO-1: Heme oxygenase-1; PEN: Pentobarbital; ISO: Isoflurane; LEAN: Lean Zucker rats; FAT: Obese Zucker rats; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

severe macrovesicular and microvesicular fatty infiltration in hepatocytes (Figure 3C and D). In contrast, we did not detect any accumulation of lipid droplets in LEAN animals (Figure 3A and B).

Effect of ISO treatment on serum levels of α -glutathione S-transferase

As shown in Figure 4, α -GST, one of the most specific serum enzymes identifying hepatocyte injury did not differ between the groups.

DISCUSSION

In the present report we demonstrate that ISO induced upregulation of *HO-1* gene expression, which was reproducibly shown in normal livers and serves as a major protective mechanism against hepatic ischemia and reperfusion injury, is abrogated in the presence of hepatic steatosis. ISO treatment of heterozygous lean Zucker rats representing a normal phenotype led to a profound induction of HO-1 mRNA and protein with a subsequent

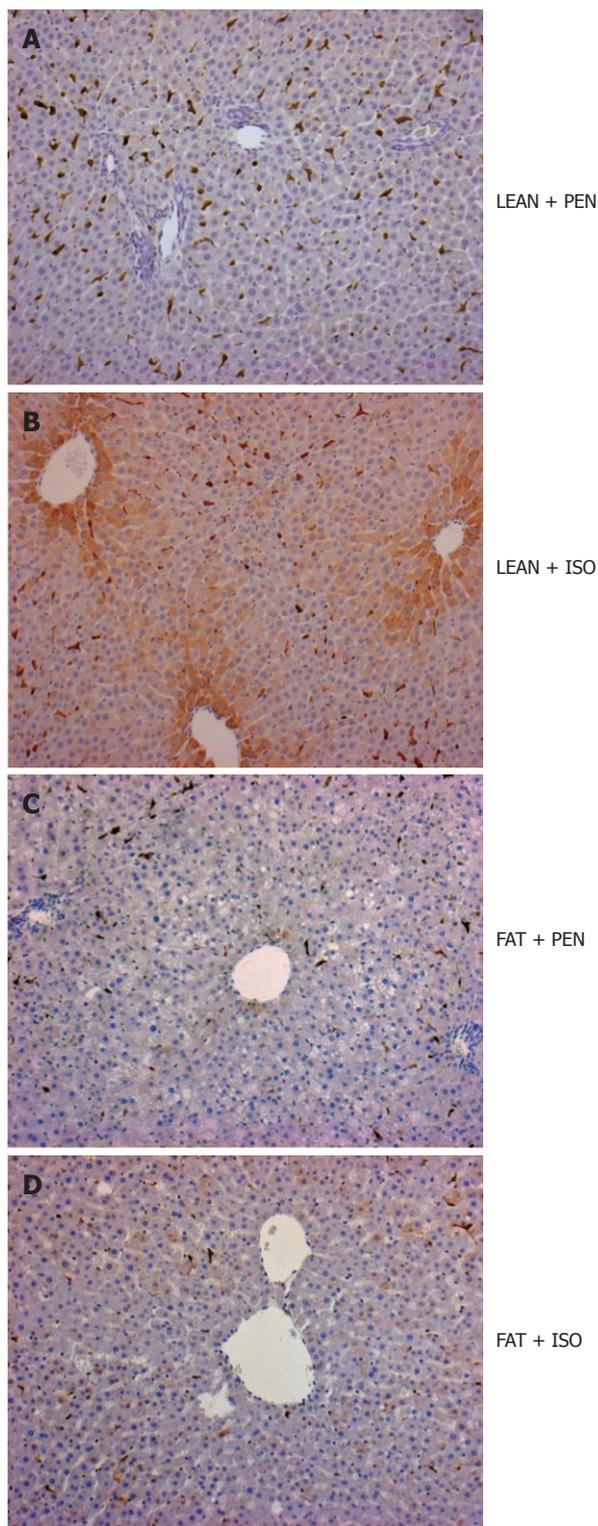


Figure 2 Heme oxygenase-1 protein expression pattern in liver sections from lean and obese Zucker rats after treatment with pentobarbital or isoflurane. A: Heme oxygenase-1 (HO-1) protein was restricted to spindle-shaped sinusoidal lining cells in pentobarbital treated animals; B: Isoflurane (ISO) administration led to markedly upregulated HO-1 protein expression in hepatocytes of the perivenular area; C and D: No detectable induction of HO-1 protein in hepatocytes of obese animals was detected after ISO treatment. PEN: Pentobarbital; LEAN: Lean Zucker rats; FAT: Obese Zucker rats.

increase in HO enzyme activity in the liver. In contrast

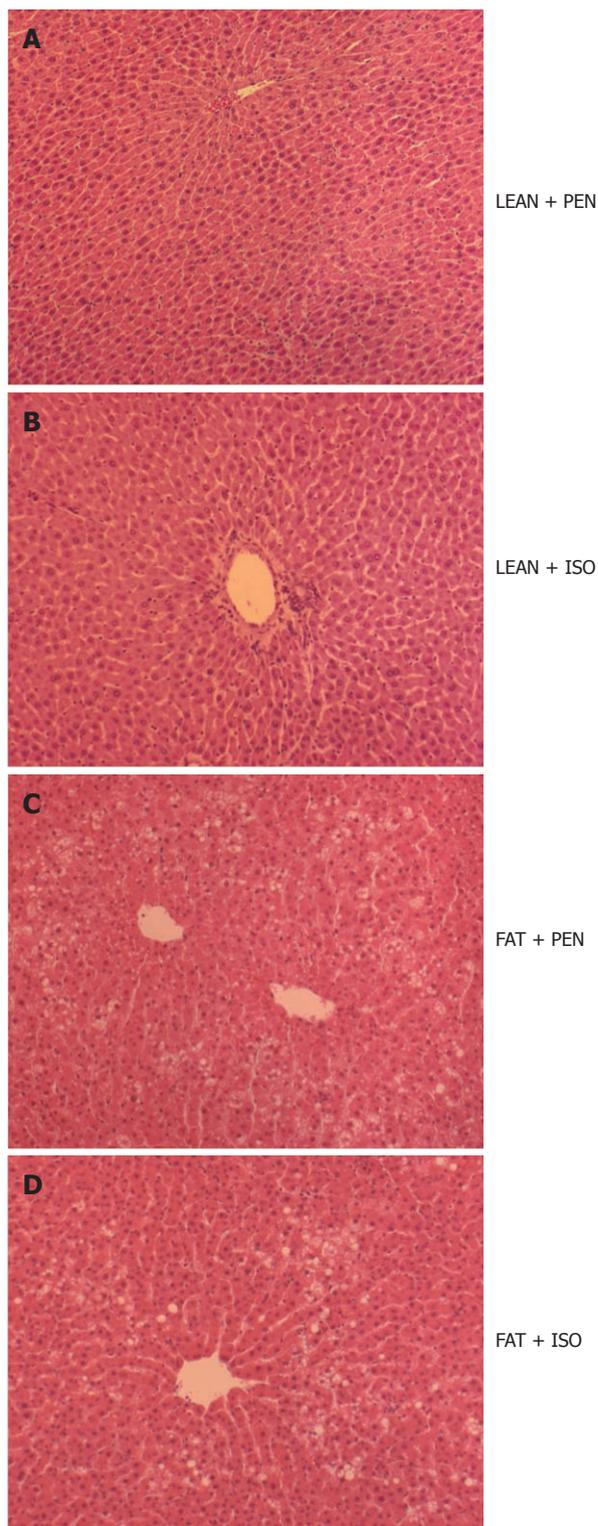


Figure 3 Hematoxylin and eosin staining of liver sections. To assess the degree of hepatic steatosis, hematoxylin-eosinstaining was performed. A, B: No lipid droplets were detected in lean Zucker rats; C, D: In obese Zucker rats numerous intracellular lipid vacuoles were observed within the liver tissue. PEN: Pentobarbital; ISO: Isoflurane; LEAN: Lean Zucker rats; FAT: Obese Zucker rats.

to these findings, which confirm earlier reports from our laboratory in Sprague Dawley rats, ISO administration to homozygous obese Zucker rats, an established animal model for steatosis hepatitis, had no effect on *HO-1* gene

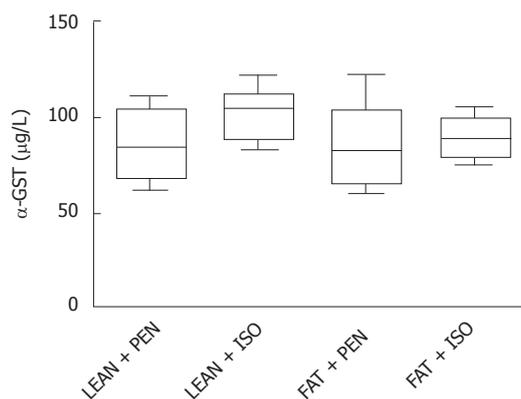


Figure 4 Determination of serum levels of α -glutathione s-transferase. There were no significant differences in α -glutathione s-transferase serum levels between the different groups. Data are presented as median (box: 25th and 75th percentiles; error bars: 5th and 95th percentiles) for $n = 5$ animals per group. PEN: Pentobarbital; ISO: Isoflurane; LEAN: Lean Zucker rats; FAT: Obese Zucker rats.

expression in the liver of these animals.

Volatile anesthetics are non-toxic inducers of hepatic *HO-1* gene expression^[16,17,19,21]. We previously showed that pretreatment with ISO leads to an improvement in hepatic macro- and microvascular blood flow and reduces portal vascular resistance in the normal liver^[17]. Furthermore, ISO induced upregulation of hepatic HO-1 is an important hepatoprotective mechanism against I/R injury. It specifically upregulates HO-1 protein in hepatocytes of the perivenular area, the primary localization of cellular injury in low flow states like I/R. HO-1 induction improves microcirculation in the early reperfusion period, decreases the oxidative burst and significantly reduces serum levels of liver enzymes and morphological signs of hepatic injury after I/R^[19]. In addition to these experimental data, Beck-Schimmer and colleagues recently published the first randomized clinical study demonstrating the hepatoprotective effect of the volatile anesthetic, sevoflurane, in patients undergoing liver resection^[18]. Interestingly, they found an even more pronounced beneficial effect of sevoflurane in patients with steatotic livers. No information is available regarding sevoflurane treatment on *HO-1* gene expression in their study. However, we hypothesize that the protective effect of sevoflurane in this trial is independent of HO-1 since the duration of pretreatment (30 min) and the concentration applied (1.5 of minimal alveolar concentration) is most likely not sufficient to upregulate hepatic *HO-1* gene expression. Therefore, volatile anesthetics might mediate liver protection by at least two different mechanisms, one dependent and one independent of HO-1. To exclude the possibility that HO-1 upregulation in homozygous Zucker rats is generally prevented by genetic modifications in these animals, we screened the literature in this regard. It has been repeatedly shown by different authors that the administration of a variety of compounds can profoundly induce HO-1 in obese Zucker rats^[22,23]. Therefore, the inability to upregulate HO-1 by ISO seems to be substance specific rather than based on a general lack of inducibility

in these animals. As indicated in Figure 1A and B, hepatic HO-1 induction in obese animals was slightly but significantly higher than in the livers of lean controls. However, this did not affect HO enzyme activity in our experiments (Figure 1C).

To exclude hepatotoxic effects of the anesthetics in our experiments, we performed serum α -GST measurements. α -GST levels, which serve as a very specific marker of hepatocyte injury, did not differ between the respective groups indicating the non-toxic action of ISO.

Obese Zucker rats develop hypertensive blood pressure values accompanied by improper autoregulation caused by an impairment of sympathetic baroreceptor reflexes^[24-26]. Therefore, anesthetics (e.g., barbiturates) may have an even more pronounced effect on blood pressure in obese rather than lean Zucker rats. This could be an explanation for the higher blood pressure in the LEAN + PEN group compared to the other animals observed in the present study.

Patients with hepatic steatosis are at higher risk for postoperative complications after major hepatic surgery including liver transplantation, and adverse outcomes have been repeatedly documented^[8,27-30]. Due to the increasing gap between the number of available organs and the number of patients awaiting an organ, the amount of so-called “marginal livers” considered for transplantation is increasing. Based on this dilemma, it is important to develop protective strategies particularly for the above-mentioned type of organs comprising severely steatotic livers to expand the pool of available liver grafts.

The present study demonstrates that ISO is a potent inducer of *HO-1* gene expression in non-steatotic livers but failed to upregulate HO-1 in steatotic organs. If validated in humans, this observation may have an impact on the anesthetic regimen in patients undergoing liver surgery.

ACKNOWLEDGMENTS

The authors are grateful to Martina de Groot and Heide Marniga for their excellent technical assistance.

COMMENTS

Background

Experimental and clinical evidence support the hypothesis that administration of volatile anesthetics could be a promising approach to limit ischemia/reperfusion (I/R) injury and to improve the outcome of patients undergoing liver surgery. The volatile anesthetic isoflurane is a potent non-toxic inducer of heme oxygenase-1 (HO-1) gene expression in the normal liver. The authors previously showed that these livers are protected from I/R injury.

Research frontiers

There are no studies currently available characterizing the inductive effects of volatile anesthetics on steatotic livers which are especially vulnerable to I/R injury. Therefore, we examined the effects of isoflurane (ISO) on hepatic HO-1 induction in lean (non-steatotic livers) and obese (steatotic livers) Zucker rats.

Innovations and breakthroughs

The findings of the present study demonstrate that isoflurane is a potent inducer of HO-1 gene expression in non-steatotic livers but failed to upregulate HO-1 in steatotic livers.

Applications

If verified in humans, this observation may have a crucial impact on the anesthetic regimen in patients undergoing liver surgery.

Terminology

HO-1 also called heat shock protein-32 and its catalytic products were recently identified as major players in cell protection in different organs. Hepatic I/R injury is a fundamental problem in major hepatic surgery including liver transplantation. The Zucker rat is a genetic research model for obesity and hypertension named after Lois M Zucker. Homozygous Zucker rats have high levels of lipids in their blood and an increased size and number of fat cells. Therefore, these animals serve as a model for steatosis hepatis.

Peer review

In this manuscript, the authors compare the effects of the volatile anesthetic isoflurane in the induction of HO-1 among lean and obese rats. They measure HO-1 expression at the mRNA and protein level, and also assess the activity of this enzyme. The overall data suggest that isoflurane efficiently induces HO-1 in lean, but not in obese animals. The study is well designed, appropriate controls are included and the experiments are of high technical quality. The conclusion is fully supported by the presented data.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Xiong L

Decreased accumulation of ultrasound contrast in the liver of nonalcoholic steatohepatitis rat model

Yuki Miyata, Takeo Miyahara, Fuminori Moriyasu

Yuki Miyata, Takeo Miyahara, Fuminori Moriyasu, Department of Gastroenterology and Hepatology, Tokyo Medical University, 6-7-1, Nishi-Shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

Author contributions: Miyata Y and Miyahara T contributed equally to this work; Moriyasu F designed research; Miyata Y and Miyahara T performed research; Miyata Y and Miyahara T contributed new reagents/analytical tools; Miyata Y wrote the paper.

Correspondence to: Fuminori Moriyasu, MD, Department of Gastroenterology and Hepatology, Tokyo Medical University, 6-7-1, Nishi-Shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan. moriyasu@tokyo-med.ac.jp

Telephone: +81-3-53256838 Fax: +81-3-53256840

Received: February 11, 2011 Revised: April 7, 2011

Accepted: April 14, 2011

Published online: October 7, 2011

of contrast ultrasonography may be valuable for non-invasive diagnosis of NASH.

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Key words: Nonalcoholic steatohepatitis; Leptin; Kupffer cell; Methionine choline-deficient diet; Contrast ultrasound

Peer reviewer: Søren Rafaelsen, MD, Consultant Radiologist, Associate Professor, Department of Radiology, Vejle Hospital, Vejle 7100, Denmark

Miyata Y, Miyahara T, Moriyasu F. Decreased accumulation of ultrasound contrast in the liver of nonalcoholic steatohepatitis rat model. *World J Gastroenterol* 2011; 17(37): 4191-4198 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4191.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4191>

Abstract

AIM: To investigate the diagnosis of nonalcoholic steatohepatitis (NASH) using contrast ultrasonography in the NASH rat model.

METHODS: The liver in methionine choline-deficient diet (MCDD) rats, a NASH model constructed by feeding an MCDD, was examined by contrast ultrasonography at weeks 2, 4, 8, 12 and 16, with late phase images of contrast ultrasonography (Kupffer imaging) in which contrast enhancement was achieved by incorporation of a contrast agent by Kupffer cells (KCs), and images were compared to those in rats taking a regular chow.

RESULTS: Decrease in contrast enhancement was observed first in MCDD rats at week 2. KCs were counted based on immunohistochemistry, but their numbers were not reduced and it was assumed that attenuation of contrast enhancement was attributable to reduced phagocytic activity of the KCs.

CONCLUSION: It is suggested that clinical application

INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is a relatively new disease entity, proposed by Ludwig in 1980^[1], which exhibits a clinical manifestation similar to that of alcoholic steatohepatitis in terms of liver histology, despite a lack of history of alcohol abuse causing hepatitis. The diagnosis of fatty liver without a history of drinking alcohol is generally termed nonalcoholic fatty liver disease (NAFLD), which is most simply fatty liver with an excellent prognosis, while NASH is a subtype of NAFLD^[2].

It is important to differentiate NASH from NAFLD at an early stage to start relevant treatment. Although there have been a number of reports aimed at differentiating NASH patients^[2], histological diagnosis based on liver biopsy is the only diagnostic method at present. However, liver biopsy is an invasive method, with a small risk of complications, and is too invasive for diagnosis of a benign disease, such as NASH.

It has been previously reported that liver-specific

Kupffer images of contrast ultrasound can be used for differential diagnosis between NASH and NAFLD^[3]. In this study, using a rat model of NASH, we carried out contrast ultrasonography to compare contrast enhancement between the NASH model and control rats that were fed a regular chow. LevovistTM (Schering AG, Berlin, Germany) and SonazoidTM (GE Healthcare, Oslo, Norway) were used as contrast ultrasonography agents. Microbubbles of LevovistTM and SonazoidTM are phagocytosed and incorporated by Kupffer cells (KCs) in the late contrast phase (Kupffer imaging)^[4]. There is a report that contrast enhancement by the contrast agent in the late contrast phase was decreased in patients diagnosed with NASH, compared to NAFLD patients^[3,5]. The reason for decreased microbubble accumulation in the NASH liver is presumed to be because phagocytosis of microbubbles by KCs is decreased.

We constructed a NASH model by feeding a methionine choline-deficient diet (MCDD, Oriental Yeast Co., Tokyo, Japan) to rats^[6-8]. Then, examining the liver using contrast ultrasonography, we compared the phagocytic activity by KCs in rats eating an MCDD and those given regular chow, using contrast enhancement in the late contrast phase.

MATERIALS AND METHODS

Animals and construction of a NASH model

Male Wistar rats weighing about 50 g at the time of purchase were used and kept in a room at 21 °C ± 2 °C with a 12-h cycle of light and darkness. The NASH model was constructed by feeding an MCDD to 15 rats, and the group was designated the MCDD group. Conversely, a diet of regular chow was fed to 5 rats and they were designated the control group. Rats were subjected to experiments at 2, 4, 8, 12 and 16 wk after beginning the diet, and three rats in the MCDD group and one in the control group were used for experiments at each time point. During the follow-up period, water and regular chow were given *ad libitum*. Body weight was measured in all rats in both groups at the designated time points. In addition, to observe the histological changes in liver tissue in MCDD rats at the designated time points, part of the resected liver was fixed in 10% formalin after the experiments, and paraffin slices were prepared and stained with hematoxylin and eosin for histological examination. All animals received humane care and the experimental protocols were approved by the Animal Ethical Committee of Tokyo Medical University.

Incorporation of the contrast ultrasonography agent

After rats were anesthetized at the designated time point by inhalation of diethyl ether (Wako Pure Chemical Industries, LTD., Tokyo, Japan), the abdomen and thighs were shaved and the fascia exposed by an excision of abdominal skin. At the same time, the thigh was incised to expose the femoral vein and a 24-gauge indwelling needle (TERUMO Corp., Tokyo, Japan) was inserted to secure

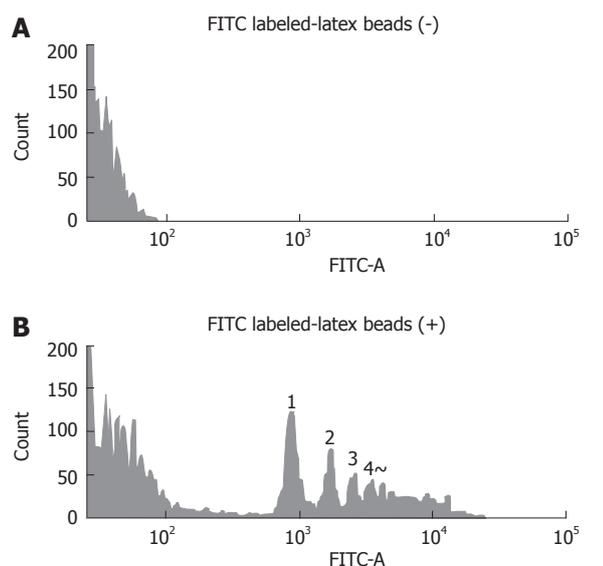
the vessel. From this site, a total of 500 µL of LevovistTM solution (10 µL/g body weight) with physiological saline was infused as a bolus. In both groups, the liver was scanned through the exposed fascia with an echo probe 10 min later. Images were scanned at mechanical index (MI) of 1.5 and then any LevovistTM remaining in the liver was destroyed by sweep scanning over the whole liver. Next, as for LevovistTM, a total of 500 µL of SonazoidTM 10 times diluted solution (1 µL/g of SonazoidTM solution/body weight) with physiological saline was infused intravenously as a bolus and, 10 min later, images were scanned and recorded at Advanced Dynamic FlowTM (ADF) ultrasound mode at MI of 1.5. An AplioTM (Toshiba Medical Systems, Tokyo, Japan) was used in this study. The frequency used was 7.5 MHz and the type of the transducer was linear. ADF was harmonic power Doppler mode from Toshiba Medical Systems.

A region of interest (ROI) was determined arbitrarily in the constant area of ultrasound images of the liver parenchyma obtained at 10 min, and contrast enhancement was quantified in the MCDD and control groups by intensity analysis software Image J (<http://rsb.info.nih.gov/ij/>). Quantified values in the MCDD group were expressed as relative values compared to those in the control group at each designated time point. An ROI was set in the region covering a wide area of the liver parenchyma, excluding large vessels as far as possible. The average intensity in the ROI was measured and relative values were compared.

In vivo administration of FITC-latex beads and isolation of KCs

After experiments using contrast ultrasound, 50 µL of FITC-latex beads (2 µm in diameter, Polyscience, Warrington, PA, United States) were mixed with 500 µL of physiological saline and infused from the tail vein to examine the phagocytic activity of KCs in rats in the MCDD and control groups. One hour later, KCs were isolated according to the following methods: Rats were anesthetized by inhalation of diethyl ether and a midline abdominal incision was made. A 20-G indwelling needle was inserted into the portal vein and after perfusion with Hanks Balanced Salt Solution (HBSS, Sigma, St. Louis, MO, United States), the portal vein was perfused with 100 mL of Dulbecco's Modified Eagle's Medium (DMEM/F-12, Sigma) containing 200 mg pronase (Roche Diagnostics Corp., Indianapolis, IN, United States), followed by 100 mL of DMEM/F-12 containing 25 mg collagenase (Nitta Gelatin Inc., Osaka, Japan). When collagenase leaked out of the blood vessel because of rupture during perfusion, and it was judged that the liver was not perfused, the procedure was stopped. After completion of perfusion, the digested liver was extracted carefully and incubated in DMEM/F-12 supplemented with 0.035% pronase and 62.5 units/mL of DNase (Sigma) for 20 min at 37 °C on a shaker incubator.

Next, the suspension was filtered through gauze and undigested liver tissue was discarded. The liver cell sus-



$$\text{KC phagocytic index} = [\text{FITC (+) cells} / \text{total KC (gated cells)}] \times 100\%$$

Figure 1 Measurement of Kupffer cells phagocytic activity by flow cytometry. A: The negative control, into which fluorescein isothiocyanate (FITC)-labeled latex beads were not injected; B: The positive control to which FITC-labeled latex beads were administered. Note the several peaks of the FITC from the beads in the positive control. The numbers on the peak show the number of latex beads phagocytosed. KC: Kupffer cell.

pension containing KCs was centrifuged for 1 min at 50 *g*/min and the supernatant collected. Then, the supernatant was centrifuged again at 50 *g*/min and parenchymal liver cells were removed as far as possible. Subsequently, the supernatant was centrifuged at 500 *g*/min for 8 min and a pellet of non-parenchymal cells was obtained. The supernatant was discarded and the pellet was resuspended with a small amount of washing buffer. The non-parenchymal liver cell suspension containing KCs was centrifuged at 900 *g*/min for 15 min with 50% and 25% Percoll (Pharmacia, Uppsala, Sweden) for density-gradient centrifugation, and the first layer from the top was collected. Next, the suspension was rinsed with 40 mL of HBSS and percentages of KCs that had phagocytosed FITC-latex beads were measured by flow cytometry. The purity of the KCs was examined by incorporation of latex beads 2 μm in size and confirmed to be always 96% or greater. Viability was examined by the trypan blue dye exclusion test and unstained cells accounted for 90% or more.

Changes in phagocytic activity of KCs

Phagocytosis by KCs isolated from each group was examined using a BD LSR-II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, United States). First, in a preliminary experiment, it was determined whether FITC-latex beads phagocytosed by KCs were measurable by the flow cytometer. The flow cytometry histogram of KCs in the control group is shown in Figure 1. The upper panel shows the flow cytometry histogram of KCs in the control rat without adding FITC-latex beads and

the lower panel shows the flow cytometry histogram of KCs in the control rat given FITC-latex beads. The amount of FITC-latex beads added was the same as in the subsequent experiments. There were peaks representing the number of FITC-latex beads, but the number of KCs that phagocytosed five beads or more was not determined. Nevertheless, it was possible to detect phagocytosed FITC-latex beads. KCs isolated in the control group were used to determine the position of gating and the gates were set a little wider. After the flow of 10 000 counts of KCs, the percentage of cells positive for fluorescence of FITC-latex beads in all gated cells was calculated in the MCDD and control groups and taken as the phagocytic activity.

Changes in KCs

Because there was a possibility that microbubble accumulation in the liver was dependent on the number of KCs in the liver, the liver was removed from one rat in each group, embedded in O.C.T. compound (Sakura Finetechnical Co. LTD, Tokyo, Japan) and snap-frozen immediately in liquid nitrogen. Later, 5 μm frozen sections were cut with a cryostat. Sections were fixed with acetone and subjected to immunohistochemistry for KCs with anti-rat macrophage antibody (ED2, SEROTEC Ltd, Oxford, United Kingdom) to count positive cells under the same magnification in the MCDD and control groups. In addition, 5 μm frozen liver specimens were cut with the cryostat and immunofluorescent latex beads were observed.

Statistical analysis

Unpaired Student's *t* test was employed for all statistical analyses and a *P* value less than 0.01 was considered statistically significant.

RESULTS

Body weight changes and liver histology in the MCDD and control groups

Body weight initially was about 50 g and increased in the MCDD group to 270 g at week 9, but decreased slightly thereafter. Although body weight reduced, general conditions were stable. In the control group, body weight increased steadily to about 440 g at week 16. General conditions also were stable (data not shown). In terms of histological changes in the liver, large-droplet fat deposition was recognized in the entire liver lobules at week 2 and later. At week 8, necrotic inflammatory changes were observed around the central vein and inflammatory findings were evident. At week 16, fibrosis was observed extending from the central vein and the MCDD group was judged to be a NASH model. In contrast, no fatty liver, inflammation, or fibrosis was observed up to week 16 in the control group (Figure 2).

Changes in contrast ultrasonographic findings

Signal intensity of the liver obtained by contrast ultrasound with LevovistTM was compared between the two groups.

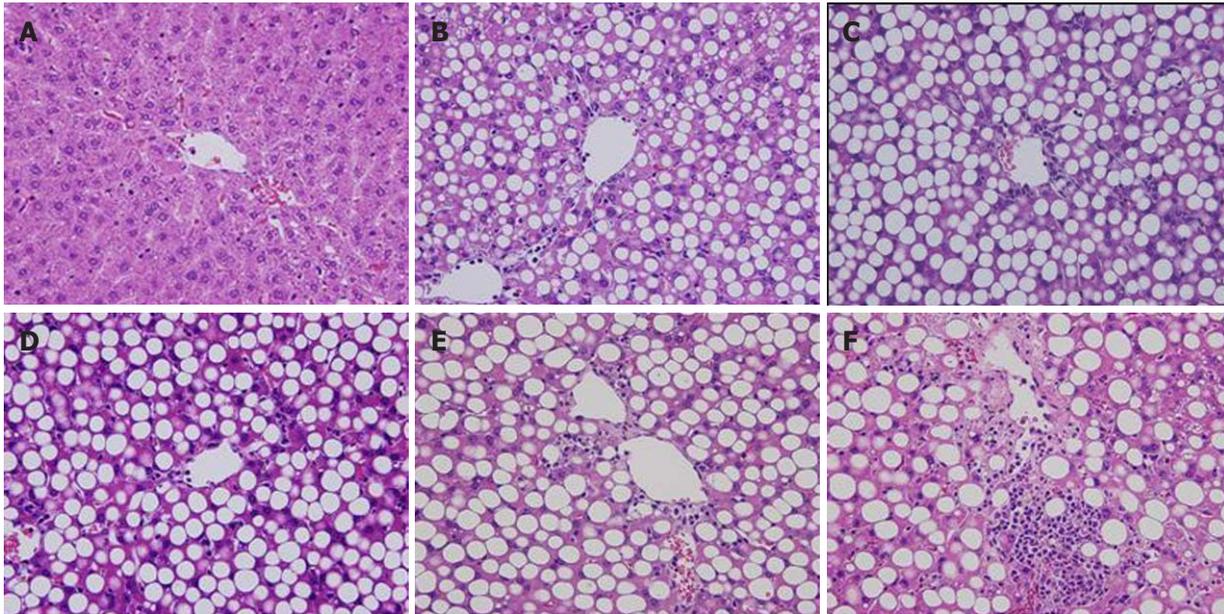


Figure 2 Histological changes of methionine choline-deficient diet-fed rat liver (hematoxylin eosin x 200). Extremely large vesicle fat deposits in almost the entire lobules were detected even as early as two weeks after the start of the methionine choline-deficient diet. By 8 wk, spotty necrosis was dispersed and by 16 wk, there was fibrosis extending from the central vein. A: Control; B: Two weeks methionine choline-deficient (MCD); C: Four weeks MCD; D: Eight weeks MCD; E: Twelve weeks MCD; F: Sixteen weeks MCD.

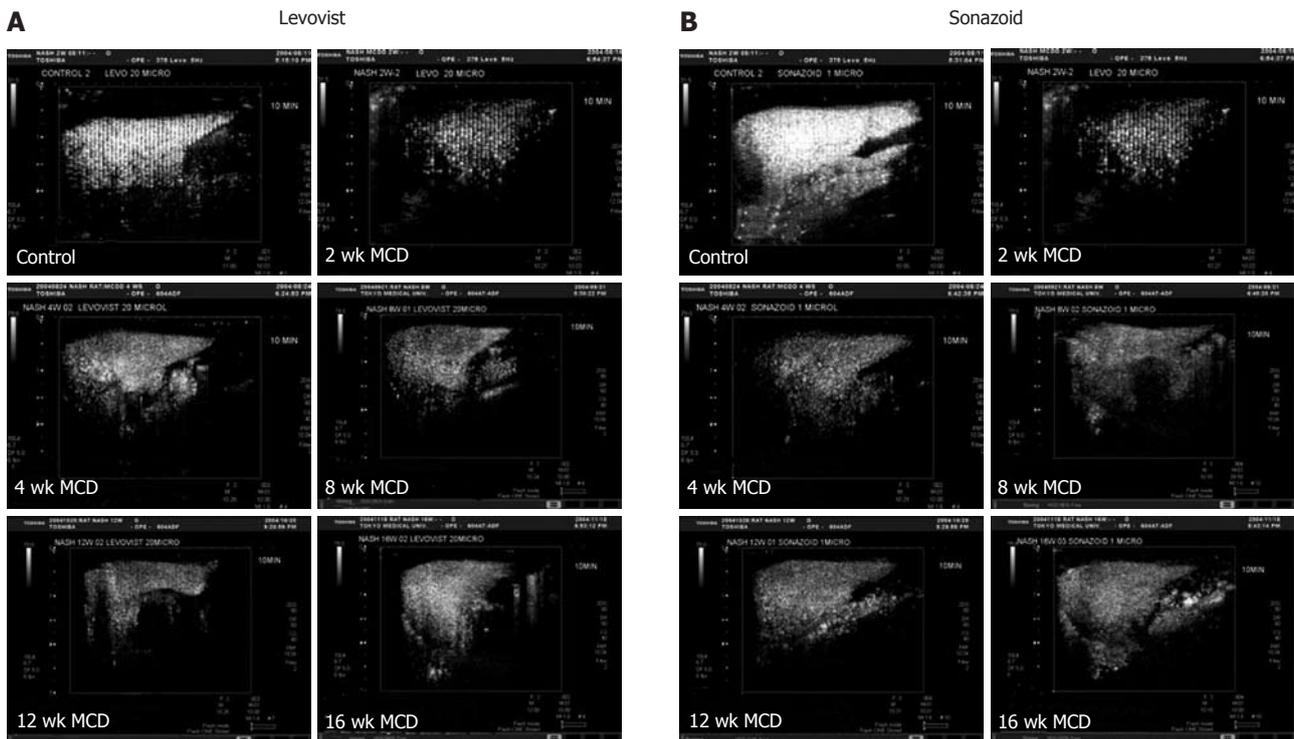


Figure 3 Ultrasound images of the liver 10 min after Levovist™ (A) and Sonazoid™ injection (B). Note signal intensity of the liver was lower in all methionine choline-deficient diet rats than in the controls. MCD: Methionine choline-deficient.

In the MCDD group, the intensity showed a significant decrease at weeks 2, 8 and 12, and a decrease, although not significant, at weeks 4 and 16 (Figures 3A and 4A). On the other hand, signal intensity of the liver obtained by contrast ultrasound with Sonazoid™ showed a significant decrease at all weekly time points in the MCDD group,

compared to the control group (Figures 3B and 4B).

Phagocytic activity of KCs in each group

Phagocytic activities (control *vs* MCDD) of KCs examined with fluorescent latex beads were 44.3% ± 12.6% *vs* 18.5% ± 9.8% (*P* < 0.01) at week 2, 55.1% ± 0.1% *vs*

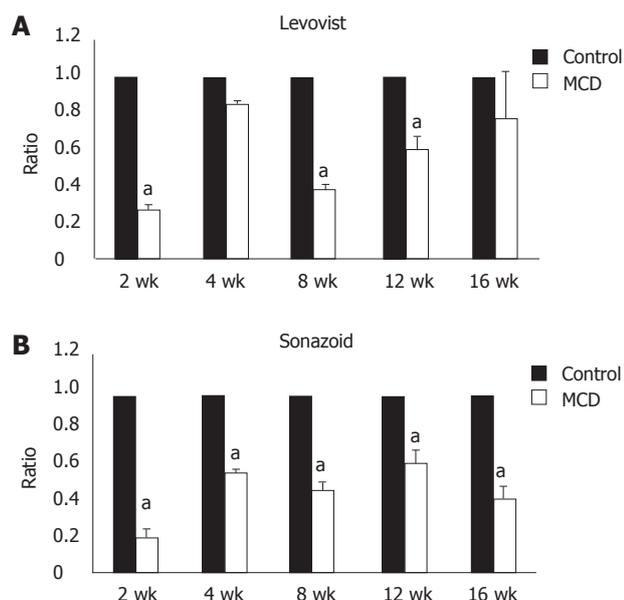


Figure 4 Changes in the signal intensity between control and methionine choline-deficient diet livers. A: Levovist™ contrast enhanced ultrasonography (CEUS). In the methionine choline-deficient diet (MCDD) liver, there was a significant decrease in Levovist™ signal intensity, compared to control livers at 2, 8 and 12 wk. While there was a lower tendency in signal intensity in MCDD liver than control liver at 4 and 16 wk, however, significance was not obtained; B: Sonazoid™ CEUS. In the MCDD liver, there was a significant decrease in Sonazoid™ signal intensity, compared to control livers at all time points. MCD: Methionine choline-deficient. ^a $P < 0.05$ vs control.

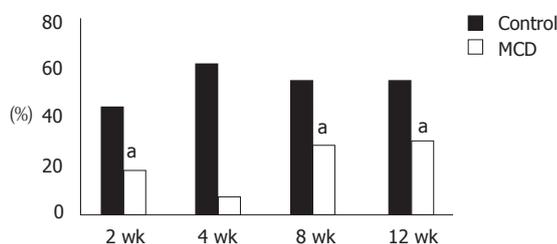


Figure 5 Decreased phagocytic activity in Kupffer cells from methionine choline-deficient diet rats compared to Kupffer cells from control rats. Control and methionine choline-deficient diet (MCDD) rats were injected with fluorescein-isothiocyanate (FITC)-labeled latex beads and Kupffer cells (KCs) were isolated 1 h later. KCs which had phagocytosed FITC-labeled latex beads were counted by flow cytometry and changes in the phagocytic activity were compared between control and MCDD KCs. Note that there was an almost 50% decrease in phagocytic activity in KCs from MCDD, compared to KCs from controls. ^a $P < 0.05$ vs control.

18.5% \pm 9.8% ($P < 0.01$) at week 8, and 57.4% \pm 3.4% *vs* 30.3% \pm 0.6% ($P < 0.01$) at week 12, and the activities in the MCDD group were about half those in the control group. At week 4, only one rat was examined but the activities were 61.9% in the control and 7.6% in the MCDD group, and again they tended to be lower in the MCDD group (Figure 5).

Counting of KCs and images of phagocytosed fluorescent latex beads

Because the decrease in the accumulation of the ultrasound contrast agent in the MCDD group potentially was

Group ($n = 10$)	¹ Total ED-2(+) cells
Control	122 \pm 14
MCDD	² 110 \pm 25

Numbers of Kupffer cells (KCs) in control and methionine choline-deficient diet (MCDD) livers were counted after immunostaining with ED-2 antibody, which recognizes residential macrophages or KCs in the liver. There was no significant difference in the numbers of KCs between controls and MCDD livers. ¹mean \pm SD; ²No significance.

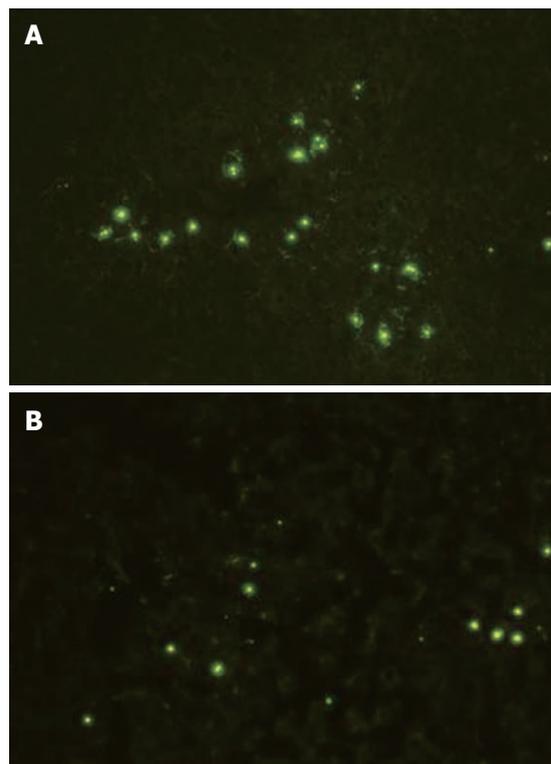


Figure 6 Fluorescein-isothiocyanate-labeled latex beads were injected through the tail veins of control and methionine choline-deficient diet rats. One hour later, small samples of the liver were snap frozen and sectioned using a cryostat. After immediate drying, unfixed tissues were observed by fluorescence microscopy. Note that there were fewer fluorescein-isothiocyanate-labeled latex beads in the livers from methionine choline-deficient diet (B) compared to the controls (A). Magnification: $\times 200$.

attributable to a decrease in the number of KCs compared to the control group, KCs were counted histologically based on immunohistochemistry with anti-rat macrophage antibody. As a result, there was no significant difference between the MCDD and control groups in the number of KCs (Table 1). In addition, fluorescence microscopy revealed a decrease in the accumulation of fluorescent latex beads in the MCDD group compared to the control group (Figure 6).

Serum leptin concentrations

Serum leptin concentrations (control *vs* MCDD) were 120.6 ng/L \pm 39.4 ng/L *vs* 31.5 ng/L \pm 77.6 ng/L ($P < 0.01$) at week 2, 396.7 ng/L \pm 95.0 ng/L *vs* 99.3 ng/L \pm

14.5 ng/L ($P < 0.01$) at week 4, 618.6 ng/L \pm 0 ng/L *vs* 79.2 ng/L \pm 7.0 ng/L ($P < 0.01$) at week 8, and 397.2 ng/L \pm 221.3 ng/L *vs* 93.7 ng/L \pm 21.5 ng/L ($P < 0.01$) at week 12, and were significantly lower in the MCDD group at all time points.

DISCUSSION

Fatty liver disease is a state in which triglycerides accumulate in hepatocytes. A manifestation of fat-related liver disease in non-drinkers is known as NAFLD, and consists of simple fatty liver and NASH accompanied by inflammatory changes. At present, it is difficult to differentiate NASH patients from a number of NAFLD patients by any diagnostic method other than liver biopsy. However, biopsy is invasive and potentially causes critical complications. Because it has been reported that 5% of NAFLD patients develop liver cirrhosis^[9], it is important to diagnose NASH at an early stage. There have been reports of differentiating NASH patients from NAFLD patients without the invasiveness of liver biopsy^[2,10]. It has been reported that contrast enhancement is decreased in the late contrast phase in NASH patients compared to healthy subjects^[3,5]. In this study, contrast enhancement was compared by injection of the contrast ultrasonography agent in a NASH model receiving MCDD and in controls taking a regular chow.

MCDD rats have been used in research to investigate the pathogenesis of NASH^[6,11,12]. We used MCDD rats as the most general NASH model developing steatohepatitis, which was induced quickly and easily by the administration of MCDD alone. In this study, the rats started to show fat deposition two weeks after the beginning of MCDD administration, steatohepatitis at week 8 and fibrosis at week 16.

LevovistTM is a first generation ultrasound contrast agent consisting of air as the inner gas within a lipid shell. SonoVueTM, OptisonTM, DefinityTM, ImagentTM and SonazoidTM are available commercially for liver imaging. LevovistTM and SonazoidTM are easily phagocytosed by KCs^[13] and have a liver-specific contrast phase. The ultrasound contrast agent LevovistTM, a high MI contrast agent consisting of microbubbles, is destroyed when exposed to ultrasound with a high acoustic pressure. Most Doppler signals are retrieved when all microbubbles in the scan volume are destroyed with high MI ultrasound. On the other hand, SonazoidTM is a new-generation ultrasound contrast agent, and develops resonance and vibration under the conditions of lower acoustic pressure than that for LevovistTM, because of an elastic phospholipid shell around the inner gas, perfluoro-butane. It is a low MI contrast agent to visualize harmonic signals. Compared to LevovistTM, SonazoidTM has the advantage of enabling evaluation of contrast enhancement in a real-time manner.

In order to quantify the accumulated microbubbles in the liver parenchyma, we destroyed microbubbles by exposing high MI ultrasound to both contrast agents, Levo-

vistTM and SonazoidTM. The period of about 2 min from the injection of the ultrasound contrast agent reflects the early vascular phase, while the time period from about 10 min later reflects the delayed parenchymal phase, or Kupffer imaging, representing the time for phagocytosis of microbubbles by KCs^[14,15]. Therefore, in the delayed parenchymal phase, it is possible to obtain information similar to that provided by superparamagnetic iron oxide (SPIO)-enhanced MRI^[16]. Clinically, about 25% of the ultrasound contrast agent injected from the peripheral vein is phagocytosed by KCs and visualized as the hepatic parenchyma-specific contrast^[17]. It has been reported that dysfunction of KCs is involved in NAFLD^[18].

In this study, images were scanned to measure the signal intensity 10 min after infusion of the ultrasound contrast agent, and accumulation of the medium by KCs was compared between the MCDD and control groups. As a result, contrast enhancement was decreased in MCDD rat livers, as in clinically diagnosed NASH patients. There are two potential mechanisms for the decrease of contrast enhancement in Kupffer imaging, which represents phagocytosis of microbubbles by KCs. These mechanisms are a reduction in phagocytosis by KCs and a decrease in the number of KCs despite normal phagocytic activity. To count the number of KCs, KCs were immunostained with anti-rat macrophage antibody, and it was found that there was no statistical difference in the number of KCs. Therefore, it was thought that decrease of contrast enhancement was attributable to a reduction in phagocytic activity of KCs, rather than a decrease in the number of KCs.

As for the pathogenesis of NASH, phagocytic activity of KCs and involvement of leptin have been suggested. Leptin is an adipocytokine produced by adipose tissue which interacts with a receptor in the hypothalamus. Leptin controls the amount of body fat by suppressing appetite and stimulating sympathetic nerve activity. NAFLD patients with obesity and insulin resistance exhibit leptin resistance and, thereby, serum leptin concentrations increase^[18].

In *ob/ob* mice defective in the leptin gene^[19], fatty liver is present in association with insulin resistance and obesity, and leptin administration improves fatty liver^[20]. In addition, *db/db* mice harboring an abnormality in the leptin receptor do not respond to leptin and develop obesity and fatty liver, as do *ob/ob* mice. Loffreda isolated and cultured macrophages from the peritoneum and bone marrow in *db/db* mice (with abnormality in the leptin receptor) and *ob/ob* mice (deficient in the leptin gene), added *Candida parapsilosis*, and examined the phagocytic activity of macrophages in the groups with and without addition of leptin. Phagocytic activity was augmented in the *ob/ob* mice but unchanged in the *db/db* mice by addition of leptin, which suggested the possibility that leptin interacted directly with KCs to regulate phagocytic activity^[21].

Sakaida *et al*^[22] examined lipopolysaccharide (LPS)-induced phagocytic activity and production of TNF- α in

the KCs isolated from *fa/fa* rats, with a mutation in the leptin receptor gene, and reported that both were reduced compared to the control (*fa/-* rats) and therefore leptin potentially affected the function of KCs. It has been presumed that leptin activated KCs and their phagocytic activity, and phagocytic activity of KCs and serum leptin concentrations were indeed decreased in MCDD rats in this study. In other words, because the amount of leptin secretion was low in MCDD rats, activation of KCs by leptin was attenuated and, thereby, the phagocytic activity of KCs was decreased. Although stimulation with leptin may be closely associated with the phagocytic activity of KCs, it is one of our experimental challenges to isolate and culture KCs from MCDD rats and determine whether addition of leptin will improve their phagocytic activity.

Although there is a report that contrast enhancement by contrast ultrasound was decreased in NASH patients compared to NAFLD and type C chronic hepatitis patients^[5], its pathogenesis has yet to be elucidated. By downregulating KCs and provoking leptin resistance, persistence of high concentrations of serum leptin levels decreases the activity of leptin and thereby reduces phagocytic activity in NASH patients. In this study, serum leptin concentrations were low and reduced leptin activity was one potential factor responsible for the pathogenesis. It should be possible to clinically diagnose NASH patients non-invasively, without liver biopsy, by quantifying contrast enhancement in contrast ultrasonography^[3]. Sensitivity and specificity for diagnosis of NASH differentiation from NAFLD are more than 90% and decrease in Levovist accumulation is the early stage of NASH.

In MCDD rats, pathological findings revealed fatty liver without inflammation or necrosis two weeks after the beginning of the MCDD, and the phagocytic activity of KCs was attenuated. These results suggest that it may be possible to diagnose NASH in human NASH patients at an early stage when inflammation, necrosis, and fibrosis specific for NASH have not developed. In addition, should early diagnosis of NASH be possible, early treatment would be feasible with prevention of progression to liver cirrhosis, and improvement of prognosis might be expected. It is necessary to accumulate clinical cases examined by a combination of contrast ultrasound and liver biopsy.

In conclusion, contrast enhancement in the late phase of contrast ultrasound was compared in MCDD rats and rats taking a regular chow. In the MCDD rats, accumulation of the contrast medium was attenuated compared to the control rats. As for the cause, a reduction in phagocytic activity by KCs was suspected, but activation by serum leptin might also be involved in the reduction of phagocytic activity.

COMMENTS

Background

Nonalcoholic steatohepatitis (NASH) is a disease that exhibits inflammation and fibrosis, and NASH may progress to liver cirrhosis and hepatocellular carcinoma. Early clinical diagnosis is important and difficult, and histological diagnosis based on liver biopsy is the only diagnostic method of NASH at the present time.

Research frontiers

Liver biopsy is somewhat invasive and may cause some complications, such as bleeding in the abdominal cavity or infection. Thus, a non-invasive method to diagnose NASH needs to be developed. It has been previously reported that liver-specific Kupffer images of contrast ultrasound can be used for differential diagnosis between NASH and nonalcoholic fatty liver disease (NAFLD).

Innovations and breakthroughs

The authors carried out a comparison of contrast ultrasonography between a NASH model and control rats. In the NASH model group, the intensity showed a significant decrease.

Applications

Contrast ultrasonography can be easily used to differentially diagnose NASH from NAFLD without liver biopsy.

Peer review

In the study, the authors demonstrated that phagocytic function of Kupffer cells from NASH liver is decreased. In this animal study, the authors also found that late phase contrast enhanced-ultrasound may be helpful in diagnosing NASH. Late phase contrast enhancement of NASH liver by ultrasound was lower compared to the simple fatty liver. The use of objective contrast quantification software appears promising. This might have implications in the diagnosis of NASH patients and in the evaluation of focal liver lesions in NASH patients; however further human studies are needed to evaluate this method.

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S- Editor Tian L L- Editor Logan S E- Editor Xiong L

Comparative outcome of stapled trans-anal rectal resection and macrogol in the treatment of defecation disorders

Ivano Biviano, Danilo Badiali, Laura Candeloro, Fortunée Irene Habib, Massimo Mongardini, Angelo Caviglia, Fiorella Anzini, Enrico S Corazziari

Ivano Biviano, Danilo Badiali, Laura Candeloro, Fortunée Irene Habib, **Fiorella Anzini**, **Enrico S Corazziari**, Department of Medicine and Medical Specialties, SAPIENZA, Rome University, 00161 Rome, Italy

Massimo Mongardini, Department of Surgical Sciences, Division of General Surgery L, SAPIENZA Rome University, 00161 Rome, Italy

Angelo Caviglia, Departmental Operative Unit of Colonproctology, Hospital San Camillo-Forlanini, 00161 Rome, Italy

Author contributions: Biviano I maintained the database and obtained the follow-up data; Badiali D supplied the patients, acquired the data, performed the anorectal manometry, and interpreted results; Habib FI acquired data, performed the defecography, and interpreted the results; Mongardini M and Caviglia A supplied patients and performed the stapled trans-anal rectal resection procedure; Candeloro L maintained the database and obtained follow up data; **Anzini F provided technical assistance**; Corazziari ES, conceived the study protocol, supplied patients, interpreted the data, and critically revised the article.

Correspondence to: Dr. Danilo Badiali, Department of Medicine and Medical Specialties, SAPIENZA, Rome University, Italy Viale del Policlinico 155, 00161 Rome, Italy. danilo.badiali@uniroma1.it

Telephone: +39-06-49978305 Fax: +39-06-49978384

Received: December 22, 2010 Revised: March 4, 2011

Accepted: March 11, 2011

Published online: October 7, 2011

Abstract

AIM: To prospectively assess the efficacy and safety of stapled trans-anal rectal resection (STARR) compared to standard conservative treatment, and whether preoperative symptoms and findings at defecography and anorectal manometry can predict the outcome of STARR.

METHODS: Thirty patients (Female, 28; age: 51 ± 9 years) with rectocele or rectal intussusception, a defecation disorder, and functional constipation were

submitted for STARR. Thirty comparable patients (Female, 30; age 53 ± 13 years), who presented with symptoms of rectocele or rectal intussusception and were treated with macrogol, were assessed. Patients were interviewed with a standardized questionnaire at study enrollment and 38 ± 18 mo after the STARR procedure or during macrogol treatment. A responder was defined as an absence of the Rome III diagnostic criteria for functional constipation. Defecography and rectoanal manometry were performed before and after the STARR procedure in 16 and 12 patients, respectively.

RESULTS: After STARR, 53% of patients were responders; during conservative treatment, 75% were responders. After STARR, 30% of the patients reported the use of laxatives, 17% had intermittent anal pain, 13% had anal leakage, 13% required digital facilitation, 6% experienced defecatory urgency, 6% experienced fecal incontinence, and 6% required re-intervention. During macrogol therapy, 23% of the patients complained of abdominal bloating and 13% of borborygmi, and 3% required digital facilitation. No preoperative symptom, defecographic, or manometric finding predicted the outcome of STARR. Post-operative defecography showed a statistically significant reduction ($P < 0.05$) of the rectal diameter and rectocele. The post-operative anorectal manometry showed that anal pressure and rectal sensitivity were not significantly modified, and that rectal compliance was reduced ($P = 0.01$).

CONCLUSION: STARR is not better and is less safe than macrogol in the treatment of defecation disorders. It could be considered as an alternative therapy in patients unresponsive to macrogol.

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Key words: Constipation; Obstructed defecation; Rectocele; Rectal intussusception; Stapled trans-anal rectal resection

Peer reviewer: Imran Hassan, MD, Assistant Professor, Department of Surgery, SIU School of Medicine, 701 North Rutledge, PO Box 19638, Springfield, IL 62794, United States

Biviano I, Badiali D, Candeloro L, Habib FI, Mongardini M, Caviglia A, Anzini F, Corazziari ES. Comparative outcome of stapled trans-anal rectal resection and macrogol in the treatment of defecation disorders. *World J Gastroenterol* 2011; 17(37): 4199-4205 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4199.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4199>

INTRODUCTION

Functional constipation affects up to 20% of the population, and about 50% of constipated patients consulting a tertiary referral practice complain of difficult evacuation^[1,2], such as straining at stools, sensation of incomplete evacuation, or ano-rectal obstruction^[3,4], and may require digitation to facilitate defecation. Rectocele and intra-rectal intussusception are frequent findings in patients with functional constipation, and are thought to play a relevant role in defecatory alterations^[5]. Surgical repair of the recto pelvic anatomy has been proposed to improve defecation. Recently, an international consensus conference^[6] proposed that “the combination of the characteristic history of disordered defecation and the anatomical finding of one or more of the following: rectocele, rectal intussusception, perineal descent, mucosal prolapse may lead a surgeon to offer the stapled trans-anal rectal resection (STARR)^[7-9] procedure, provided that the individual has failed medical management”.

The STARR procedure consists of the trans-anal resection of the distal rectum using a double-stapler. Semi-circular purse-string sutures are applied on the prolapsed rectal wall, including mucosa, submucosa, and rectal muscle wall, at 2 cm above the hemorrhoidal apex so as to include the rectocele and the internal rectal prolapse.

So far both favorable^[10] and unfavorable results^[11] of the procedure have been reported. However, **no study** has assessed the efficacy and safety of the STARR procedure in comparison with evidence based conservative treatment for the management of functional constipation and defecatory disorders.

The main aim of the present study was to assess the efficacy and safety of the STARR procedure in the treatment of patients with chronic constipation complaining of defecatory disorders and with defecographic evidence of rectocele and intra-rectal intussusception. An additional aim of the study was to assess whether preoperative symptoms, and manometric or defecographic findings, can predict the long-term outcome of the STARR procedure.

MATERIALS AND METHODS

Population

Consecutive outpatients referred for refractory chronic

constipation in a 48-mo period by two surgical and one gastroenterological centers, underwent a diagnostic work up, including anorectal manometry and defecography. Patients with refractory chronic constipation were identified as those who did not respond to the usual conservative treatment and still complained of difficult and/or incomplete evacuation, despite the use of high daily doses of contact laxatives, enemas, or digital evacuation. Inclusion criteria were the following: (1) diagnosis of functional constipation according to the Rome III criteria during the preceding three months, with onset at least 6 mo prior to the diagnosis (in the absence of laxatives and/or enemas) of two or more of the following complaints: less than three bowel movements (BM) per week; straining at defecation and/or sense of incomplete evacuation and/or hard stools and/or sensation of anorectal obstruction/blockage and/or manual maneuvers to facilitate defecation on at least 25% of occasions; (2) difficult evacuation defined as either straining or sensation of obstruction/blockage; (3) age between 18 and 75 years; (4) no previous anorectal surgery; (5) no abnormality at barium enema or colonoscopy; (6) normal laboratory routine tests; (7) evidence of rectocele and/or intra-rectal intussusceptions at defecography; (8) no previous treatment with oral macrogol solution; (9) no pregnancy and efficacious birth control methods; (10) absence of systemic disease; and (11) absence of therapy affecting intestinal function. Chronic anxiolytic and antidepressive treatment were admitted provided the dosage was not modified during the study period. Exclusion criteria were the following: (1) no diagnosis of functional constipation; (2) previous anorectal surgery; (3) age less than 18 and above 75 years; (4) absence of rectocele and intra-rectal intussusception at defecography; (5) abnormality at barium enema or colonoscopy; (6) abnormal laboratory tests; (7) previous treatment with oral macrogol solution; (8) pregnancy and no use of efficacious birth control methods; (9) presence of systemic disease; and (10) presence of therapy affecting intestinal function.

Study protocol

At referral, all patients were interviewed with a standardized questionnaire, and had a physical examination. The questionnaire inquired about bowel habit: frequency of defecation, straining, stool consistency, sensation of incomplete evacuation, sensation of anal obstruction/blockage, digital facilitation to evacuate, anal pain, and anal incontinence.

On a different day, a colonoscopy was performed in patients over 50 years of age who had not had a colonoscopy or a barium enema in the previous five years. Patients were then submitted on different days for defecography and anorectal manometry.

Thirty consecutive patients, reporting an unsatisfactory response to the conservative treatment of constipation with different types of laxatives, were enrolled by the two surgical units; sixteen by AC and fourteen by

MM, and were then submitted to the STARR procedure according to a standardized and previously published method^[10]. In the same time period, thirty additional and consecutive patients referred to the gastrointestinal center were assigned conservative treatment with oral macrogol solution. These patients were instructed to consume 1 sachet (8.75 g) of macrogol dissolved in 125 mL of water *bid.*, with the option to either reduce the dose to *od* or increase it up to *qid.* to obtain evacuation of soft stools.

After the STARR procedure patients, were subjected to a second rectoanal manometry and defecography.

All patients were re-assessed at least 24 mo after the surgical procedure or the medical prescription.

At follow-up, the patients were interviewed, either face to face or by telephone, with the same standardized questionnaire used at referral, which included additional items related to treatment satisfaction and adverse events. Patients were invited to declare whether they were “totally dissatisfied with the treatment”, or “partially satisfied with the treatment” when at least one symptom of constipation and/or side effects were present, or “fully satisfied with the treatment”.

The degree of constipation was evaluated with the Wexner score^[12]. The study was approved by the local ethics committee.

Defecography

A cleansing non-medicated water enema was performed the night before the radiological examination. About 200 milliliters of barium paste were injected into the rectum, through an anal catheter. Continuous injection of the contrast during slow withdrawal of the catheter rendered the anal canal opaque. Patients were then seated on a radiolucent commode. The entire evacuation sequence was recorded on videotape (JVC SR-VS30E Mini DV/S-VHS). Latero-lateral radiograms were taken at rest and during mid-evacuation, as previously reported^[13].

Anorectal manometry

After a cleansing enema, rectal sensitivity and anorectal manometry were evaluated using a multilumen polyethylene catheter with four open tips disposed radially, and 0.5 mm apart longitudinally, continuously perfused (0.5 mL/min) with bubble-free distilled water by means of a pneumo-hydraulic infusion system (Arndorfer, Milwaukee, Wisconsin, United States), and connected to Beckman 611 external transducers. A fifth lumen ended in a latex balloon attached to the tip of the catheter. Intraluminal pressure variations were transmitted from the transducers to a polygraph (R612 Dynograph Recorder Sensor-Medics Italia srl) for recording.

The resting pressure profile of the anal canal was recorded with a pull-through technique. Thereafter, the manometric probe was positioned in the anal canal with the recording holes and the deflated balloon in the rectum. The patient was then asked to squeeze and to strain. Lastly the intra-rectal balloon was intermittently inflated

with progressive volumes of air to elicit the recto-anal inhibitory reflex (RAIR). To assess the threshold of rectal sensitivity, patients were instructed to refer the first sensation of rectal distension and/or the urge to defecate during the incremental intrarectal balloon inflations^[14].

Analysis of data

The primary endpoint of the study was relief of constipation, i.e., when the patient no longer met the Rome III criteria for functional constipation and was considered a responder.

Secondary endpoints were: the assessment of (1) the constipation improvement by means of the Wexner score; and (2) symptoms, defecographic, and manometric findings in predicting the outcome of the STARR procedure.

Analysis of defecographic data: **Frame by frame** analysis of the sequences recorded on the videotape assessed timing and dynamics of evacuation, rectal emptying, and presence of anatomical alterations of the rectal wall. Anorectal angle (ARA) widening, anal canal opening, pelvic floor (PF) location at rest, and its mobility were assessed on the latero-lateral radiograms. Rectocele was defined as an outpouching of the anterior rectal wall into or across the rectovaginal septum, rectal intussusception was defined as an enfolding of the rectal wall, which may (intra-anal) or may not (intra-rectal) protrude through the anal canal. Pelvic floor dyssynergia was defined as: anorectal angle (ARA) widening $< 10^\circ$ and/or the opening of anal canal < 10 mm, and/or anal canal opening > 10 mm in more than 30 s or interrupted by repetitive squeezing contractions. Contrast rectal residue was assessed by a semi-quantitative evaluation of the rectal residue as small ($< 40\%$), intermediate (40% - 70%), and abundant ($> 70\%$).

Analysis of manometric data: **Maximal resting and squeezing pressures** were identified as the maximal steady value observed for 20 s in basal condition and during maximal voluntary anal contraction. The thresholds of RAIR and rectal sensitivity were defined as the smallest inflated volumes of the intrarectal balloon inducing, respectively, a fall in anal pressure of at least 12 mmHg^[14], and the urge to evacuate. Rectal compliance was calculated as the ratio between intrarectal balloon volume inflated with 100 mL of air and intrarectal pressure. During straining, a decrease of anal pressure of more than 20% was considered a normal relaxing pattern; the absence of a decrease of less than 20%, or the increase, of the anal pressure were considered as dyssynergic patterns.

Statistical analysis

Results are reported as means and standard deviation (mean \pm SD). Descriptive statistical techniques were used to compare the two groups of patients. A χ^2 test and Fisher's exact test were used to compare the frequency of symptoms in the different study groups; Student's *t* test was used to compare the two groups for

continuous variables.

RESULTS

Sixty patients were enrolled: 30 patients underwent STARR, and 30 patients were treated with macrogol. The two study groups were comparable for gender, age, and symptom presentation (Table 1).

Macrogol group: The follow up period was 44 ± 11 mo. Twenty-two (75%) patients were classified as responders; constipation by the Wexner score decreased significantly (13.9 ± 1.5 vs 5.9 ± 4.5 ; $P < 0.001$). Seven patients (27%) had discontinued macrogol and were using either contact or other osmotic laxatives; two patients still required digital facilitation (Figure 1).

STARR group: The follow up period was 38 ± 18 mo. Sixteen (53%) patients were classified as responders. No outcome difference was observed between patients of the two surgical units (responders: 8/16 vs 6/14). Constipation by the Wexner score decreased significantly (13.4 ± 3.2 vs 7.32 ± 5.76 ; $P < 0.001$); nine (30%) patients still used laxatives and four (13%) digital facilitation (Figure 1). After the surgical procedure, 14 patients reported at least one side effect and two (6%) required re-intervention; the first for relapse of rectocele and the second for fecal incontinence. The presenting symptoms at referral did not differ between responders and non-responders (Table 2).

Comparison between the study groups: The two groups did not differ statistically for response to the treatment according to the Rome criteria for functional constipation, for improvement of constipation evaluated by means of the Wexner score, and for degree of satisfaction (Table 3). Bowel symptoms at follow-up were similar in the two study groups (Figure 1).

Adverse events in the STARR procedure group, at the end of follow up, were: staining/leakage (13%), fecal incontinence (6%), urgency (6%), intermittent anal pain (17%), and re-intervention (6%). Adverse effects in the macrogol therapy group were abdominal bloating (23%), and borborygmi (13%).

Defecography

Defecography was performed at referral and at 7 ± 4 mo after STARR in 16 patients. In comparison to the pre-surgical condition, defecographic variables did not vary after surgery, except for a significant reduction in rectal diameter (7.5 ± 1.2 cm vs 5.6 ± 1.2 cm; $P < 0.001$), and size of rectocele (3.9 ± 1.3 cm vs 1.4 ± 1.5 cm; $P < 0.001$). The size of intussusceptions was reduced, but the variation was not statistically significant (3.2 ± 1.7 cm vs 2.4 ± 1.3 cm; ns).

Responders and non-responders did not statistically differ for any defecographic variable assessed before surgery (Table 3). Before the operation, the mean ARA variation during evacuation was not statistically different between non-responders and responders (Table 4).

Table 1 Clinical presentations of the two study groups *n* (%)

	STARR <i>n</i> = 30 (F28)	Macrogol <i>n</i> = 30 (F30)	<i>P</i> value
Mean age (yr)	51 ± 9	53 ± 13	
Duration of constipation > 10 yr	24 (80)	26 (87)	0.7
Straining	28 (93)	29 (97)	1
Hard stools	27 (90)	29 (97)	0.6
Incomplete evacuation	27 (90)	27 (90)	0.7
Anal blockage	27 (90)	27 (90)	0.7
Digital facilitation	11 (37)	8 (27)	0.6
< 3 BM/wk	23 (77)	26 (87)	0.5
Laxatives	27 (90)	30 (100)	0.2
Rectal bleeding	13 (43)	7 (23)	0.2
Rectocele > 3 cm	23 (77)	15 (50)	0.06
Rectal intussusception	15 (50)	20 (67)	0.3

BM: Bowel movements; STARR: Stapled trans-anal rectal resection.

Table 2 Presenting symptoms in responders and non-responders to stapled trans-anal rectal resection *n* (%)

	Responders	Non-responders	<i>P</i> value
Straining	16 (100)	12 (86)	0.4
Hard stools	14 (87)	13 (93)	0.9
Incomplete evacuation	16 (100)	11(78)	0.2
Anal blockage	15 (94)	12 (86)	0.9
Digital facilitation	4 (25)	7 (50)	0.3
< 3 BM/wk	13 (81)	10 (71)	0.8
Laxatives	14 (87)	13 (93)	0.9

BM: Bowel movements.

Table 3 Number of responders, degree of satisfaction, and Wexner constipation score variation during macrogol and after stapled trans-anal rectal resection treatment *n* (%)

	Macrogol	STARR	<i>P</i> value
Responders	22 (73)	16 (53)	0.2
Satisfaction			0.4
Total	18 (60)	16 (53)	
Partial	5 (17)	9 (30)	
Not satisfied	7 (23)	5 (17)	
Δ Wexner score, mean ± SD	8 ± 5	6 ± 5.2	0.1

STARR: Stapled trans-anal rectal resection; Δ: Difference.

After the operation, it was significantly less in the non-responders compared to the responders (28 ± 16 vs 8 ± 20 degrees; $P < 0.05$).

Before STARR, defecographic evidence of pelvic floor dyssynergia was detected in three patients; equally represented in responders (one patient) and non-responders (two patients). After STARR, defecographic evidence of pelvic floor dyssynergia was detected in three patients; equally represented in responders (one patient) and non responders (two patients).

Ano-rectal manometry

Ano-rectal manometry was performed at referral and 24 ± 4 mo after STARR in 12 patients. After STARR, rectal compliance was significantly reduced (5.4 ± 1.9 vs $3.7 \pm$

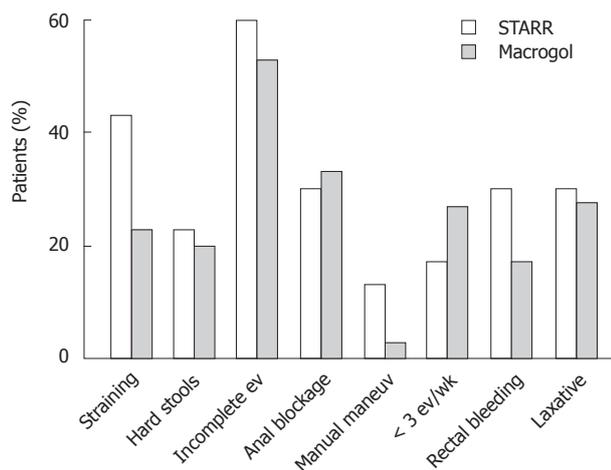


Figure 1 Percentage of patients with constipation symptoms, rectal bleeding, and use of laxatives (other than macrogol in the macrogol treatment group) after stapled trans-anal rectal resection and during macrogol treatment. No statistical difference was observed between the two study groups. STARR: Stapled trans-anal rectal resection. ev: Evacuations.

1.2 mL/mmHg; $P = 0.01$), all other manometric variables did not vary. Responders and non-responders did not differ for any manometric findings before and after the procedure.

Before STARR, manometric evidence of pelvic floor dyssynergia was detected in four patients, equally represented in responders (two patients) and non-responders (two patients). After STARR, manometric evidence of pelvic floor dyssynergia was detected in seven patients, equally represented in responders (four patients) and non-responders (three patients).

DISCUSSION

No previous study has compared the STARR procedure with conservative pharmacological therapy. This study evaluated, over a long time period, the outcome of STARR treatment for constipation, defined with standardized and validated criteria. The surgical procedure was performed following the standardized and previously published procedure^[9] by two experienced coloproctology units that obtained comparable postsurgical outcomes. The patients submitted to the STARR procedure reported an unsatisfactory response to a usual, but not standardized, laxative treatment.

Previous studies have reported the postsurgical outcomes of the STARR procedure or of other modified techniques, but no study has compared the efficacy and safety of STARR procedure versus the standardized conservative treatment.

In the present investigation, we compared the outcome of STARR procedure with the gold standard treatment of constipation based on macrogol in a prospective, parallel group, longitudinal study. This therapy for functional constipation is supported by level 1 evidence and grade A recommendation^[15]. This is a high molecular weight (3350 or 4000) non-absorbable, non-metabolized soluble

Table 4 Defecographic findings in responders and non-responders submitted to stapled trans-anal rectal resection (mean \pm SD)

Variables	Before		After	
	Non-responders	Responders	Non-responders	Responders
Rectal diameter (cm)	7.7 \pm 1.4	7.3 \pm 1	5.5 \pm 0.9	5.8 \pm 1.5
Δ ARA evacuation ($^\circ$)	16 \pm 8	25 \pm 18	8.1 \pm 20.3	28 \pm 16.2 ^a
PF at rest (cm)	5.1 \pm 1.5	4.8 \pm 1.3	4.3 \pm 1.5	4.6 \pm 1
PF during squeezing (cm)	1.4 \pm 0.7	1.3 \pm 0.9	1.8 \pm 0.9	1.2 \pm 0.8
Δ PF evacuation (cm)	3.1 \pm 1.6	3 \pm 1.3	3.6 \pm 1.1	3.1 \pm 1.5
Rectocele (cm)	3.8 \pm 1.8	4.1 \pm 0.6	1.1 \pm 1.6	1.7 \pm 1.5
Anal diameter (cm)	1.4 \pm 0.6	1.4 \pm 0.8	1.3 \pm 0.8	1.1 \pm 0.4

ARA: Ano-rectal angle; PF: Pelvic floor; Δ : Difference; ^a $P < 0.05$ vs non-responders.

polyethylene, which forms hydrogen bonds with water in the gut; it is used with orthograde whole-gut irrigation in preparing for colon investigation, and in small-volume daily doses (125-250 mL) to treat functional constipation. Macrologol therapy is reported to be effective in about 80% of constipated patients, is well tolerated, and devoid of serious side effects, even in long term treatment^[16,17].

All the patients of these study groups met the Rome III criteria for functional constipation and had defecographic evidence of rectocele and or intrarectal intussusception. Patients were evaluated with a standardized questionnaire before and after treatment, with a mean follow-up of 38 mo, which is, to our knowledge, one of the longest reporting STARR outcome^[18-31]. Some long-term studies reported sustained improvement of defecation score, but provided conflicting results about side effects, relapse, and complications^[32-34].

The present study demonstrated that the surgical treatment was less, but not statistically so, efficacious than the conservative one, as indicated by the finding that 75% of the macrogol group and 53% of the STARR group did not present any Rome criteria for functional constipation. In addition, after the STARR operation, about 30% of the patients still consumed laxatives and 13% were using digital manipulation to evacuate.

Of note is that during a mean follow up of three years after the STARR operation, intermittent bleeding was present in 24% of the patients, anal pain in 17%, and anal incontinence of variable severity in 25%. Furthermore, two patients required a re-intervention. The observed prevalence of these complications in the present study is similar to that reported in other studies^[11,25]. The European STARR register reports perioperative and postoperative complications in about 36% of the patients, and defecatory urgency in 20% of the cases at one year of follow-up^[35]. In our study, a few adverse events of STARR persisted in this long-term follow up with possible detrimental effects on daily living.

The degree of satisfaction expressed by patients parallels that of the improvement of constipation achieved by the two treatments. Only 15% of the patients were

not satisfied with the STARR procedure, despite the presence of some persistent symptoms or the previously mentioned complications.

Defecography was performed in all patients before and, in a subgroup of them, after the STARR procedure. No defecographic finding before surgery predicted the outcome of the STARR operation. As expected, after surgery there was a significant reduction of rectal diameter and the size of the rectocele, but such variations were no different between responders and non-responders.

After STARR, the mean value of ARA variation during evacuation in non-responders was significantly reduced in comparison to the preoperative value, and was significantly less than in responders. This finding indicates that the STARR procedure may affect the relaxation pattern of the puborectalis muscle during evacuation. It remains to be established how the STARR procedure induces this effect on the puborectalis. It is reasonable to assume that a reduced ARA variation during defecation may negatively affect evacuation, nonetheless it is not possible to conclude from this study whether it has any role in the poor clinical outcome of non-responders.

According to the STARR consensus conference, the inclusion criteria for surgical treatment are based on clinical presentation of difficult evacuation and/or straining, in the presence of rectocele and/or intussusception. In this study, the inclusion criteria were based on the consensus conference recommendations; however, no presenting symptom was predictive of the outcome of STARR, nor of the procedure adverse events.

In addition, anorectal manometry was not useful in predicting the outcome of STARR. A previous study reported that altered compliance could be predictive of positive outcome^[10]. This observation was not confirmed by the present study; the discordance could be due to the small sample in this study or to the different method used to evaluate compliance. In the previous study, compliance was assessed by the ratio between volume and pressure at the threshold of rectal sensitivity, whereas in this study, it was calculated using the fixed volume of 100 mL of air, to avoid possible subjective differences of rectal sensitivity. However, a study designed to investigate whether rectal compliance is altered in females with obstructed defecation, showed that the compliance of the rectal wall is normal^[36].

Several structural and functional alterations of the rectum and/or pelvic floor are considered to markedly impair the act of defecation; however, the findings of this study indicate that stool consistency is a major factor in chronic constipation. Indeed, the favorable response to macrogol treatment in the non-surgical group indicated that reducing stool consistency, reported at referral to be hard by more than 90% of the patients, effects resolution of constipation, despite the persistence of the structural and functional alterations. Thus, it seems reasonable to consider a surgical procedure only after the failure of a standardized macrogol treatment.

In conclusion, the results of this prospective study

suggest that STARR is not better and is less safe than conservative therapy in the treatment of defecation disorders in functional constipation patients. Preoperatively, no presenting symptom, or defecographic, and manometric variables were useful to indicate STARR and predict its results. Postoperatively, a reduced widening of ARA during evacuation was associated with an unfavorable outcome of the procedure. The STARR procedure could be considered as an alternative treatment in patients with constipation and defecatory disorders who are unresponsive to conservative macrogol treatment.

COMMENTS

Background

Constipation is a common problem. It is not clear whether a defecation disorder commonly known as obstructed defecation syndrome (ODS) is due to anatomical abnormalities. However, some surgeons propose a new type of surgery [stapled trans-anal rectal resection (STARR)], which, by correcting the anatomical changes, should solve the constipation. Surgery should be considered for those patients who do not benefit from conservative treatment. The gold standard pharmacological treatment of constipation is based on macrogol, which is also effective in patients with altered defecation.

Research frontiers

Ideally, a randomized double blind clinical trial would have more properly assessed the efficacy and safety of the STARR procedure, but the comparison between a non-invasive treatment and an invasive procedure is an objective obstacle to plan a proper protocol, and such studies have not been previously performed. No previous study has so far compared the STARR procedure with conservative pharmacological therapy.

Innovations and breakthroughs

This study evaluated, over a long time period, the outcome of STARR treatment for constipation in comparison with macrogol therapy. The study has shown that STARR is not better, and is less safe, than macrogol therapy. In addition, no preoperative findings could predict the outcome of surgery.

Applications

The authors believe that macrogol should be used before considering surgery in cases of lack of response to conservative treatment

Terminology

Rectocele is a protrusion of part of the rectum into the vagina. Rectal intussusception is a protrusion of the rectal mucous membrane into the lower rectum.

Peer review

The manuscripts reports the comparative analysis of two strategies in managing functional constipation. The authors have compared outcomes of patients that underwent treatment with a conservative regimen that consisted of macrogol and patients that underwent the STARR procedure. This is a well done study; despite not being randomized it provides useful information and should be published.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Zhang DN

Comparison of Milan and UCSF criteria for liver transplantation to treat hepatocellular carcinoma

Tarkan Unek, Sedat Karademir, Naciye Cigdem Arslan, Tufan Egeli, Gulsen Atasoy, Ozgul Sagol, Funda Obuz, Mesut Akarsu, Ibrahim Astarcioglu

Tarkan Unek, Sedat Karademir, Naciye Cigdem Arslan, Tufan Egeli, Gulsen Atasoy, Ibrahim Astarcioglu, Department of General Surgery, Dokuz Eylul University, School of Medicine, Izmir 35340, Turkey

Ozgul Sagol, Department of Pathology, Dokuz Eylul University, School of Medicine, Izmir 35340, Turkey

Funda Obuz, Department of Radiology, Dokuz Eylul University, School of Medicine, Izmir 35340, Turkey

Mesut Akarsu, Department of Gastroenterology, Dokuz Eylul University, School of Medicine, Izmir 35340, Turkey

Author contributions: Unek T designed the research/study, analyzed the data, performed the study and wrote the paper; Karademir S designed the research/study, analyzed the data, performed the study and wrote the paper; Arslan NC performed the study, collected the data and wrote the paper; Egeli T collected the data; Atasoy G collected the data; Sagol O examined pathologic specimens; Obuz F collected the data; Akarsu M collected the data and performed the study; Astarcioglu I performed the study.

Correspondence to: Dr. Sedat Karademir, Department of General Surgery, Dokuz Eylul University, School of Medicine, Izmir 35340, Turkey. sedatkarademir@gmail.com

Telephone: +90-232-4122908 Fax: +90-232-2772666

Received: May 12, 2011 Revised: August 1, 2011

Accepted: August 8, 2011

Published online: October 7, 2011

Abstract

AIM: To assess the validity of the Milan and University of California San Francisco (UCSF) criteria and examine the long-term outcome of orthotopic liver transplantation (OLT) in patients with hepatocellular carcinoma (HCC) in a single-center study.

METHODS: This study is a retrospective review of prospectively collected data. Between 1998 and 2009, 56 of 356 OLTs were performed in patients with HCC. Based on pathological examination of liver explants,

patients were retrospectively categorized into 3 groups: Milan + ($n = 34$), Milan -/UCSF + ($n = 7$) and UCSF - ($n = 14$).

RESULTS: Median follow-up period was 39.5 (1-124) mo. The 5-year overall survival rates in the Milan +, Milan -/UCSF + and UCSF-groups were 87.7%, 53.6% and 33.3%, respectively ($P < 0.000$). Within these groups, tumor recurrence was determined in 5.8%, 14.3% and 40% of patients, respectively ($P < 0.011$). Additionally, the presence of microvascular invasion within the explanted liver had a negative effect on the 5-year disease free survival (74.7% vs 46.7%, $P < 0.044$).

CONCLUSION: The Milan criteria are reliable in the selection of suitable candidates for OLT for the treatment of HCC. For cases of OLT involving living donors, the UCSF criteria may be applied.

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Key words: Hepatobiliary radiology; Hepatobiliary surgery; Hepatobiliary pathology; Hepatocellular carcinoma; Liver malignancy; Liver transplantation; Living donor liver transplantation; Living related liver transplantation; Oncologic surgery; Survival; Transplant

Peer reviewer: Cuneyt Kayaalp, MD, Professor, Department of General Surgery, Staff Surgeon of Gastrointestinal Surgery, Turgut Ozal Medical Center, Inonu University, Malatya 44315, Turkey

Unek T, Karademir S, Arslan NC, Egeli T, Atasoy G, Sagol O, Obuz F, Akarsu M, Astarcioglu I. Comparison of Milan and UCSF criteria for liver transplantation to treat hepatocellular carcinoma. *World J Gastroenterol* 2011; 17(37): 4206-4212 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4206.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4206>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world, and is associated with the third highest number of cancer-related deaths^[1]. Moreover, for 70%-90% of HCC cases, HCC develops on a background of cirrhosis or chronic liver inflammation^[2]. Currently, there are three potentially curative therapeutic options for HCC, liver resection, orthotopic liver transplantation (OLT), and local ablative therapies^[3]. Although liver resection treats localized HCC, it is not optimal for treating multifocal HCC, and has no efficacy in preventing *de novo* HCC that can develop in the remnants of a cirrhotic liver. Alternatively, liver transplantation is an established therapy which offers the potential advantage of removing both the tumor and the organ at risk for developing future malignancies^[4].

In order to identify the best candidates for OLT, a set of criteria were proposed, referred to as the "Milan" criteria. According to these guidelines, patients with cirrhosis and a solitary tumor with a diameter less than 5 cm, or patients who have up to 3 tumor nodules, each of which is smaller than 3 cm and are not characterized by vascular invasion or extrahepatic metastasis (according to preoperative radiologic findings), are patients that have a higher probability of obtaining a successful outcome following OLT. For example, the 5-year recurrence-free survival rate for a set of patients who fulfilled the Milan criteria was reported to be 83%^[5]. The "Milan criteria" were subsequently adopted by the United Network for Organ Sharing (UNOS) in 2002 as the optimal criteria for determining the use of OLT to treat HCC^[6]. However, an expanded set of criteria proposed by the University of California San Francisco (UCSF), referred to here as the "UCSF" criteria, allows patients with a solitary tumor smaller than 6.5 cm, or patients having 3 or fewer nodules, with the largest lesion being smaller than 4.5 cm or having a total tumor diameter less than 8.5 cm without vascular invasion, to undergo OLT. Based on the comparable success of this set of criteria in selecting patients for OLT, it has been suggested that the Milan criteria may be too stringent^[7]. Therefore, the aim of this study was to examine the long-term outcome of patients undergoing liver transplantation to treat HCC, and to compare the use of the current criteria (both the Milan and UCSF) for the selection of HCC patients for possible OLT.

MATERIALS AND METHODS

Between 1998 and 2009, 56 of 356 (15.7%) OLTs were performed in patients with HCC at the Dokuz Eylul University Hospital (Izmir, Turkey). Of these, 50 were diagnosed with HCC prior to transplantation, and 6 (10.7%) were diagnosed during OLT. According to pre-OLT imaging and post-OLT pathological evaluation, 56 patients were retrospectively classified into 3 groups: Milan +, Milan -/UCSF + and UCSF - (Table 1).

Following the pathological examination of liver ex-

Table 1 Number of patients associated with each criteria depending on pre-orthotopic liver transplantation imaging and post-orthotopic liver transplantation pathology results *n* (%)

Diagnostic approaches	Milan +	Milan -/UCSF +	UCSF -
pre-OLT imaging	44 (78.0)	5 (8.9)	7 (12.5)
post-OLT pathology	34 (60.7)	7 (12.5) ¹	15 (26.8) ²

¹6/7 patients were classified as "Milan +" based on pre-OLT imaging; ²4 patients each were classified as "UCSF +/Milan -" and "Milan +" based on pre-OLT imaging. UCSF: University of California San Francisco; OLT: Orthotopic liver transplantation.

plant specimens, 14 (25.0%) patients were reclassified due to underestimates of tumor size, and 7 (12.5%) patients were reclassified due to the tumor number being greater than expected (false negative rate: 25%) (Table 1). For the applied Milan and UCSF criteria, false negative rates of pre-OLT radiological evaluations were 22.7% (10/44) and 16.3% (8/49), respectively. In summary, 8 patients met the UCSF criteria prior to undergoing OLT, and exceeded the UCSF criteria following pathologic evaluation of the explants obtained.

Pre-OLT workup

All patients included in this study had cirrhosis due to various etiologies. A pre-operative diagnosis of HCC was based on a patient's medical history, a physical examination, laboratory studies, α -fetoprotein (AFP) levels, and the results of one or more imaging studies [i.e., abdominal ultrasonography, contrast-enhanced computed tomography (CT), angiographic CT, or abdominal magnetic resonance imaging (MRI)]. Tumor biopsies were not performed to confirm each diagnosis. Chest CT, cranial CT, and technetium-99 m bone scintigraphy were used to detect the potential incidence of extrahepatic disease, and distant or lymph node metastases were not detected in any of the patients. Pre-OLT adjuvant therapies, including radiofrequency ablation (RFA), transarterial hepatic chemoembolization (TACE), percutaneous ethanol injection (PEI), and liver resection were performed in selected patients. In the absence of medical contraindications, patients who fulfilled the Milan criteria in pre-OLT evaluations were qualified to receive a transplant from either a living or deceased donor. Alternatively, for patients who did not fulfill the Milan criteria, these patients were qualified to receive organs from living donors only. In our series, 31 (55.3%) living and 25 (44.7%) deceased donor liver grafts were utilized. In the latter group, 3 marginal liver grafts from deceased donors were transplanted to recipients who did not fulfill the Milan criteria.

Pre-OLT locoregional therapy

Thirteen out of 50 (26%) patients received locoregional treatment prior to OLT, which included either TACE (*n* = 9), liver resection (*n* = 2), PEI (*n* = 1), or RFA (*n* = 1). Moreover, complete radiological regression was associated with all patients who underwent TACE, PEI, or RFA.

Table 2 Patient characteristics according to Milan and University of California San Francisco criteria determined from post-orthotopic liver transplantation imaging and post-orthotopic liver transplantation pathological evaluations *n* (%)

Variables	Milan +	Milan -/UCSF +	UCSF -	<i>P</i> value
No. of patients	34	7	15	
Gender (M/F)	29/5	7/0	14/1	
Age (yr)	55.1 ± 6.6	51.0 ± 4.5	56.4 ± 5.5	0.222
CTP (A/B/C)	8/18/8	3/1/2003	10/4/2001	
MELD	13.3 ± 4.9	12.7 ± 4.3	13.4 ± 4.3	0.803
AFP (ng/dL)	4.9	6.1	11.9	0.953
No. nodules	1.4 ± 0.6	2.4 ± 1.3	5.7 ± 4.4	0.000
Max diameter of largest nodule (mm)	22.5 ± 11.6	45.9 ± 11.5	47.9 ± 23.8	0.000
Grade				
Well	17 (73.9)	2 (28.6)	4 (26.7)	
Moderate	17 (53.1)	5 (71.4)	10 (66.7)	
Poor			1 (6.6)	
Microvascular invasion	6 (17.6) ^a	2 (28.6)	7 (46.7) ^a	0.034 ^a

M: Male; F: Female; UCSF: University of California San Francisco; CTP: Child-Turcot-Pugh; MELD: Model of End-stage Liver Disease; AFP: α -fetoprotein. ^a*P* < 0.05.

Furthermore, two patients were successfully downstaged to the Milan criteria following treatment with TACE. For the two patients who underwent curative resection for HCC, both suffered intrahepatic recurrences one year later and were scheduled to undergo OLT. Due to the use of local ablative procedures, the incidence of major morbidity was 0%.

Immunosuppressive regimen and antiviral prophylaxis

OLTs involving deceased or living donors were performed by the same surgical team, and standard techniques were used. Briefly, patients received an immunosuppressive regimen of calcineurin inhibitors (i.e., cyclosporine A or tacrolimus), mycophenolic acid, and corticosteroids in the early post-operative period. The latter were tapered and eventually discontinued during the second month following each OLT. For patients with hepatitis B virus (HBV), peri- and post-operative hepatitis B immunoglobulin and an antiviral were administered. Lamivudine-resistant patients were treated with tenofovir. During the follow-up period, serum hepatitis B antibody levels were kept above 200 IU/L, and interferon and ribavirin treatments were initiated if hepatitis C recurred.

Pathological evaluation

All explants were examined by an experienced hepatopathologist (Sagol O), and were categorized depending on the size, number, distribution, HCC histologic grade, and vascular invasion associated with each tumor.

Post-OLT monitoring

Post-operative death was defined as death within 3 mo post-OLT. All patients underwent regular follow-up examinations in the outpatient clinic. Both the surgical

team and an experienced hepatologist maintained surveillance for tumor recurrence or metastasis based on AFP levels and chest CT scans, as well as by contrast-enhanced abdominal CT scans performed once every 3 mo for the first year post-OLT, then once a year thereafter. The minimum follow-up period was 12 mo.

Statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Chicago, Illinois, United States). Data are expressed as the mean ± SD, and median and range values are provided when appropriate. Quantitative variables were compared using the Kruskal-Wallis test. Comparisons between groups with regard to qualitative variables were performed using the chi-square test and Fisher's exact test, if necessary. Survival was calculated using Kaplan-Meier estimates, with comparisons made using the log rank test. *P* < 0.05 was considered statistically significant.

RESULTS

The demographic characteristics of the patients included in this study are presented in Table 2. OLT was performed for patients who had been on a waiting list for a median of 62 d. Furthermore, the interval between when the patients were listed for transplantation and when the patients underwent transplantation was similar for both deceased and living donor transplantations (i.e., 60 vs 68 d, respectively). The average rates of graft weight/body weight for both OLT groups were also 1.09% (range, 0.69-1.8) and 1.82% (range, 0.76-2.58), respectively.

The mean hospital stay for patients was 31.2 ± 21.5 d, and complications associated with surgery were experienced by 10 (17.8%) recipients. Four (7%) recipients presented with biliary leak, with two of the cases resolving and two of the cases resulting in death due to sepsis. In addition, 3 (5.3%) recipients acquired pneumonia post-operatively. Two of these patients recovered, while the other died from respiratory arrest. One recipient died due to intra-abdominal sepsis and another developed intra-abdominal hemorrhage post-operatively and underwent a second operation. Only one patient experienced a wound infection. In contrast, a total of 4 (7%) patients died due to surgical complications, while another patient died from duodenal ulcer perforation with sepsis. The overall mortality for this study was 8.9% (5/56).

Pre-operative AFP levels ranged from 1.72 to 3630 ng/dL (median, 158.7 ng/dL), with the normal range being 0.5-5 U/L. Only 6/56 (10.7%) patients had an AFP level greater than 200 ng/dL. Furthermore, the mean AFP levels during the pre-OLT period for patients with incidental HCC was 15.5 ± 26.6 ng/dL (range, 2.45-63.1).

Tumor characteristics are described in Table 2. In particular, the number of nodules per patient and the diameter of the largest nodule were significantly lower in the Milan + group compared to the Milan -/UCSF + and UCSF-groups, respectively (*P* < 0.000).

Adjuvant chemotherapy was an option for 13 (23.2%) patients who were medically eligible for chemotherapy

Table 3 Follow-up data for patients who underwent orthotopic liver transplantation for hepatocellular carcinoma *n* (%)

Variables	Milan +	Milan -/UCSF +	UCSF -
No. of patients	34	7	15
Post-operative death	2 (5.9)	-	1 (4.5)
Death	5 (14.7)	2 (28.6)	11 (73.3)
HCC recurrence	2 (5.8)	1 (14.3)	6 (40.0)
Median follow-up	51.5 (1:124)	32 (1:66)	14 (3:66)

UCSF: University of California San Francisco; HCC: Hepatocellular carcinoma.

Table 4 Causes of mortality associated with cases in this study

Causes of death	<i>n</i> (%)
Sepsis (late postoperative period)	7 (36.8)
Lung metastasis	5 (26.3)
Sepsis (early postoperative period)	3 (15.8)
Recurrent fulminant hepatitis B	2 (10.5)
Duodenal ulcer perforation	1 (5.3)
Intracranial hemorrhage	1 (5.3)

and was administered post-OLT according to pathological tumors with a diameter of > 2 cm and/or microvascular invasion or post-OLT in case of recurrence. These patients received 5-fluorouracil in combination with epirubicin, mitomycin C, or cisplatin. None of the patients died as a result of chemotherapy-related complications. However, for three patients, grade 4 hematologic toxicity was reported, two patients experienced grade 3 gastrointestinal toxicity (i.e., excessive nausea and vomiting), and one patient exhibited grade 3 neurotoxicity.

Recurrence

Tumor recurrence was experienced by 9 (16%) patients (Table 3). Recurrence rates were 5.8%, 14.3% and 40% in the Milan +, Milan -/UCSF + and UCSF-groups, respectively. Five patients presented solely with distant metastasis in the lung (*n* = 3), in both the lung and bone (*n* = 1), and in the bone and skin (*n* = 1). The remaining 4 patients suffered intrahepatic tumor recurrence with (*n* = 2) or without (*n* = 2) extrahepatic metastasis (i.e., lung, adrenal gland, bone). None of the 6 recipients with incidental HCC recurred. For the treatment of tumor recurrence, chemotherapy was the only therapeutic option administered.

Survival

The median follow-up period was 39.5 mo (range, 1-124), and at this point, 19 (33.9%) patients had died. Causes of death are listed in Table 4. Correspondingly, the 1-, 3- and 5-year overall survival (OS) rates of the whole series were 80.4%, 68.9% and 65.3%, respectively. The disease-free survival rates for the same categories were 78.6%, 67.1% and 67.1%, respectively.

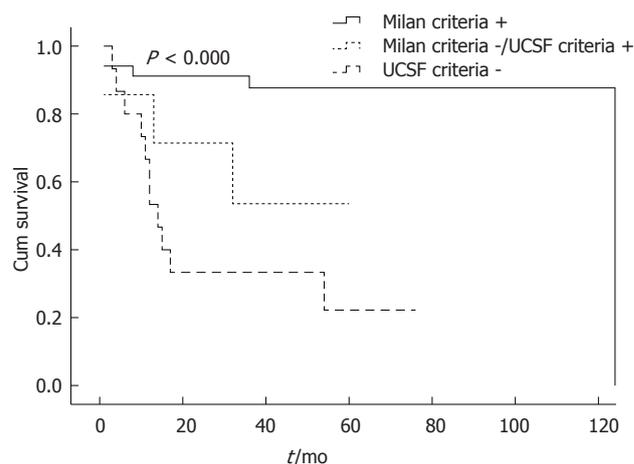


Figure 1 Kaplan-Meier overall survival curves for Milan +, Milan -/UCSF + and UCSF-patients (post-orthotopic liver transplantation). UCSF: University of California San Francisco.

Overall survival in the groups

When OS rates were calculated according to the criteria used, the 1-, 3- and 5-year OS rates for the Milan + group were 91.2%, 87.7% and 87.7%, respectively. The mean survival time was 110.3 ± 7.2 mo (95% CI: 96.1-124.4) for this group. In contrast, the 1-, 3- and 5-year OS rates for Milan -/UCSF + patients were 85.7%, 53.6% and 53.6%, respectively. The mean OS period was 39.8 ± 9.1 mo (95% CI: 22.1-57.6). The OS rates for UCSF-patients were 66.7%, 33.3% and 22.2%, respectively, with a mean survival time of 29.8 ± 7.4 mo (95% CI: 15.3-44.4) ($P < 0.000$) (Figure 1).

Disease-free survival in the groups

The rates of disease-free survival at 1-, 3- and 5-years post-OLT were 91.2%, 87.7% and 87.7%, respectively, for Milan + patients. Furthermore, the mean disease-free survival period was 109.3 ± 7.2 mo (95% CI: 95.2-123.1). In contrast, the 1- and 3-, and 5-year disease-free survival rates for Milan -/UCSF + patients were 71.4%, 53.6% and 53.6%, respectively, and the mean disease-free survival period was 39.6 ± 9.2 mo (95% CI: 21.6-57.5). The disease-free survival rates for the UCSF-group were 33.3%, 25.0% and 25.0%, respectively, and the mean disease-free survival period was 26.1 ± 7.8 mo (95% CI: 10.9-41.4) ($P < 0.000$) (Figure 2).

Microvascular invasion

When OS rates were calculated for patients with and without microvascular invasion, the 1-, 3- and 5-year OS rates for each category were 87.8%, 74.7% and 74.7%, and 73.3%, 53.3% and 35.6%, respectively ($P < 0.029$) (Figure 3). Furthermore, disease-free survival rates were 82.9%, 74.7% and 74.7% for patients without microvascular invasion, and 53.3%, 46.7% and 46.7% for patients with microvascular invasion ($P < 0.044$) (Figure 4). Moreover, we found that the presence of microvascular invasion was significantly higher in UCSF - than Milan +

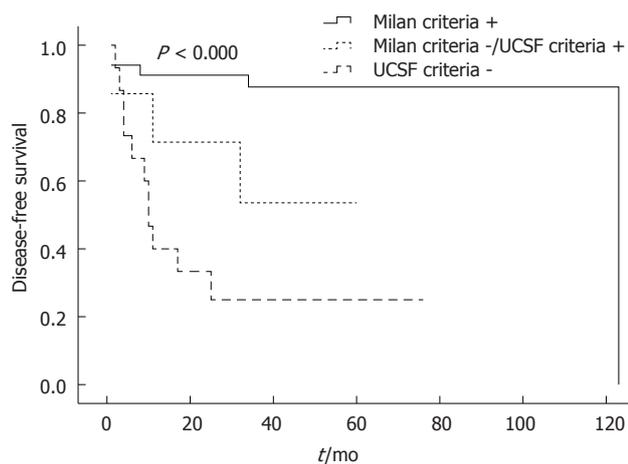


Figure 2 Kaplan-Meier disease-free survival curves for Milan +, Milan -/UCSF + and UCSF-patients (post-orthotopic liver transplantation). UCSF: University of California San Francisco.

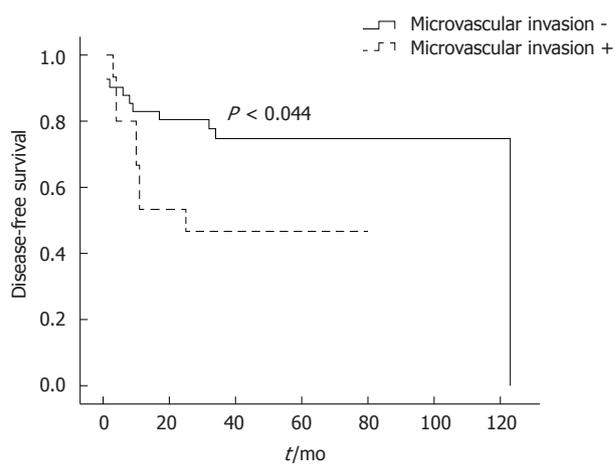


Figure 4 Kaplan-Meier disease-free survival curves for patients with microvascular invasion (post-orthotopic liver transplantation).

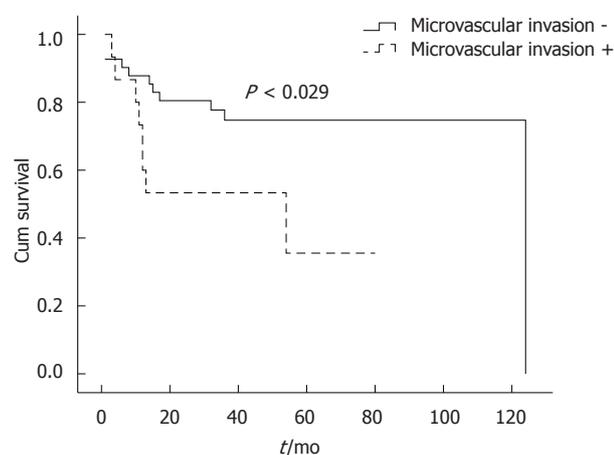


Figure 3 Kaplan-Meier overall survival curves for patients with microvascular invasion (post-orthotopic liver transplantation).

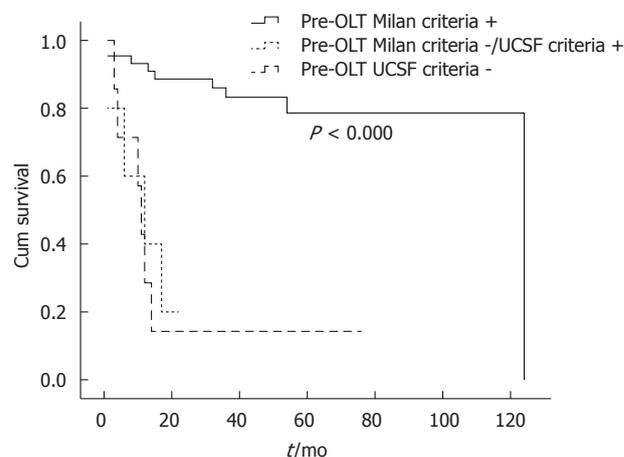


Figure 5 Kaplan-Meier overall survival curves for Milan +, Milan -/UCSF + and UCSF-patients based on pre-orthotopic liver transplantation imaging results. UCSF: University of California San Francisco. OLT: Orthotopic liver transplantation.

patients ($P < 0.034$).

Survival analysis based on pre-OLT imaging

In the three groups which were classified based pre-OLT imaging, OS rates are shown in Figure 5. Among these, the 1-, 3- and 5-year OS rates for Milan + patients were 93.2%, 83.3%, and 78.6%, respectively. The mean survival period was 102.7 ± 7.2 mo (95% CI: 88.5-116.9 mo). In contrast, the OS rates for UCSF-patients were 28.6%, 14.3%, and 14.3%, respectively, with a mean survival period of 18.6 ± 8.9 mo (95% CI: 1.0-36.2 mo) ($P < 0.000$). When the same evaluations were made for Milan -/UCSF + patients ($n = 5$), only the 1-, and 2-year OS rates were available and were 40% and 20%, respectively, and the mean survival period was 11.6 ± 3.4 mo (95% CI: 5.0-18.2 mo) (Figure 5).

DISCUSSION

This retrospective study sought to examine the overall reliability of the Milan and UCSF criteria as clinical tools

for the selection of HCC patients to be treated with OLT. Currently, the best liver transplant outcomes for HCC are obtained using the Milan criteria. For these patients, the 5-year survival rates are greater than 70% and the recurrence rate is 15%^[8-10]. In 2002, UNOS adopted the “Milan criteria” as the optimal criteria for selecting patients for possible OLT due to HCC^[6]. However, it was subsequently proposed that the Milan guidelines be expanded based on the comparable survival rates that were being achieved for patients undergoing selection based on the UCSF criteria^[7]. Therefore, to investigate whether the Milan criteria are too restrictive for the selection of patients who could otherwise benefit from OLT, a series of HCC cases, who were confirmed by pathology studies of explanted liver specimens, were analyzed. In particular, Milan + patients had significantly better 5-year OS rates than both Milan -/UCSF + and UCSF-patients (87.7% *vs* 53.6% and 33.3%; $P < 0.039$ and $P < 0.000$, respectively). Additionally, Milan -/UCSF + patients who would be expected to obtain the maximum benefit from the proposed expanded criteria had

no significant difference in survival rates compared to UCSF-patients (53.6% *vs* 33.3%, $P < 0.239$).

In most cases, patient selection criteria are based on radiological imaging performed to assess the extent of intrahepatic disease present, and to exclude extrahepatic spread. However, pre-OLT imaging studies have been shown to underestimate tumor stage in 20%-30% of cases^[4,11,12]. Consistent with these results, pre-OLT imaging associated with the series of cases evaluated in this study underestimated either the size, or the number, of tumors present in 14/56 (25%) patients. As a result, 80% of patients identified as Milan -/UCSF + prior to OLT were reclassified as UCSF-following pathological evaluations of the explants obtained. In addition, the 5-year survival rate of these reclassified patients was 25%. Thus, the Milan criteria appear to provide a wider “safe” margin and reduce the negative influence of underestimates of tumor stage by pre-OLT imaging.

As the interval between imaging studies performed and the date of transplantation increases, the patient undergoing transplantation is potentially at a higher risk for tumor recurrence. This could be avoided by shortening the waiting time for a transplant by increasing the number of organ donors, or better utilization of living donors. In this series, OLT was performed a median of 62 d after the patient was placed on a waiting list. In addition, due to the limited number of deceased donors available in Turkey, transplant centers have agreed to allocate deceased donor liver grafts to HCC patients who meet the Milan criteria. Although living donors are currently utilized as a source of liver grafts for the treatment of HCC, the primary concern for transplant programs is minimization of donor morbidity and mortality. Today, living donor hepatectomies are performed safely, and for countries experiencing a shortage in deceased donors, OLTs from living donors shorten the time that patients spend on a waiting list^[13]. In our study, 55.4% of the grafts used were obtained from living donors. However, despite all efforts, the morbidity and mortality of living donors following resection of the right lobe of the liver is approximately 0.5% and 35.0%, respectively^[14-15]. Thus, considering the safety of living donors and the poor long-term survival rates associated with recipients exceeding the UCSF criteria, it is recommended that the UCSF criteria be followed in order to select HCC patients with the highest likelihood of survival following OLT.

Overall, the recurrence rate (16%) associated with this study was consistent with previous reports^[16]. According to the patient groups, the recurrence rates were 5.8%, 14.3% and 40.0% for the Milan +, Milan -/UCSF +, and UCSF-patients, respectively. We hypothesize that these low recurrence rates are associated with the use of the Milan criteria in patient selection and especially for the allocation of deceased donor grafts.

Microvascular invasion is a key step in HCC metastasis. However, as a characteristic of tumor growth that must be determined pathologically, it is impossible to

know pre-operatively if it exists. According to previous studies, the presence of microvascular invasion is considered a negative factor for OLT in the treatment of HCC. Correspondingly, in our study microvascular invasion was associated with a significant decrease in 5-year OS rates from 74.7% to 35.6% ($P < 0.029$). Moreover, we found that the presence of microvascular invasion was significantly higher in UCSF -than Milan + patients ($P < 0.034$).

There are cases where HCC is detected in explanted livers incidentally, and transplant centers worldwide have reported variable incidences of this situation. In particular, Chui *et al*^[17] and Loinaz *et al*^[18] reported the unexpected incidence of HCC to be 1.4% and 2.8%, respectively. In other series, slightly higher incidences of 7% and 8% have been reported^[19,20]. This discrepancy could be partly due to the thickness of the liver sections used for pathologic examination. In the present series, the rate of unexpected HCC incidence was 10.7% (6/56). The mean tumor diameter associated with these cases was 14.7 mm, and the maximum nodule size was less than 20 mm. Furthermore, none of these tumors exhibited radiological features that are typically associated with HCC. Serum AFP levels for 5/6 of these patients were also within the normal range, while one patient had an AFP level of 63.1 ng/dL. However, previous studies have demonstrated that AFP levels are not a reliable indicator for the diagnosis of HCC^[8], and this was consistent with our results. In addition, confirmatory biopsies were not performed for these cases since almost all of the patients had diagnostic findings identified in the imaging studies conducted. Therefore, our results indicate that the current guidelines of the American Association for the Study of Liver disease can provide a reliable diagnosis of HCC^[8].

In conclusion, pre-OLT imaging continues to have a relatively high false negative rate for HCC patients considered for transplantation. The inaccuracy of imaging modalities for identification of tumor characteristics such as size and number may result in the selection of patients with unfavourable survival outcome for OLT. Based on the cases analyzed in this study, it would appear that the Milan criteria are very useful and safe in selecting recipients who will benefit from OLT. Therefore, given the limited number of deceased liver grafts available, the Milan criteria should be followed in the selection of suitable candidates for OLT for the treatment of HCC. In contrast, for cases of OLT involving living donors, the UCSF criteria may be applied. In addition, future advancement in imaging modalities may further improve the reliability and applicability of these selection criteria.

COMMENTS

Background

Liver transplantation offers the best long-term effective treatment for patients with hepatocellular carcinoma (HCC) in cirrhosis. Patients who fulfill the Milan criteria may have a 5-year survival of up to 88%. However, application of the

Milan criteria might lead to the exclusion of patients who otherwise would benefit from orthotopic liver transplantation (OLT). Several studies have evaluated more liberal criteria for tumor staging which could be adopted without significant impairment of patient survival or tumor recurrence. However, the expansion of tumor-specific criteria for transplantation raises concerns about rational use of scarce deceased donor organs and safety issues in living donation.

Research frontiers

The role of OLT in patients with HCC beyond the Milan criteria is a matter of debate. Several different selection criteria have been proposed to reach an optimum survival outcome after OLT. Improvements in imaging modalities and markers of aggressive tumor biology may help in selecting patients with better outcome after OLT.

Innovations and breakthroughs

In a substantial portion of HCC patients, pre-OLT imaging studies underestimate the extent of the disease. Patients who were reclassified to higher tumor stages after pathological evaluations of the explants had poor survival after OLT. The Milan criteria appear to provide a wider safe margin and reduce the negative influence of underestimates of tumor stage by pre-OLT imaging.

Applications

In the face of low deceased donor organ availability and safety issues in living donation, transplanting patients with HCC who meet the Milan criteria appears to have optimum benefit on patient survival. Better understanding of tumor behavior may help in selecting patients who may benefit from OLT even with higher tumor burden (expanded selection criteria).

Peer review

Despite several studies in the literature about the Milan and University of California San Francisco (UCSF) criteria, this study for the first time suggests Milan criteria for safer preoperative radiological staging over the UCSF criteria.

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S- Editor Tian L L- Editor Webster JR E- Editor Zhang DN

Efficacy of premedication with activated Dimethicone or N-acetylcysteine in improving visibility during upper endoscopy

Seyed Mohammad Kazem Hosseini Asl, Gholam Reza Sivandzadeh

Seyed Mohammad Kazem Hosseini Asl, Gholam Reza Sivandzadeh, Department of Internal Medicine, Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, 7193711351 Fars, Iran

Author contributions: Both Hosseini Asl MK and Sivandzadeh GhR designed and performed the research; Hosseini Asl MK and Sivandzadeh GhR wrote the manuscript.

Correspondence to: Gholam Reza Sivandzadeh, MD, Department of Internal Medicine, Shiraz University of Medical Sciences, Nemazee Hospital, Zand St., Shiraz, 7193711351 Fars, Iran. ghsivand@sums.ac.ir

Telephone: +98-711-6125610 Fax: +98-711-6474316

Received: December 28, 2010 Revised: January 18, 2011

Accepted: January 25, 2011

Published online: October 7, 2011

Abstract

AIM: To assess the efficacy of N-acetylcysteine (NAC) and activated Dimethicone in improving endoscopic mucosal visibility.

METHODS: A total of 148 patients were randomly allocated into four groups to receive one of the following premedications: group A: 100 mL water alone; group B: activated Dimethicone plus water (up to 100 mL); group C: NAC plus water (up to 100 mL); and group D: activated Dimethicone and NAC plus water (up to 100 mL). A single endoscopist blinded to the patients group assessed the gastric mucosal visibility scores (range 1-4) at four sites. The sum of the scores from the four sites was considered as the total mucosal visibility score (TMVS).

RESULTS: The patients in group B showed a significantly lower TMVS than those of groups A and C ($P < 0.001$). The TMVS in patients of group D was significantly lower than that of groups A and C ($P < 0.001$). The TMVS did not significantly differ between groups B and D ($P > 0.05$). The difference between TMVS of groups C and A was not significant ($P > 0.05$).

CONCLUSION: Premedication with activated Dimethicone 20 min prior to the upper endoscopy leads to the best visibility. NAC does not improve visualization by itself.

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Key words: Dimethicone; N-acetylcysteine; Simethicone; Upper endoscopy

Peer reviewer: Dr. György M. Buzás, Department of Gastroenterology, Ferencváros Health Center, IX. District Polyclinic, Mester u 45, 1095 Budapest, Hungary

Hosseini Asl MK, Sivandzadeh GhR. Efficacy of premedication with activated Dimethicone or N-acetylcysteine in improving visibility during upper endoscopy. *World J Gastroenterol* 2011; 17(37): 4213-4217 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4213.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4213>

INTRODUCTION

Studies recently demonstrated a declining trend in gastric cancer incidence throughout the world; yet, it is still the second most common cause of mortality due to malignant diseases^[1]. As detecting the cancer at the early stage has a great impact on its potential curability, mass screening programs are implementing in Japan with the highest rate of the disease. Although the real effect of this approach on mortality is said to be little by some, studies conducted in Japan favor endoscopic mass screening especially by the advent of new minimally invasive procedures such as endoscopic mucosal resection for cancers detected at early stages^[2-5].

Foam, bubbles, and mucus accumulated in the upper gastrointestinal tract can interfere with clear mucosal visualization and pose potential risk of missing early or subtle lesions. That is why anti-foam and bubble-bursting

agents are widely used in gastrointestinal endoscopic centers particularly in Japan where it is common. This is a routine practice neither in the country where this study was conducted nor in the West, probably in fear of some presumed risk of pulmonary aspiration^[6].

Simethicone [Dimethylpolysiloxane (DMPS) or activated Dimethicone] was proved to be a good defoaming agent for pre-endoscopic usage to remove bubble and mucus^[7,8]. Pronase, a proteolytic enzyme isolated in 1962 from the culture filtrate of *Streptomyces griseus*, is another agent whose efficiency as a premedication for improving the visual field of endoscopy devoid of foam and mucus has been investigated and is now being used routinely in Japan's endoscopic centers. It is better to be used in combination with DMPS and bicarbonate to yield more improvement in visibility^[9,10].

Other than upper endoscopy, Simethicone has been studied to be used in colonoscopy as an additive to other bowel preparations to eliminate bubbles^[11,12], in capsule endoscopy as small bowel preparation for the same goal^[13,14], and in endoscopic ultrasonography to reduce artefacts and increase the accuracy of the modality^[15,16].

Currently, N-acetylcysteine (NAC), a mucolytic agent, in combination with DMPS has shown to be effective in elimination of gastric mucus and bubbles when used 20 min prior to endoscopy, improving visualization of the gastric mucosa^[17]. Owing to the lack of any study surveying the efficiency of NAC independently, the present study aimed to compare the effect of this compound and activated Dimethicone (Simethicone) with placebo and together as premedications for gastroscopy.

MATERIALS AND METHODS

This double-blind, randomized, placebo-controlled study was carried out from April to August 2010. Amongst all the consecutive patients referred to our out-patient endoscopy clinic, 148 patients were enrolled in the study after giving a written informed consent. The patients with a history of upper gastrointestinal surgery, gastric cancer, gastrointestinal bleeding, caustic ingestion, pregnancy, diabetes mellitus, asthma, and allergic reactions were excluded from the study. This study was approved in the ethics committee of the local university.

The patients were randomly allocated into four different groups (using random blocks) with peculiar liquid premedication for each one: (1) group A, 100 mL water; (2) group B, 100 mg, 2.5 mL, activated Dimethicone (Dimetin, Tolid Daru co., Tehran, Iran) plus water up to 100 mL; (3) group C, 600 mg N-acetylcysteine (ACC, Hexal AG, Holzkirchen, Germany) in water up to 100 mL; and (4) group D, 100 mg, 2.5 mL, activated Dimethicone and 600 mg N-acetylcysteine plus water up to 100 mL.

All the liquid solutions were prepared in the same opaque bottles and taken about 20 min prior to endoscopic procedure under supervision of an informed attendant nurse. All patients were unaware of their groups and the type of liquid solutions. Then the patient awaited

endoscopy in sitting position in the endoscopy waiting room.

All the endoscopic procedures were performed by a single, experienced endoscopist blinded to the patient's group and premedication. The endoscopies were done at a relatively fixed period of time in a clinic affiliated with Shiraz University of Medical Sciences, using a video endoscope (EPK 1000 PENTAX, Japan).

During endoscopy, four distinct domains of the stomach including the antrum, the upper part of the greater curvature, the lower part of the greater curvature, and the gastric fundus were evaluated separately for mucosal visibility. Scoring from 1 to 4 for each domain, known as visibility score, was defined based on the modified form of Kuo *et al.*^[9] scoring system like the one used by Chang *et al.*^[17] as follows: (1) score 1, no adherent mucus on the gastric mucosa; (2) score 2, little amount of mucus on the gastric mucosa, but no obscuring vision; (3) score 3, large amount of mucus on the gastric mucosa, with less than 50 mL of water to clear; and (4) score 4, large amount of mucus on the gastric mucosa, with more than 50 mL of water to clear.

The sum of visibility scores of all four domains is considered as the TMVS for each patient.

Statistical analysis

The demographic characteristics were assessed using a χ^2 test, ANOVA, or one-way analysis of variance. The visibility scores of all the four groups were analyzed using Kruskal-Wallis and Mann-Whitney pairwise comparisons. *P* value < 0.05 was considered statistically significant.

RESULTS

From a total of 148 patients enrolled in the study, 77 (52%) were male and 71 (48%) female. Then, 38, 37, 37 and 36 patients were randomly assigned to groups A, B, C and D, respectively. The mean (\pm SD) age was 42.2 ± 13.9 in group A, 44.3 ± 18 in group B, 44.6 ± 16.4 in group C, and 41.8 ± 17.5 in group D. The mean age in the whole study population was 43.2 ± 16.4 . The most common reason for endoscopy in all the groups and also in the total population was dyspepsia (65.5% in total). Moreover, the second most common cause in all the patients was acid reflux (12.8%). All demographic data encompassing sex distribution per group and reason for endoscopy are shown in Table 1. There was no statistically significant difference (*P* > 0.05) among groups regarding age and gender.

The mean of TMVS in group A was 9.50 ± 2.55 , in group B 5.11 ± 1.28 , in group C 8.41 ± 2.10 , and in group D 5.39 ± 1.71 . The total mean ranks in groups A, B, C and D were 109.96, 41.69, 98.39 and 46.24, respectively (the lower the rank, the better the visibility). The difference among the mean ranks was statistically significant (*P* < 0.001). Group B showed the least visibility scores at different locations of the stomach and also the least

Table 1 Demographic characteristics of patients in each group

Group	A	B	C	D
Number	38	37	37	36
Age(yr; mean ± SD)	42.2 ± 13.9	44.3 ± 18.0	44.6 ± 16.4	41.8 ± 17.5
Female: Male (<i>n</i>)	18:20	19:18	16:21	18:18
Cause of endoscopy				
Dyspepsia	25	28	21	23
Reflux	6	5	4	4
Screening for cancer	7	0	5	4
Others	0	4	7	5

No significant difference between each two groups regarding age and gender. Group A received water, group B received activated Dimethicone plus water, group C received N-acetylcysteine plus water, and group D received activated Dimethicone and N-acetylcysteine plus water.

Table 2 The mean rank¹ of any group of patients in distinct domains of stomach

Group	A	B	C	D
Antrum	102.82	48.91	91.74	53.19
Lower part of the greater curvature	103.43	51.24	88.00	53.99
Upper part of the greater curvature	100.17	48.49	92.43	55.71
Fundus	102.21	51.43	90.96	52.04

¹The lower the mean rank, the better the visibility.

Table 3 Mean mucosal visibility score at different sites and total mean mucosal visibility scores in any group separately (mean ± SD)

Group	A	B	C	D
Antrum	2.39 ± 0.94	1.22 ± 0.53 ^{a,b}	2.05 ± 0.78	1.28 ± 0.51 ^{c,d}
Lower part of the greater curvature	2.26 ± 0.89	1.14 ± 0.34 ^{a,b}	1.89 ± 0.87	1.19 ± 0.46 ^{c,d}
Upper part of the greater curvature	2.47 ± 0.79	1.38 ± 0.54 ^{a,b}	2.35 ± 0.94	1.53 ± 0.69 ^{c,d}
Fundus	2.37 ± 0.75	1.38 ± 0.54 ^{a,b}	2.11 ± 0.65	1.39 ± 0.54 ^{c,d}
Total (TMVS)	9.50 ± 2.55	5.11 ± 1.28 ^{a,b}	8.41 ± 2.10	5.39 ± 1.71 ^{a,b}

^a*P* < 0.001 vs group A; ^b*P* < 0.001 vs group C; ^c*P* < 0.05 vs group A; ^d*P* < 0.05 vs group C; a, b, c, d: Kruskal-Wallis and Mann-Whitney pairwise comparisons. TMVS: Total mucosal visibility score.

mean TMVS which were all significantly lower than those of groups A and C (*P* < 0.001). The patients in group D had significantly lower visibility scores for separate gastric domains (*P* < 0.05) and showed lower mean TMVS than group A and C too (*P* < 0.001). Groups B and D did not differ significantly in scores (*P* > 0.05). Despite the fact that patients in group C achieved lower scores than group A patients, the difference was not significant at all (*P* > 0.05). The mean rank, the mean mucosal visibility scores at separate sites, and the mean TMV scores in distinct groups are depicted in Table 2 and 3, respectively. No adverse reaction was detected during the study in any group.

DISCUSSION

Esophagogastroduodenoscopy or upper endoscopy re-

mains commonplace for the evaluation of upper gastrointestinal tract disorders. One of the major applications of this modality is to discern gastric cancer at early stages. This is of paramount importance because of the direct effect of early diagnosis of gastric cancer on patients' future survival, quality of life, and management. A case series from Britain, reported by Sue-Ling *et al*^[18] showed a 5 year survival rate of 98% for patients detected at early stages of gastric cancer and survived operation. On the other hand, rapid diagnosis is not guaranteed by doing upper endoscopy alone even in a wide range. Suvakovic *et al*^[19] in 1997 remarked that open-access gastroscopy by itself was not sufficient to increase early gastric cancer pick-up; moreover, more awareness from general practitioners, more experience in endoscopy, and high sensitivity for biopsying are important among others too. Besides, foam, bubbles, and mucus accumulated on gastric mucosa are postulated to play a role by blurring visual field during endoscopy. So, it seems prudent to make use of some agents before endoscopy to eliminate these troubles and enhance the precision and accuracy of endoscopy in showing subtle abnormalities.

Simethicone (activated Dimethicone or activated Methylpolysiloxane), commonly used for relief of bloating and gas with no significant adverse reaction or interaction^[20], is a safe adjunct to endoscopic premedications. It works via decreasing the surface tension of bubbles of air and dispersing them without remarkable absorption in the gastrointestinal system^[20]. The effectiveness of Simethicone has already been proved in some other trials as a defoaming agent^[7,8]. Recently, Keeratchananont *et al*^[22], though using a different grading scale and including the esophagus and duodenum in their study, concluded that 133.3 mg (2 mL) of liquid Simethicone in 60 mL water 15-30 min prior to procedure could improve the visibility and reduce the number of flushings required for removing the mucus significantly. They also showed that using Simethicone prior to endoscopy would cut down the duration of the procedure and consequently lead to more satisfaction to both physician and patient. Similarly in our study, those patients in group B who received 100 mg activated Dimethicone in water showed better visualization compared to group A that received only simple water as placebo. The amount of water to be given with Simethicone had been a matter of debate. We used of a fixed volume of water in all our patient groups to remove the possible role thereof; however, in two clinical trials it was shown that there was no significant difference in visibility between those who received Simethicone alone or with 100 mL water^[9,17].

Pronase is a proteolytic enzyme commonly used in Japan as a premedication in combination with bicarbonate and Gascon (Simethicone)^[6]. Fujii *et al*^[10] came to the conclusion that the solution of 100 mL water, 20 000 units Pronase, 1 gm bicarbonate, and 80 mg DMPS was more effective than DMPS alone in improving visibility during conventional endoscopy and chromoendoscopy. They showed that this would decrease duration of endoscopy. Kou *et al*^[9] in a similar study proved that Pronase would

improve visualization much better than DMPS only when used in combination with bicarbonate and DMPS. They vividly concluded that Pronase without DMPS was of no use. Pronase is not routinely used in this country and was not the scope of the study.

NAC is a mucolytic and antioxidant agent acting via its free sulfhydryl group to lower the mucus viscosity^[21]. Nor significant interaction neither adverse reaction has been reported with oral preparations. In this study, those patients with a history of asthma and Diabetes Mellitus were excluded. This study is the only one in which the effect of NAC alone has been investigated and compared to Dimethicone and placebo. The patients in group C who received 600 mg NAC in 100 mL water did not show any betterment in visibility scores (8.41 ± 2.10 vs 9.50 ± 2.55 in group A). Combination of NAC and Dimethicone in group D demonstrated better visualization than simple water in group A. But this combination was not superior to Dimethicone alone in group B. We supposed that this was the effect of Dimethicone appearing in group D as in group B and NAC had no effect. In contrast to our results, Chang *et al*^[17] concluded that the mixture of 400 mg NAC and 100 mg DMPS plus water up to 100 mL is more effective than DMPS alone or DMPS in water in a significant manner. They also recommended that NAC could be a substitute for Pronase where it was unavailable. In their study, the mean of the total visibility score in the patients who received NAC plus DMPS was 6.5 ± 2.2 (vs 5.39 ± 1.71 in this study) and in those receiving DMPS with water 7.6 ± 2.6 (vs 5.11 ± 1.28 in this study). The scoring system was exactly similar in the two studies though performed by different endoscopists. All these compounds were proved not to affect the result of rapid urease tests using Campylobacter-like organism tests^[9,17].

In conclusion, regarding the lower cost of Dimethicone (activated) (one third that of NAC per patient herein) and lack of Pronase, we suggest the routine use of 100 mg activated Dimethicone in water up to 100 mL twenty min prior to upper endoscopy here and all other areas where Pronase is not available. To clarify the exact benefits of NAC requires further trials.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Nasrin Shokrpour at the Center for Development of Clinical Research of Nemazee Hospital for editorial assistance. The authors also thank Dr. Nader F for her suggestions in methodology, and Dr. Vosoughi M for the statistical interpretations

COMMENTS

Background

Upper endoscopy is a routine and widely-used procedure to evaluate upper gastrointestinal tract. Since foam and air bubbles can impair visibility, some anti-foam agents such as Simethicone and Pronase are used as premedications prior to endoscopy.

Research frontiers

Activated Dimethicone (Simethicone) has been shown to be effective in reducing

foam and bubbles in the stomach. N-acetylcysteine (NAC) is a mucolytic drug that is supposed to be efficacious too. This study aims to compare the efficacy of these agents in improving visibility.

Innovations and breakthroughs

Activated Dimethicone was shown to be effective. In contrast to prior findings our study showed that NAC was not able to improve visibility when used alone. Furthermore, combination of NAC and Dimethicone did not differ from Dimethicone alone in providing more clear visualization. This is the first study in which the efficacy of NAC was investigated independently as a premedication.

Applications

Usage of activated Dimethicone prior to upper endoscopy is of benefit for improving mucosal visibility; however, N-acetylcysteine seems not to be effective if used alone. Thus, activated Dimethicone should be considered as a premedication before upper endoscopy especially in areas where other agents are lacking.

Peer review

This is a nice study. It is well composed, balanced, documented and the English spelling is good.

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S- Editor Tian L L- Editor O'Neill M E- Editor Xiong L

Usefulness of fecal lactoferrin in predicting and monitoring the clinical severity of infectious diarrhea

Chien-Chang Chen, Chee-Jen Chang, Tzou-Yien Lin, Ming-Wei Lai, Hsun-Chin Chao, Man-Shan Kong

Chien-Chang Chen, Ming-Wei Lai, Hsun-Chin Chao, Man-Shan Kong, Division of Gastroenterology, Department of Pediatrics, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan, China

Chien-Chang Chen, Ming-Wei Lai, Hsun-Chin Chao, Man-Shan Kong, Department of Medicine, Chang Gung University, College of Medicine, Taoyuan 333, Taiwan, China

Chee-Jen Chang, Graduate Institute of Clinical Medical Sciences, Clinical Informatics and Medical Statistics Research Center, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan, China

Chee-Jen Chang, Department of Medicine, Chang Gung University, College of Medicine, Taoyuan 333, Taiwan, China

Tzou-Yien Lin, Division of Infectious Disease, Department of Pediatrics, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan, China

Tzou-Yien Lin, Department of Medicine, Chang Gung University, College of Medicine, Taoyuan 333, Taiwan, China

Author contributions: Chen CC, Chang CJ and Kong MS performed the majority of the experiments; Chang CJ, Lin TY, Lai MW and Chao HC provided the vital reagents and analytical tools and were also involved in editing the manuscript; Chen CC and Kong MS designed the study and wrote the manuscript.

Supported by Chang Gung Memorial Hospital research project grants CMRPG470051-470052

Correspondence to: Man-Shan Kong, MD, Assistant Professor of Pediatrics, Attending physician, Division of Gastroenterology, Department of Pediatrics, Chang Gung Memorial Hospital; Department of Medicine, Chang Gung University, College of Medicine, 12L, 5 Fu-Hsing St, Kwei-Shan, Taoyuan 333, Taiwan, China. pedegl1969@gmail.com

Telephone: +886-3-3281200 Fax: +886-3-3288957

Received: May 3, 2011 Revised: July 11, 2011

Accepted: July 18, 2011

Published online: October 7, 2011

ranging from 3 mo to 10 years in age were enrolled, and one to three stool samples from each subject were collected. Certain parameters, including white blood cells /differential count, C-reactive protein, fecal mucus, fecal pus cells, duration of fever, vomiting, diarrhea and severity (indicated by Clark and Vesikari scores), were recorded and analyzed. Fecal lactoferrin was determined by enzyme-linked immunosorbent assay and compared in different pathogen and disease activity. Generalized estimating equations (GEE) were also used for analysis.

RESULTS: Data included 226 evaluations for 117 individuals across three different time points. Fecal lactoferrin was higher in patients with *Salmonella* ($11.17 \mu\text{g/g} \pm 2.73 \mu\text{g/g}$) or *Campylobacter* ($10.32 \mu\text{g/g} \pm 2.94 \mu\text{g/g}$) infections and lower in patients with *rotavirus* ($2.82 \mu\text{g/g} \pm 1.27 \mu\text{g/g}$) or *norovirus* ($3.16 \mu\text{g/g} \pm 1.18 \mu\text{g/g}$) infections. Concentrations of fecal lactoferrin were significantly elevated in patients with severe ($11.32 \mu\text{g/g} \pm 3.29 \mu\text{g/g}$) or moderate ($3.77 \mu\text{g/g} \pm 2.08 \mu\text{g/g}$) disease activity compared with subjects with mild ($1.51 \mu\text{g/g} \pm 1.36 \mu\text{g/g}$) disease activity ($P < 0.05$). GEE analysis suggests that this marker could be used to monitor the severity and course of gastrointestinal infections and may provide information for disease management.

CONCLUSION: Fecal lactoferrin increased during bacterial infection and with greater disease severity and may be a good marker for predicting and monitoring intestinal inflammation in children with infectious diarrhea.

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Key words: Lactoferrin; Diarrhea; Generalized estimating equations; Vesikari scores; Clark scores

Peer reviewer: Victor E Reyes, PhD, Professor, Departments of Pediatrics and Microbiology and Immunology, Director, GI Immunology Core, Texas Gulf Coast Digestive Diseases Center, Technical Director, Child Health Research Center, University of Texas Medical Branch, 301 University Blvd., Children's Hospital, Galveston, TX 77555-0366, United States

Abstract

AIM: To explore the value of fecal lactoferrin in predicting and monitoring the clinical severity of infectious diarrhea.

METHODS: Patients with acute infectious diarrhea

Chen CC, Chang CJ, Lin TY, Lai MW, Chao HC, Kong MS. Usefulness of fecal lactoferrin in predicting and monitoring the clinical severity of infectious diarrhea. *World J Gastroenterol* 2011; 17(37): 4218-4224 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4218.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4218>

INTRODUCTION

Infectious diarrhea caused by pathogens may induce gastroenteritis, bloody stool, or severe intraabdominal infections, which spreads disease, especially among infant and child populations, and increases the economic burden. Viral infection is a leading cause of diarrhea among children in developed and developing countries. Moreover, several bacterial pathogens, including *Salmonella spp.*, *Campylobacter spp.*, and *Shigella spp.*, can cause invasive diarrhea. These pathogens have the capacity to invade the mucosa of the distal small intestine and colon, stimulate local and systemic inflammatory responses, and may sometimes cause hemorrhaging and ulceration of the mucosa. Although acute infectious diarrhea is a common clinical disease in children, few reliable and noninvasive diagnostic tools have been used as biological markers in patients with acute infectious diarrhea or persistent digestive symptoms.

Lactoferrin, an 80 kDa iron-binding glycoprotein produced by many exocrine glands, is a major whey protein with a major constituent in the secondary granules of neutrophilic leukocytes. Lactoferrin displays diverse biological activities, ranging from the activation of innate immunity^[1,2], microbicidal effects^[3], and anti-cancer cell responses^[4]. Exposure of host cells to lactoferrin may modulate subsequent cellular functions, such as cytokine gene activation^[5], cytotoxicity^[6], and T cell^[7] or B cell^[8] maturation. Lactoferrin may affect innate immunity by stimulating macrophages through interaction with toll-like receptor pathways^[2]. Because diarrheal illnesses are extremely common in communities and hospitals throughout the world, a noninvasive inflammatory marker may be helpful for disease management. The presence of lactoferrin in bodily fluids, including intestinal lumen, is proportional to the flux of neutrophils, and its assessment can provide a reliable biomarker for inflammation. Neutrophils have been shown to be involved in the perpetuation of inflammation in the gut in acute infections caused by *Shigella* and *Salmonella* and inflammatory bowel disease (IBD)^[9-11]. Guerrant *et al.*^[12] confirmed increased fecal lactoferrin in 96% (25/26) of samples from patients with shigellosis and concluded that fecal lactoferrin was a useful marker for fecal leukocytes.

Few scales are available for evaluating gastroenteritis disease severity. The most commonly used scoring scales are the Vesikari 20-point scale, in which an episode of gastroenteritis with a score ≥ 11 is considered severe^[13] (≥ 11 moderate, ≥ 16 severe), and the Clark 24-point scale, in which an episode of gastroenteritis with a score

≥ 16 is considered severe^[14]. Our present prospective study was conducted to explore the role of fecal lactoferrin in gastrointestinal infection, including (1) predicting bacterial or viral infection; (2) ascertaining the extent to which values may be associated with the severity of gastroenteritis in the above scales; and (3) monitoring the severity and course of gastrointestinal infection, which may provide information for disease management.

MATERIALS AND METHODS

This prospective study enrolled and analyzed children being treated in Chang Gung Children's Hospital located in Northern Taiwan. All subjects provided written informed consent, and three fecal samples were collected from each subject.

Enrollment was conducted between September 2008 and May 2010. Diarrhea was defined as three or more outputs of loose or liquid stools per day. Inclusion criteria were 3 mo to 10 years of age and hospitalization with infectious diarrhea. Exclusion criteria were immunodeficiency and history of IBD or gastrointestinal tract surgery. The study protocol was approved by the Institutional Review Board of Chang Gung Memorial Hospital. Informed consent was obtained from the parents of all eligible children. The study was performed in accordance with the Declaration of Helsinki.

Upon entering the study, hospitalized patients received treatment consisting of intravenous fluid and oral rice water or half-strength formula. The severity of diarrhea was evaluated according to the following parameters: volume of stools, fecal consistency and frequency. Other clinical symptoms, including fever, vomiting, abdominal pain, daily intake, and appetite, were also assessed. All participants underwent first-step hematology and biochemistry tests [including blood cell counts, serum C-reactive protein (CRP), and electrolytes] as well as fecal pus cell and mucus analysis. Disease severity was recorded using the severity scoring methods of the Vesikari 20-point scale and Clark 24-point scale. In the Vesikari 20-point scale, an episode of gastroenteritis with a score ≥ 11 is considered moderate or severe (< 11 mild, ≥ 11 moderate, ≥ 16 severe)^[5], and in the Clark 24-point scale, an episode of gastroenteritis with a score ≥ 16 is considered severe. Fecal samples of some patients were collected at three different time points, including the initial stage of infectious diarrhea, 3-5 d later and 7-10 d later. Series follow-ups of fecal lactoferrin were measured by enzyme-linked immunosorbent assay. Their control group comprised 15 children (mean age, 3.7 years; range, 1-10 years) without diarrhea. We compared and analyzed the levels of fecal lactoferrin collected from the different patients at the same time point.

Identifying pathogens

To assess the etiology of infectious diarrhea, fecal specimens were collected to detect *Salmonella*, *Shigella* or *Campylobacter* colonies on specifically prepared agar plates.

Table 1 Patient characteristics

Gender	
Female	52
Male	65
Age (mean), yr	3.23 (3 mo-10 yr)
Pathogen identified	
Rotavirus	41
Norovirus	28
Salmonella	31
Campylobacter	17
Disease severity (Vesikari scoring scale)	
Mild	42
Moderate	50
Severe	25
Duration of diarrhea (median), h	73.8 (14-169)
Vomiting, (d)	2.1 (0-5)
Fever, (d)	2.9 (0-7)
WBC counts (10 ⁶ /L)	12 658 ± 2364
CRP (mg/L)	34.7 ± 22.1
Hemoglobin (g/dL, n = 117)	11.6 (8.2-15.3)
Platelets (10 ⁹ /L, n = 117)	232 (134-585)

Range in brackets. WBC: White blood cells; CRP: C-reactive protein.

The fecal specimens were also sent for evaluating *rotavirus* antigen levels by ELISA and *norovirus* RNA by real-time polymerase chain reaction.

Lactoferrin assay

The stool samples were prepared and analyzed for lactoferrin according to the manufacturer's instructions (AssayMax Human Lactoferrin ELISA Kit, St. Charles, MO, United States). This assay employs a quantitative sandwich enzyme immunoassay technique that measures lactoferrin in 4 h. A polyclonal antibody specific for lactoferrin was pre-coated onto a microplate. Lactoferrin standards and samples were sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for lactoferrin recognized by a streptavidin-peroxidase conjugate. Absorbance was read at 450 nm. Lactoferrin was expressed as µg/g of feces.

Statistical analysis

Simple univariate correlation coefficients (Spearman rank correlation) were calculated using baseline data only. Independent associations between the variables of interest were investigated by generalized estimating equations (GEE). GEE is a regression technique that allows the investigation of longitudinal data while adjusting for within-patient correlations. GEE requires a predefined working correlation structure for the dependent variable (lactoferrin), and based on first level and follow-up data, an exchangeable correlation structure was chosen here. The GEE approach was developed to correct for repeated outcomes within the same subject^[15]. When using data from more than two time points, the GEE analysis was employed for longitudinal analysis (associations).

A univariate comparison between groups was performed with a *t* test for repeated measures, and the χ^2 test

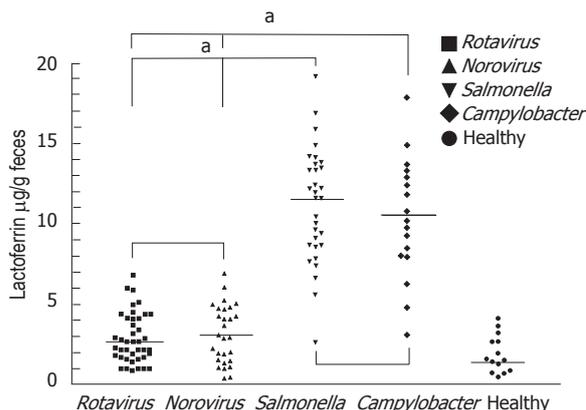


Figure 1 Grouped samples of fecal lactoferrin (µg/g feces) in healthy children and children with gastroenteritis caused by different pathogens, including rotavirus, norovirus, Salmonella and Campylobacter infection. The mean level of fecal lactoferrin was higher in patients with *Salmonella* or *Campylobacter* infections but lower in patients with *rotavirus* or *norovirus* infections. Horizontal line: Mean; ^a*P* < 0.05.

and Fisher's exact test were used with categorical data. Analyses were performed on the intention-to-treat population. A *P* value less than 0.05 was considered significant, and the statistical tests were two-tailed. The GraphPad Software Prism 3.03 (GraphPad Software, Inc., San Diego, CA, United States) and SPSS Software, version 15.0 (SPSS Inc., Chicago, IL, United States), were used for the statistical analysis.

RESULTS

Description of samples

A total of 154 participants were screened between September 2008 and May 2010. From that cohort, 37 patients were excluded from further study because no definite pathogen was identified from the stool examination. Among the individuals included in the study, *rotavirus* infection was diagnosed in 41 patients, and *norovirus* infection was diagnosed in 28 patients. In addition, *Salmonella* infection was diagnosed in 31 patients and *Campylobacter* infection in 17 patients. Demographic details are shown in Table 1.

The data include a total of 226 evaluations for 117 individuals across three different time points. The pattern of assessment was as follows: 43 subjects (36.9%) had three assessments, 23 (19.6%) had two assessments, and 51 (43.5%) had one assessment. The mean age of the subjects was 3.23 years (SD 2.15, range 3 m/o-10 y/o), and 65 (55.5%) were male.

Fecal lactoferrin

The mean ± SD of the fecal lactoferrin concentration was 11.17 µg/g ± 2.73 µg/g in patients with *Salmonella* infections, 10.32 µg/g ± 2.94 µg/g in patients with *Campylobacter* infections, 2.82 µg/g ± 1.27 µg/g in patients with *rotavirus* infections and 3.16 µg/g ± 1.18 µg/g in patients with *norovirus* infections (Figure 1). Concentrations of each fecal marker for patients with either form of bacterial infection were significantly elevated compared

Table 2 The Vesikari and Clark severity scoring scales for the evaluation of gastroenteritis in children

Severity scoring scales	Point value		
	1	2	3
Vesikari ^[5]			
Duration of diarrhea (d)	1-4	5	≥ 6
Maximum number of diarrhea stools/24 h	1-3	4-5	≥ 6
Duration of vomiting (d)	1	2	≥ 3
Maximum number of vomiting episodes/24 h	1	2-4	≥ 5
Temperature (°C)	37.1-38.4	38.5-38.9	≥ 39.0
Dehydration	-	Mild	Moderate to severe
Treatment	Rehydration	Hospitalization	-
Clark ^[6]			
Diarrhea			
Number of stools/d	2-4	5-7	≥ 8
Duration in days	1-4	5-7	≥ 8
Vomiting			
Number of emeses/d	1-3	4-6	≥ 7
Duration in days	2	3-5	≥ 6
Rectal temperature			
Temperature (°C)	38.1-38.2	38.3-38.7	≥ 38.8
Duration in days	1-2	3-4	≥ 5
Behavioral symptoms/signs			
Description	Irritable/less playful	Lethargic/listless	Seizure
Duration in days	1-2	3-4	≥ 5

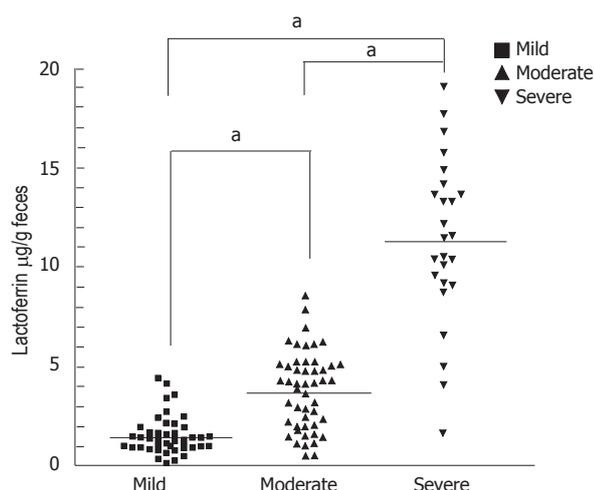


Figure 2 Fecal lactoferrin level ($\mu\text{g/g}$ feces) in children with mild, moderate or severe disease activity according to the Vesikari score (< 11 mild, ≥ 11 moderate, ≥ 16 severe). Levels of fecal lactoferrin were elevated in moderate and severe disease activities. Horizontal line: Mean; ^a $P < 0.05$.

with those of virus-infected patients. The P values for lactoferrin were < 0.05 . No statistical differences were observed in fecal lactoferrin concentrations between the clinically confirmed *Salmonella* and *Campylobacter* infections. The P values for lactoferrin was 0.71.

The mean \pm SD of fecal lactoferrin concentration was $11.32 \mu\text{g/g} \pm 3.29 \mu\text{g/g}$ in patients with severe disease activity (Vesikari score ≥ 16 , Table 2), $3.77 \mu\text{g/g} \pm 2.08 \mu\text{g/g}$ in patients with moderate disease activity (Vesikari score ≥ 11) and $1.51 \mu\text{g/g} \pm 1.36 \mu\text{g/g}$ in patients with mild disease activity (Vesikari score < 11 , Figure 2). Concentrations of each fecal marker for patients with either form (viral or bacterial) of severe or moderate disease activity were significantly elevated compared with those of mild

disease activity. The P values for lactoferrin were < 0.05 .

Univariate linear regression analysis

Certain parameters associated with the level of fecal lactoferrin, including white blood cells/differential count, C-reactive protein, fecal mucus, fecal pus cells, duration of fever, vomiting, diarrhea and severity (as indicated by Clark and Vesikari scores), were recorded and analyzed. To determine the correlation between these parameters and fecal inflammatory markers, we performed a univariate linear regression analysis.

The univariate linear regression analysis revealed that the Vesikari score, Clark score, fecal pus cells, CRP, vomiting and dehydration were all correlated with lactoferrin level (Table 3).

GEE analysis results

Table 4 reveals the results of the multivariate analysis of the predictive value of fecal lactoferrin with time variations. Subjects with higher Vesikari severity scores had higher fecal lactoferrin levels initially (when time = 0), and the levels of fecal lactoferrin may have decreased when followed-up at different time points (when time > 0).

(Lactoferrin = $3.9289 + 3.2257 \times \text{Vesikari score} - 0.1835 \times \text{time} - 0.1575 \times \text{time} \times \text{Vesikari score}$)

However, there was no significant relationship between fecal lactoferrin and the Clark score with time variations. On the contrary, subjects with higher band form (%) had higher fecal lactoferrin levels initially (when time = 0), and the levels of fecal lactoferrin may have decreased when followed-up at different time points (when time > 0).

(Lactoferrin = $3.6654 + 1.9759 \times \text{Band} + 1.627 \times \text{time} - 1.5261 \times \text{Band} \times \text{time}$)

Table 3 Univariate linear regression outcome: Lactoferrin ($y = \alpha + \beta x$)

	β	Standard error	95% CI		P value
			Lower	Upper	
WBC	-0.05	0.10	-0.25	0.14	0.581
Segment	0.00	0.02	-0.05	0.05	0.941
Band	0.47	0.27	-0.07	1.01	0.089
CRP	0.02	0.01	0.00	0.04	0.043 ^a
Fecal pus cell					
None	0.00				
Present	0.38	1.55	-2.74	3.49	0.809
Fecal occult blood					
None	0.00				
Present	1.71	1.22	-0.73	4.15	0.165
Fecal mucus					
None	0.00				
Positive	-2.21	1.46	-5.14	0.71	0.135
Vesikari scoring scale					
Non-severe < 11	0.00				
Moderate \geq 11	2.76		-1.50	7.02	0.191
Severe \geq 16	2.81	1.13	0.56	5.07	0.015 ^a
Clark scoring scale					
Non-severe < 16	0.00				
Severe \geq 16	3.13	1.10	0.93	5.32	0.006 ^a
Body temperature	0.29	0.30	-0.31	0.89	0.341
Abdominal pain	0.88	0.63	-0.39	2.15	0.169
Abdominal distension	-0.30	1.01	-2.32	1.71	0.764
Dehydration	3.13	1.38	0.37	5.89	0.027 ^a
Oral intake	1.13	1.23	-1.33	3.59	0.362
Activity	1.13	1.00	-0.87	3.13	0.262
Fever day	0.09	0.28	-0.46	0.65	0.739
Diarrhea day	0.41	0.29	-0.60	1.41	0.423
Vomiting day	-0.99	0.53	-1.93	-0.04	0.041 ^a

^aP < 0.05. WBC: White blood cells; Band: Band form neutrophil; CRP: C-reactive protein.

DISCUSSION

An intense intestinal infection involves intense infiltration of neutrophils, macrophages, mast cells, lymphocytes, natural killer cells, other inflammatory cells in the epithelial lining and the lamina propria of the colonic mucosa^[16]. Cells in the innate immune system secrete various enzymes and metabolites, including myeloperoxidase and lactoferrin, produced by activated neutrophils. Lactoferrin is found mainly in the oral cavity and intestinal tract where it can come into direct contact with pathogens, such as viruses and bacteria. The noninvasive fecal marker lactoferrin may prove useful in screening for inflammation in patients with abdominal pain and diarrhea^[17]. Our study demonstrates the usefulness of fecal lactoferrin for detecting colonic inflammation in children with gastrointestinal symptoms, such as enteritis or enterocolitis.

The most significant function of lactoferrin in mucosal defense is its antimicrobial activity. Lactoferrin can also amplify the actions of lysozyme and secretory immunoglobulin A^[18]. *In vitro* studies have shown lactoferrin's bactericidal effects on *V. cholerae*, *Salmonella enterica* subsp. *enterica* serovar mutants, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans*^[19]. Thus, increased stool levels of lac-

Table 4 Generalized estimating equations analysis for series follow-up of fecal lactoferrin

Parameter	Estimate	Standard error	95% confidence interval		P value
Clark score					
Intercept	5.1523	0.7490	3.6843	6.6202	< 0.0001 ^a
Clark score	2.0393	1.7397	-1.3704	5.4489	0.2411
Time	-0.3729	0.2530	-0.8688	0.123	0.1405
Time x Clark	0.5826	0.8342	-1.0525	2.2177	0.485
Vesikari score					
Intercept	3.9289	0.8437	2.2752	5.5825	< 0.0001 ^a
Vesikari score	3.2257	1.3018	0.6741	5.7773	0.0132 ^a
Time	-0.1835	0.2715	-0.7157	0.3487	0.4992
Time x Vesikari	-0.1575	0.5222	-1.1811	0.8660	0.0429 ^a
Band (band form neutrophil)					
Intercept	3.6654	1.0750	1.5585	5.7723	0.0007 ^a
Band	1.9759	0.6951	0.6135	3.3383	0.0045 ^a
Time	1.6270	0.8206	0.0186	3.2354	0.0474 ^a
Band x time	-1.5261	0.4677	-2.4428	-0.6094	0.0011 ^a

^aP < 0.05.

toferrin in acute shigellosis may suggest increased degranulation of neutrophils upon stimulation that may promote the killing of *Shigella* in the colonic mucosa. Interaction of lactoferrin with immune system cells induces a regulated release of cytokines, such as interleukin 6 and tumor necrosis factor alpha^[19], which has also been observed during acute *Shigella* infection in adults^[20,21] and children.

Fecal lactoferrin has been reported as a promising biomarker in active Crohn's disease^[22] and ulcerative colitis^[23], requiring the exclusion of patients enrolled with a history of the above IBDs. Indeed, in patients without known IBDs suspected of having a bacterial diarrheal illness, fecal lactoferrin may be useful in evaluating bacterial gastrointestinal infections in which antimicrobial therapy may be prescribed (e.g., *Salmonella*, *Shigella*, *Campylobacter*, and pathogenic *Escherichia coli* spp.) and aid in following the inflammatory activity of bacterial infection. Previous study has suggested that fecal lactoferrin could serve as a screening tool for deciding when to perform a stool culture^[24]. Fifty-five patients were enrolled in the Choi *et al*^[24] study, and the researchers reported that fecal lactoferrin was higher in invasive bacterial pathogens and might greatly enhance a cost-effective approach for evaluating infectious diarrhea^[24]. Fecal lactoferrin could be a more sensitive test than fecal leukocytes for evaluating patients with acute diarrhea. Scerpella *et al*^[25] reported that 94% of travelers with invasive pathogens had positive fecal lactoferrin, while only 69% had fecal leukocytes. The other study has also found that fecal lactoferrin was better than methylene blue for detecting invasive pathogens^[26]. According to our study, the fecal lactoferrin level is higher in bacterial gastrointestinal infections, such as *Salmonella* and *Campylobacter*, but lower in patients with rotavirus or norovirus infections. The above results are similar to the findings of Choi *et al*^[24] (higher fecal lactoferrin in *Salmonella*, *Campylobacter* and *Shigella* infections but lower in rotavirus infections).

In some meta-analyses of the sensitivity and specific-

ity of different markers of intestinal inflammation associated with invasive pathogens (e.g., fecal leukocytes, occult blood in stool, and fecal lactoferrin), fecal lactoferrin was recommended as having the best diagnostic accuracy^[27-29]. In enteroaggregative *Escherichia coli* infectious diarrhea, mucosal inflammation included heavy mucus formation, intimate cell adherence, and secretion of toxins, and the common finding was higher fecal lactoferrin, which suggests a diffuse colonic inflammatory process^[30,31]. Our study has demonstrated that fecal lactoferrin is higher in infections caused by *Salmonella* and *Campylobacter* and in moderate or severe disease severity.

In our study, the data include 226 evaluations for 117 individuals across three different time points. Concentrations of fecal lactoferrin were significantly elevated in patients with severe or moderate disease activity compared with those with mild disease activity ($P < 0.05$ for each marker). Univariate linear regression analysis revealed that the Vesikari and Clark scores, fecal pus cells, CRP, vomiting and dehydration were all correlated with the lactoferrin level. The parameters of the Vesikari and Clark scores included body temperature, severity of dehydration, and the number of instances and duration of diarrhea and vomiting. Fecal pus cells are usually positive in bacterial infection. Increased CRP may be related to intestinal mucosal inflammation caused by pathogens. Taken together, fecal lactoferrin might correlate with disease activity, which may include the number of instances and duration of diarrhea or vomiting, severity of fever or dehydration, fecal pus cells and CRP.

GEE is a regression technique that allows the investigation of longitudinal data and corrects for the repeated outcomes within the same subject. GEE requires a pre-defined working correlation structure for the dependent variable (lactoferrin) and is based on first level and follow-up data. In our study, we found that fecal lactoferrin on the first evaluation and follow-up levels were highly associated with Vesikari scores. The above results indicate that fecal lactoferrin may be useful in monitoring the severity of infectious diarrhea during the course of the disease and may provide information for the management of gastrointestinal infection. In addition, fecal lactoferrin levels at the first evaluation and at follow-up were also associated with the band-form percentile. This result suggests that fecal lactoferrin may play a role in monitoring the disease activity and providing guidance for treating infectious diarrhea. According to our study, the measurement of fecal lactoferrin may be a useful noninvasive test for evaluating intestinal infectious or inflammatory situations. For children with persistent diarrhea or recurrent digestive symptoms after one episode of gastrointestinal infection, fecal lactoferrin could be a helpful tool for providing treatment and management information for physicians.

In conclusion, the non-invasive marker fecal lactoferrin was able to predict bacterial or viral infection, and the relative values may be associated with the severity of gastroenteritis, corresponding to Vesikari and Clark scores. Furthermore, fecal lactoferrin may be useful in monitor-

ing the severity and course of gastrointestinal infections, which may provide information for disease management and follow-up.

ACKNOWLEDGMENTS

The authors thank Shao-Yu Lin for assistance with the statistical analysis.

COMMENTS

Background

This study provides increasing evidence that acute gastrointestinal infection is a common clinical disease in children. Few reliable, noninvasive and painless diagnostic tools have been used as biological markers in patients with acute gastroenteritis.

Research frontiers

How to predict the infectious pathogens (virus or bacteria) that caused acute diarrhea has not been fully clarified. The use of fecal leukocytes, pus cells and serum C-reactive protein has been attempted but was not fully effective. Fecal lactoferrin could be involved in the inflammation caused by the intestinal infectious pathogen. We attempt to investigate a useful noninvasive fecal marker for predicting and monitoring intestinal inflammation in children with infectious diarrhea.

Innovations and breakthroughs

The study design measures the level of fecal lactoferrin during acute infectious diarrhea. The authors also investigated the clinical information and certain parameters of patients, as well as used univariate linear regression analysis and generalized estimating equations to (1) predict bacterial or viral infection; (2) ascertain the extent to which values may be associated with the severity of gastroenteritis; and (3) monitor the severity and course of gastrointestinal infection, which may provide information for disease management.

Applications

This study found that fecal lactoferrin is higher in patients with *Salmonella* infection or *Campylobacter* infections but lower in patients with *rotavirus* infection or *norovirus* infections. Fecal lactoferrin increased during bacterial infection and with greater disease severity and may be a good marker for predicting and monitoring intestinal inflammation in children with infectious diarrhea.

Peer review

The study by Chen and colleagues presents the value of using fecal lactoferrin to predict and monitor the clinical severity of infectious diarrhea. The study is well planned, includes a robust sample size, is well controlled and the results are clearly interpretable. Perhaps, one benefit, which they argue for the approach in using fecal lactoferrin is that it allows for diagnosis and monitoring without using invasive approaches.

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S- Editor Sun H L- Editor Rutherford A E- Editor Xiong L

Aberrant methylation of the 3q25 tumor suppressor gene *PTX3* in human esophageal squamous cell carcinoma

Jun-Xiong Wang, Yuan-Long He, Sheng-Tao Zhu, Shuo Yang, Shu-Tian Zhang

Jun-Xiong Wang, Medical and Health Center, Beijing Friendship Hospital Affiliated to the Capital Medical University, Beijing 100050, China

Yuan-Long He, Department of Gastroenterology, Qingdao Municipal Hospital, Qingdao 266071, Shandong Province, China

Sheng-Tao Zhu, Shu-Tian Zhang, Department of Gastroenterology, Beijing Friendship Hospital Affiliated to the Capital Medical University, Beijing 100050, China

Shuo Yang, Laboratory Medicine Department, Peking University Third Hospital, Beijing 100050, China

Author contributions: Wang JX and He YL performed the majority of the experiments; Zhu ST and Yang S provided vital reagents, analytical tools and were also involved in editing the manuscript; Zhang ST designed the study; Wang JX and He YL contributed equally to this study.

Supported by National High Technology Research and Development Program of China (863 Program), No. 2007AA02Z4Z4; China Postdoctoral Science Foundation, No. 20090460394 and Beijing Municipal Natural Science Foundation, No. 7072022

Correspondence to: **Shu-Tian Zhang, Professor**, Department of Gastroenterology, Beijing Friendship Hospital Affiliated to the Capital Medical University, No. 95 Yong'an Road, Xuanwu District, Beijing 100050, China. jxwang88@gmail.com

Telephone: +86-10-63138702 Fax: +86-10-63138076

Received: December 23, 2010 Revised: March 24, 2011

Accepted: March 31, 2011

Published online: October 7, 2011

Abstract

AIM: To identify the novel methylation-silenced gene *pentraxin 3 (PTX3)* in esophageal squamous cell carcinoma (ESCC).

METHODS: *PTX3* mRNA expression was examined in six human ESCC cell lines, one human immortalized normal esophageal epithelial cell line, primary ESCC tumor tissue, and paired adjacent nontumor tissue using reverse transcription polymerase chain reaction (RT-PCR). Semi-quantitative immunohistochemistry was used to examine cellular localisation and protein levels. Methylation specific PCR and bisulphite genomic se-

quencing were employed to investigate the methylation of the candidate gene.

RESULTS: In the majority of ESCC cell lines, we found that *PTX3* expression was down-regulated due to gene promoter hypermethylation, which was further confirmed by bisulphite genomic sequencing. Demethylation treatment with 5-aza-2'-deoxycytidine restored *PTX3* mRNA expression in ESCC cell lines. Methylation was more common in tumor tissues (85%) than in adjacent nontumor tissues (25%) ($P < 0.01$).

CONCLUSION: *PTX3* is down-regulated through promoter hypermethylation in ESCC, and could potentially serve as a biomarker of ESCC.

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Key words: Tumor suppressor gene; Pentraxin 3; Microarray; DNA methylation; Esophageal squamous cell carcinoma

Peer reviewer: Mehmet Fatih CAN, Assistant Professor, Department of General Surgery, Gulhane School of Medicine, Gulhane Askeri Tıp Akademisi, Genel Cerrahi AD, Etlik, Ankara, 06018, Turkey

Wang JX, He YL, Zhu ST, Yang S, Zhang ST. Aberrant methylation of the 3q25 tumor suppressor gene *PTX3* in human esophageal squamous cell carcinoma. *World J Gastroenterol* 2011; 17(37): 4225-4230 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4225.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4225>

INTRODUCTION

Esophageal cancer is the sixth most common cause of cancer death worldwide, with over 400 000 new cases diagnosed each year^[1]. Esophageal squamous cell carcinoma (ESCC) has a high morbidity and mortality rate in

China. However, the molecular mechanisms underlying ESCC development remain poorly understood.

Human carcinogenesis is a multi-stage process in which genetic and epigenetic changes lead to oncogene activation and tumor suppressor gene inactivation^[2]. Epigenetic changes, such as promoter DNA methylation, can induce the inactivation of tumor suppressor genes. DNA methylation plays a crucial role in the development of nearly all types of cancer^[3]. Recently, a growing list of aberrantly methylated genes has been reported in ESCC, including esophageal cancer related gene 4^[4], p16^[5], adenomatous polyposis coli^[6], transmembrane protein endothelial factor^[7], deleted in liver cancer 1^[8], ubiquitin carboxy-terminal hydrolase 1^[9], testis-specific Y-like protein 5, and human protein phosphatase-1 regulatory subunit-14A^[10]. Nevertheless, most of these tumor suppressor genes exhibit a relatively low frequency of methylation in ESCC. Thus, further studies of a greater number of genes involved in the disease pathogenesis and progression are needed to identify putative epigenetic biomarkers for this tumor type.

The *pentraxin 3 (PTX3)* gene at 3q25 is a member of the pentraxin superfamily. PTX3 expression is induced in response to inflammatory signals, and is produced at the site of inflammation by several cell types, primarily monocytes/macrophages, dendritic cells (DCs), endothelial cells, smooth muscle cells (SMCs), and fibroblasts. PTX3 can combine with a variety of soluble receptor ligands, and plays multiple biological roles, such as immune defense, female reproductive fertility, atherosclerosis, apoptosis, and the regulation of angiogenesis^[11-14]. To date, there has been no reported study concerning *PTX3* gene promoter methylation.

In the present study, we examined reactivation of epigenetically silenced genes using an oligonucleotide microarray in ESCC cell lines. We also investigated the gene expression profiles of tumor tissue and nontumor tissue in ESCC. The genes markedly up-regulated by 5-aza-2'-deoxycytidine (5-Aza-dC) treatment in an ESCC cell line and markedly decreased in tumor tissue compared with nontumor tissue were considered genes of interest. Bisulphite sequencing and methylation-specific polymerase chain reaction (MSP) analyses were carried out on these genes to confirm the presence of aberrantly methylated CpG dinucleotides. Using the methods mentioned above, we successfully identified PTX3 as a new epigenetically silenced hypermethylated gene in ESCC.

MATERIALS AND METHODS

Cell lines and tissue samples

Six human ESCC cell lines were utilized in this study (TE-11, KYSE-30, KYSE-410, KYSE-510, EC-109, and EC-9706) [from American type culture collection (ATCC) and Sciencell]. One human immortalized normal esophageal epithelial cell line (Het-1A) (from ATCC) was used as the "normal" control for ESCC. The ESCC cell lines were cultured in Roswell Park Memorial Institute 1640

supplemented with 10% fetal bovine serum (Hyclone, United States) and antibiotics (100 U/mL penicillin G and 100 µg/mL streptomycin) at 37 °C in a humidified 5% CO₂ incubator. Het-1A cells were maintained in bronchial epithelial basal media with growth supplements (Clonetics, United States).

Twenty primary ESCC and paired adjacent nontumor tissues were obtained from the Beijing Friendship Hospital, Beijing, China. Specimens were snap-frozen in liquid nitrogen and subsequently stored at -80 °C. Formalin-fixed, paraffin-embedded samples of 79 primary ESCC cancer tissue specimens and paired adjacent nontumor tissues were also obtained from the Beijing Friendship Hospital. All patients from whom we obtained the study specimens gave informed consent to participate in this study. All case samples were collected from the primary surgical resection in patients with no prior history of ESCC and adjuvant therapy. Pathological diagnosis was performed and confirmed in the Pathology Department. Tumors were histopathologically classified according to TNM (tumor node metastasis) criteria.

Treatments with 5-aza-2'-deoxycytidine

ESCC cell lines and Het-1A were treated with 10 µmol/L of the DNA demethylating agent 5-Aza-dC (Sigma-Aldrich, United States) for 4 d.

RNA extraction and reverse transcription-PCR

Total RNA was extracted from cell line pellets and tissues using Trizol (Invitrogen, United States). Reverse transcription was performed using total RNA (1 µg) with Reverse Transcription System (Applied Biosystems, United States). The PTX3 mRNA expression levels were detected by conventional RT-PCR with Taq polymerase (Takara, Japan). RT-PCR was performed for 35 cycles at an annealing temperature of 55 °C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control of RNA integrity. Primer sequences for PTX3 are as follows: (1) PTX3-F: 5'-TCTCTGGTCTGCAGTGTGG-3'; (2) PTX3-R: 5'-TGAAGAGCTTGTCCCATTC-3'; (3) GAPDH-F: 5'-CGGAGTCAACGGATTGGTCGTAT-3'; and (4) GAPDH-R: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

Isolation and bisulphite modification of genomic DNA

Genomic DNA was extracted from cells and tissues by standard phenol-chloroform extraction. Bisulphite modification of DNA was produced with a Zymo DNA Modification Kit (Zymo Research, United States) according to the manufacturer's protocol.

MSP

Bisulphite-treated DNA was amplified with the methylation-specific primer set, PTX3-MF: 5'-CGTTTGCGGT-TAGGAGTATTC-3', and PTX3-MR: 5'-CAAAACGTC-GTCCGTAACCTTA-3', or the unmethylation-specific primer set, PTX3-UF: 5'-TGTGTTTGTGGTTAGGAG-TATTTG-3' and GPX3-UR: 5'-CAAAACATCATC-

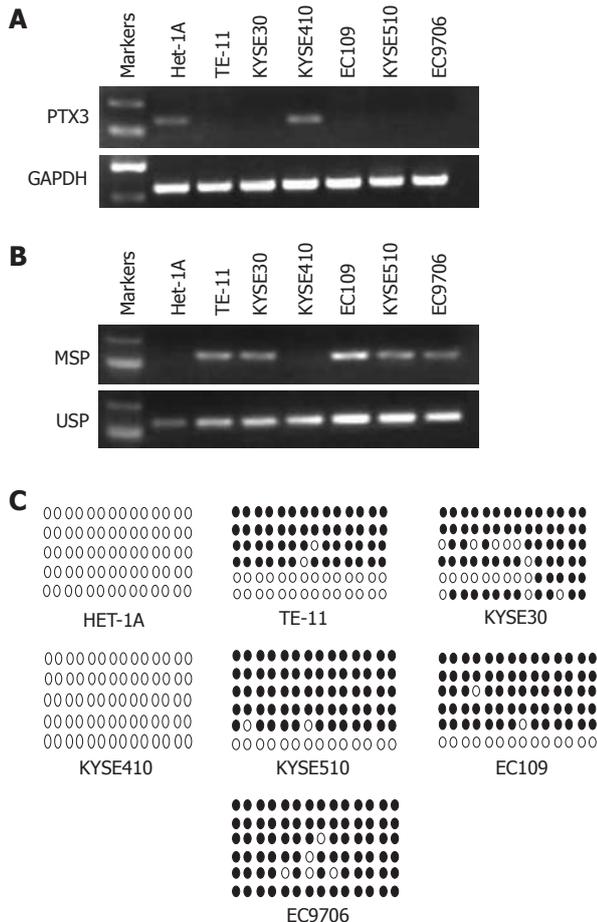


Figure 1 Promoter methylation contributes to pentraxin 3 downregulation in esophageal squamous cell carcinoma cell lines. **A:** Pentraxin 3 (PTX3) mRNA expression was detected by reverse transcription polymerase chain reaction; **B:** The methylation status of PTX3 promoter was determined by methylation-specific polymerase chain reaction. **C:** Representative high-resolution analyses of the methylation status of individual CpG sites in the PTX3 promoter by bisulphite genomic sequencing. Each row represents one bacterial clone with one circle symbolizing one CpG site. Methylated and unmethylated CpG sites were indicated by black and white circles, respectively. **PTX3:** Pentraxin 3; **GAPDH:** Glyceraldehyde-3-phosphate dehydrogenase; **MSP:** Methylation-specific polymerase chain reaction; **USP:** Unmethylation.

CATAACTTA-3', in a total volume of 20 μ L using a 0.5 unit of hot-start Taq-polymerase (Takara, Japan) per reaction. The size of the unmethylated amplicon is 105 bp, and the methylated amplicon is 103 bp. Cycle conditions were: initial denaturation and hot start for 10 min at 95 $^{\circ}$ C, then 35 cycles consisting of 30 s at 94 $^{\circ}$ C, 30 s at 59 $^{\circ}$ C (for methylated reactions) or 50 $^{\circ}$ C (for unmethylated reactions), and 45 s at 72 $^{\circ}$ C, and a final extension of 10 min at 72 $^{\circ}$ C.

Bisulphite genomic sequencing

For the bisulphite genomic sequencing technique, the targeted fragment was amplified from bisulphite-treated DNA, cloned, and sequenced to obtain an accurate map of the distribution of CpG methylation. Forward: 5' TTTTGTGAGATATTTATATGTTGTTTTT-3' and Reverse: 5'AAACACTAATCAACCTAACCTCTAC-3'.

PCR was performed for 40 cycles at an annealing temperature of 54 $^{\circ}$ C. The PCR products were then cloned into the pEasy-T1 vector (Transgene, China), and eight to ten colonies were randomly chosen and sequenced.

Immunohistochemistry

Expression of PTX3 was investigated in 79 primary ESCC cancer tissue specimens and in paired adjacent nontumor tissues using immunohistochemistry. The immunohistochemical expression of PTX3 was examined with light microscopy. The percentage of positive tumor cells was determined semi-quantitatively by assessing the entire tumor section, and each sample was assigned to one of the following categories: 0 (0%-4%), 1 (5%-24%), 2 (25%-49%), 3 (50%-74%), or 4 (75%-100%)^[15]. The intensity of the immunostain was categorised as 0 (no stain), 1+ (weak stain), 2+ (medium stain), and 3+ (strong stain). Additionally, an immunoreactive score was calculated by multiplying the percentage of positive cells and the stain intensity. In instances of heterogeneous staining intensity within one sample, each component was scored independently and the results were summed. Using this system, the maximum score was 12. For statistical analysis, the criteria were combined as follows: 0, negative; 1 to 4, weakly positive; 5 to 8, moderately positive; and 9 to 12, strongly positive^[16].

Statistical analysis

Statistical analysis was performed using the Student's *t* test and the χ^2 test; a value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Transcriptional silencing of PTX3 in most ESCC cell lines

Using RT-PCR with glyceraldehyde 3-phosphate dehydrogenase as a control, we tested the mRNA expression of PTX3 in the immortalized normal esophageal epithelial cell line Het-1A, and in 6 ESCC cell lines (TE-11, KYSE-30, KYSE-410, KYSE-510, EC-109, and EC-9706). The PTX3 transcript was silenced in 5 ESCC cell lines (TE-11, EC109, EC9706, KYSE30, and KYSE510), but not in KYSE-410 and Het-1A (Figure 1A).

As DNA methylation is an important regulator at the transcription level, we hypothesized that the silencing of PTX3 could be caused by methylation. Thus, we performed the MSP assay to measure the methylation level in these ESCC cell lines. The MSP results indicated that partial methylation was observed in the ESCC cell lines TE-11, EC109, EC9706, KYSE30, and KYSE510, whereas the unmethylated promoter was detected in KYSE-410 and in the immortalized normal esophageal epithelial cell line (Het-1A) (Figure 1B). The methylation results were in concordance with the transcription results of these ESCC cell lines.

We performed bisulphite genomic sequencing analysis to confirm the MSP result, and as shown in Figure 1C,

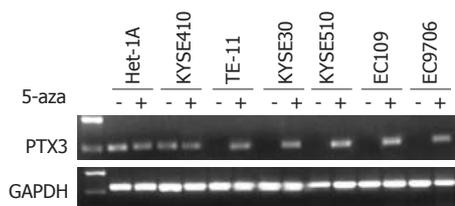


Figure 2 The mRNA expression of pentraxin 3 was restored after treatment with demethylation agent 5-aza-2'-deoxycytidine in esophageal squamous cell carcinoma cell lines. PTX3: Pentraxin 3; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.



Figure 3 Representative methylation-specific polymerase chain reaction results of esophageal squamous cell carcinoma primary tumors (T) and paired adjacent nontumor tissues (N). Numbers 1-8: Sample number. M: Methylation; U: Unmethylation.

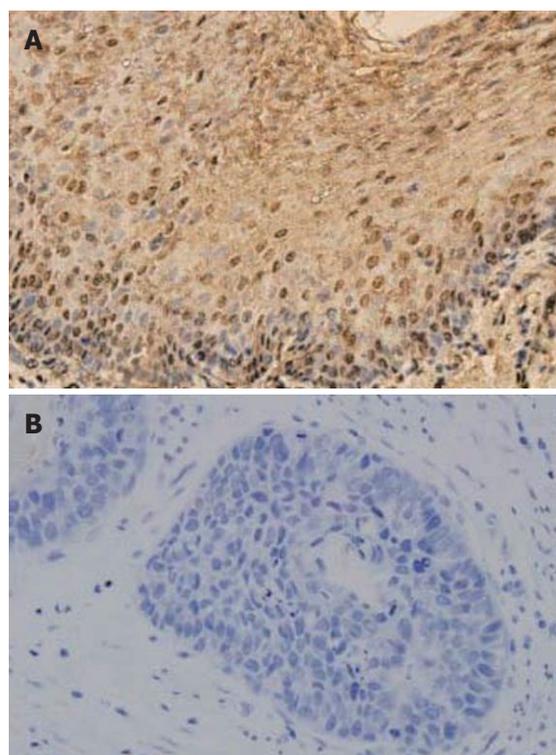


Figure 4 Pentraxin 3 expression assessed by immunohistochemistry staining in esophageal squamous cell carcinoma tumor tissues and adjacent nontumor tissues. A: Significant expression of PTX3 was detected in adjacent nontumor tissues (x 40); B: Negative or weak expression of pentraxin 3 was observed in esophageal squamous cell carcinoma (x 40).

the BGS result was consistent with our MSP data: no methylated CpG sites detected in KYSE-410 and Het-1A, but methylated CpG sites in the other ESCC cell lines (TE-11, EC109, EC9706, KYSE30 and KYSE510). The results indicated that transcriptional silencing of PTX3 was associated with methylation in ESCC cells.

PTX3 expression is up-regulated in ESCC cell lines treated with 5-aza-2-deoxycytidine

The results of RT-PCR indicated that five ESCC cell lines did not express PTX3 mRNA (Figure 1A). As mentioned above, there was a correlation between PTX3 silencing and DNA methylation in ESCC cell lines. To determine whether PTX3 expression could be reactivated by pharmacological demethylation of genomic DNA, all cell lines above were treated with the demethylating agent 5-Aza-dC. After treatment with 5-Aza-dC at 10 μmol/L for 4 d, the five silenced ESCC cell lines resulted in an obvious increase in PTX3 expression (Figure 2), further supporting the role of methylation as a primary mechanism of PTX3 silencing.

Methylation of PTX3 promoter is observed in most ESCC tumor tissues

To assess whether the PTX3 promoter hypermethylation observed in cell lines was relevant to ESCC, we further examined PTX3 methylation in 20 primary ESCC tumors with paired adjacent nontumor tissues using MSP. We found that 80% of ESCC tumor samples (16 of 20) exhibited statistically different methylation within the PTX3 promoter region, whereas the paired nontumor tissues exhibited only 20% (5 of 20) ($P < 0.001$).

The MSP results of ESCC primary tumors (T) and paired adjacent nontumor tissues (N) are shown in Figure 3.

Decreased expression of PTX3 protein in ESCC

We then analyzed 79 primary ESCC specimens and their corresponding adjacent nontumor tissues using immunohistochemical staining. PTX3 protein was detected in 24 of 79 (30.38%) ESCC specimens. In the non-malignant tissues, 67 of 79 (84.81%) samples showed positive detection of PTX3 protein.

In adjacent nontumor tissues, intense immunostaining for PTX3 was observed consistent with cytoplasmic distribution (Figure 4), whereas absent or weak immunostaining was detected in tumor tissues. Immunohistochemical results revealed that the expression of PTX3 protein in ESCC tumor tissues was significantly lower compared to adjacent nontumor tissues ($P < 0.01$).

The clinicopathological features of these patients and the results of PTX3 expression are summarized in Table 1. Statistical analysis indicated that PTX3 protein expression exhibited no correlation with the patients' age, gender, smoking habit, depth of invasion, or lymph node metastasis ($P > 0.05$). However, we found a higher frequency of promoter hypermethylation of PTX3 in the early stages of cancer (I and II) compared to advanced stages, suggesting that PTX3 hypermethylation occurs during a relatively early stage of the multi-step esophageal carcinogenesis.

DISCUSSION

Tumorigenesis is a multistep process caused by the accumulation and interplay of genetic and epigenetic alterations. DNA methylation is a key regulator of gene

Table 1 Clinical features of esophageal squamous cell carcinoma patients and their expression status for pentraxin 3 *n* (%)

Clinicopathological features	PTX3 expression		P value
	Positive	Negative	
Cases	24 (30)	55 (70)	
Gender			
Male	16 (30)	38 (70)	0.831
Female	8 (32)	17 (68)	
Age (mean, yr)	59.6	61.2	0.510
Smoker	12 (32)	26 (68)	0.823
Non-smoker	12 (29)	29 (71)	
Depth of invasion			0.315
T1, T2	16 (35)	30 (65)	
T3, T4	8 (24)	25 (76)	
Lymph node metastasis			0.311
Positive	4 (20)	16 (80)	
Negative	20 (34)	39 (66)	
TNM classification			0.012
I	9 (23)	20 (77)	
II a	10 (35)	19 (65)	
II b	2 (29)	5 (71)	
III	1 (11)	8 (89)	
IV	2 (67)	3 (23)	

TNM: TNM classification of malignant tumours, T describes the size of the tumor and whether it has invaded nearby tissue, N describes regional lymph nodes that are involved, M describes distant metastasis.

transcription and genomic stability. Alteration of DNA methylation is one of the most consistent epigenetic changes that silence tumor suppressor genes in human cancers^[17]. Additionally, aberrant methylation results in increased gene mutagenicity, due to the deamination of 5-methylcytosine to thymine^[17].

We have previously analyzed expression microarray data prior to and post treatment using a demethylating agent in the EC9706 cell line, and we speculated that the *PTX3* gene is potentially down-regulated by promoter hypermethylation. *PTX3* is involved in the regulation of innate resistance to pathogens, the inflammatory reaction, and possibly the clearance of self-components and female fertility. Transfection of *PTX3* into breast cancer cells lacking expression led to a reduction in endothelial cell invasion and capillary tube formation, as well as prevention of tumor formation in athymic nude mice^[18]. In this study, we examined the methylation status of the *PTX3* promoter region in ESCC. As far as we understand, this is the first study to report on *PTX3* gene promoter methylation in ESCC.

Silencing of *PTX3* in ESCC

Down-regulation of *PTX3* mRNA and protein was detected by RT-PCR and immunostaining. We found that *PTX3* was silenced in most ESCC cell lines (5 of 6 ESCC cell lines that we tested) and also silenced in tumor tissues. Reduced expression of *PTX3* suggests that *PTX3* plays a tumor suppressive role in ESCC.

Methylation of *PTX3* promoter

PTX3 promoter hypermethylation was confirmed by

methylation specific PCR and bisulphate genomic sequencing methylation in 83.3% of ESCC cell lines (5/6) and 80% of primary esophageal squamous cell carcinoma tissues (16/20), suggesting that promoter hypermethylation is a major, if not the only, mechanism for *PTX3* down-regulation in ESCC.

Up-regulation of *PTX3* expression after treatment with 5-Aza-dC

Treatment of ESCC cell lines *in vitro* with 5-Aza-dC, a nucleoside analogue inhibitor of DNA methyltransferase, reversed *PTX3* CpG island hypermethylation and restored *PTX3* expression; this confirmed that *PTX3* hypermethylation serves as the principal mechanism for *PTX3* downregulation in ESCC. *PTX3* hypermethylation is involved in the development and progression of esophageal cancer.

Clinico-pathological significance of *PTX3* protein expression

In our study, *PTX3* protein expression was not associated with patients' age, gender, smoking habit, depth of invasion, and lymph node metastasis. We found a high frequency of promoter hypermethylation of *PTX3* in the early tumor stages (I and II) of ESCC, indicating that aberrant methylation is a relatively early event in esophageal carcinogenesis.

Methylation-mediated inactivation is reversible, and up-regulation of *PTX3* by 5-aza-dC may reverse the malignant phenotype of tumor cells. Therefore, *PTX3* could serve as a novel target for gene therapy in ESCC treatment. In the future, further studies to elucidate the function of *PTX3* in ESCC are warranted.

In summary, our study provides the first documentation that *PTX3* is a novel tumor suppressor gene epigenetically silenced in most ESCC tumors. Our results suggest that methylation of the *PTX3* promoter region occurs at an early stage of ESCC pathogenesis and may provide a suitable biomarker for ESCC diagnosis.

COMMENTS

Background

Esophageal cancer is the sixth most common cause of cancer death worldwide, with > 400 000 new cases diagnosed each year. The molecular mechanisms underlying esophageal squamous cell carcinoma (ESCC) development remain poorly understood. DNA methylation plays a crucial role in the development of ESCC.

Research frontiers

Oligonucleotide Microarray Analysis can be used to identify novel genes that are aberrantly methylated in ESCC. Genes that were significantly upregulated after 5-aza-2'-deoxycytidine (5-Aza-dC) treatment in ESCC cells and significantly downregulated in tumor tissue compared with paired nontumor tissue were selected as hypermethylated candidate genes. Subsequently, bisulphite sequencing and methylation-specific PCR were performed to confirm the presence of aberrantly methylated CpG dinucleotides. In this study, the authors successfully identified *pentraxin 3* (*PTX3*) as a new methylation-silenced gene in ESCC.

Innovations and breakthroughs

The study provides the first documentation that *PTX3* is a novel candidate tumor suppressor gene epigenetically silenced in most ESCC tumors. The results

suggest that methylation of the PTX3 promoter region occurs at an early stage of ESCC pathogenesis and may be used as a biomarker for ESCC diagnosis.

Applications

Up-regulating PTX3 by 5-aza-dC may reverse the malignant phenotype of tumor cells. Therefore, PTX3 could be used as a novel target for gene therapy in ESCC treatment. PTX3 functions extracellularly as a secreted protein. PTX3 plays a tumor suppressive role in ESCC; thus, PTX3 protein could potentially be used directly as an anticancer drug therapy.

Terminology

PTX3 gene at 3q25 is a member of the pentraxin super-family. PTX3 expression is induced in response to inflammatory signals. PTX3 is able to combine with a variety of soluble receptor ligands and play multiple biological roles, such as immune defense, female reproductive fertility, atherosclerosis, apoptosis, and regulation of angiogenesis.

Peer review

This study reports the newly recognized tumor suppressor features of the gene *PTX3*, a gene that encodes a protein referred to as pentraxin 3 that serves as a protector against pathogens in tissues and aids in the control of autoimmunity. As an acute phase reactant, its blood level increases significantly during sepsis and severe inflammation, correlating with the severity of the disease and making the protein a useful biomarker for many inflammatory diseases. The authors of this study suggest that PTX3 could also be utilized as a tumor marker in ESCC.

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S- Editor Sun H L- Editor O'Neill M E- Editor Xiong L

Role of Kasai procedure in surgery of hilar bile duct strictures

Jin-Bo Gao, Li-Shan Bai, Zhi-Jian Hu, Jun-Wei Wu, Xin-Qun Chai

Jin-Bo Gao, Li-Shan Bai, Zhi-Jian Hu, Jun-Wei Wu, Xin-Qun Chai, Department of Hepatobiliary Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China
Author contributions: Gao JB and Chai XQ wrote the manuscript; Chai XQ designed the study; Chai XQ, Gao JB and Bai LS performed all of the procedures; and Hu ZJ and Wu JW analyzed the clinical data.

Correspondence to: Xin-Qun Chai, Professor, Department of Hepatobiliary Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China. xinqunc@hotmail.com
Telephone: +86-27-85351623 Fax: +86-27-85351623
Received: April 13, 2011 Revised: June 2, 2011
Accepted: June 9, 2011
Published online: October 7, 2011

Abstract

AIM: To assess the application of the Kasai procedure in the surgical management of hilar bile duct strictures.

METHODS: Ten consecutive patients between 2005 and 2011 with hilar bile duct strictures who underwent the Kasai procedure were retrospectively analyzed. Kasai portoenterostomy with the placement of biliary stents was performed in all patients. Clinical characteristics, postoperative complications, and long-term outcomes were analyzed. All patients were followed up for 2-60 mo postoperatively.

RESULTS: Patients were classified according to the Bismuth classification of biliary strictures. There were two Bismuth III and eight Bismuth IV lesions. Six lesions were benign and four were malignant. Of the benign lesions, three were due to post-cholecystectomy injury, one to trauma, one to inflammation, and one to inflammatory pseudotumor. Of the malignant lesions, four were due to hilar cholangiocarcinoma. All patients underwent Kasai portoenterostomy with the placement of biliary stents. There were no perioperative deaths.

One patient experienced anastomotic leak and was managed conservatively. No other complications occurred perioperatively. During the follow-up period, all patients reported a good quality of life.

CONCLUSION: The Kasai procedure combined with biliary stents may be appropriate for patients with hilar biliary stricture that cannot be managed by standard surgical methods.

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Key words: Kasai procedure; Hilar bile duct; Stricture; Surgery

Peer reviewer: Sophoclis Alexopoulos, MD, Assistant Professor of Surgery, Department of Surgery, Division of Hepatobiliary/Pancreatic and Abdominal Organ Transplant Surgery, University of Southern California, 1510 San Pablo Street, Suite 200, Los Angeles, CA 90033-4612, United States

Gao JB, Bai LS, Hu ZJ, Wu JW, Chai XQ. Role of Kasai procedure in surgery of hilar bile duct strictures. *World J Gastroenterol* 2011; 17(37): 4231-4234 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4231.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4231>

INTRODUCTION

Surgical management of hilar biliary strictures remains a great challenge. The etiology of hilar biliary stricture is diverse, including benign and malignant lesions. The most common benign lesion associated with hilar biliary stricture is secondary to intraoperative injury; most commonly after laparoscopic cholecystectomy. Benign biliary strictures can also occur after hilar bile duct trauma and cholangitis. Malignant hilar biliary stricture can be caused by primary hilar cholangiocarcinoma; a cancer that involves confluence by contiguous spread (e.g., gallbladder and liver cancer), and metastatic cancer

to hilar lymphatic nodes^[1]. Surgery for hilar bile duct stricture is difficult and not without risk. Several repair procedures have been described; none of which are fully satisfactory. Surgical complications are frequent and life-threatening, primarily related to anastomotic leak in the early postoperative period, and biliary strictures in the long term^[2-4]. We therefore recently implemented the Kasai procedure with the use of biliary stents to repair hilar bile duct strictures. We report our experience of using this approach for hilar biliary strictures.

MATERIALS AND METHODS

Patients

We retrospectively analyzed 10 patients (five male, five female) with hilar bile duct strictures who underwent the Kasai procedure in our department from January 2005 to January 2011. The mean age was 52 years (range: 37-64 years). Clinical characteristics are shown in Table 1. Postoperative variables included complications and mortality. Long-term outcomes were retrieved from follow-up visit information.

Operative technique

Radical resections were performed for all malignant hilar lesions. For benign lesions, hilar bile duct dissection was performed, and healthy, non-scarred ducts were exposed for reconstruction. The hepatic quadrate lobule was removed at the level of the hilar plate to expose adequately the bile ducts. A Roux-en-Y portoenterostomy was performed. The afferent limb was approximately 50 cm, which was secured to the hepatic parenchyma, which surrounded the transected hepatic ducts, with 4-0 absorbable braided suture. Fine silicone catheters were used as intrahepatic duct stents to minimize the risk of bile duct restenosis. These were externalized through the stump of the intestinal Roux-en-Y loop and left *in situ* for 5 mo. Intra-abdominal drainage catheters were routinely placed at the anastomosis.

RESULTS

Two lesions were classified as Bismuth III, and eight as Bismuth IV^[5]. Four patients had biliary strictures secondary to bile duct injury: three due to cholecystectomy, and one secondary to abdominal trauma. Four patients had malignant biliary strictures caused by hilar cholangiocarcinoma: one had an inflammatory hilar bile duct stricture secondary to cholangitis; and one had a hilar inflammatory pseudotumor (Table 2).

Of 10 patients, four underwent one or two prior biliary operations. Radiological modalities for evaluation of these patients included ultrasonography ($n = 7$), contrast-enhanced computed tomography ($n = 1$), magnetic resonance cholangiopancreatography (MRCP) ($n = 10$), and endoscopic retrograde cholangiopancreatography ($n = 1$, Figure 1). All patients underwent Kasai portoenterostomy with biliary stenting. Other surgical operations

Table 1 Patient demographics and clinical characteristics

Variables	<i>n</i> (%)
Sex	
Female	5 (50)
Male	5 (50)
Symptoms	
Jaundice	7 (70)
Abdominal pain	4 (40)
Fever	2 (20)
Symptom-free	2 (20)
Physical signs	
Jaundice	7 (70)
Tenderness	2 (20)

Table 2 Etiology and Bismuth classification of bile duct stricture

Etiology	Bismuth classification	
	III	IV
Injurious biliary stricture		
Abdominal trauma		1
Laparoscopic cholecystectomy		2
Open cholecystectomy	1	
Inflammatory biliary stricture		1
Inflammatory pseudotumor		1
Hilar cholangiocarcinoma	1	3
Total	2	8

were performed simultaneously, including hepatic quadrate lobectomy in 10 patients and hepatic left lobectomy in one.

There were no perioperative deaths. One patient experienced a postoperative anastomotic leak and was successfully managed conservatively with drainage and antibiotics. The liver functions of patients were returned to normal postoperatively. Surgical margins at the bile duct cut surfaces were clear in all four patients with hilar cholangiocarcinoma. No other complications such as hemorrhage, abdominal abscess, and wound infection were noted during the perioperative period.

All patients were followed up for a median period of 25.3 mo (range: 2-60 mo). All patients reported a good quality of life. No recurrence or metastasis was found in patients who underwent the Kasai procedure for malignant lesions. Moreover, cholangitis, anastomotic stricture, and cholelithiasis were not observed in any patients.

DISCUSSION

Surgical treatment of hilar bile duct strictures is one of the most challenging areas for hepatobiliary surgeons due to the anatomic complexity and diversity of lesions. Moreover, inappropriately managed biliary strictures can predispose patients to recurrent cholangitis, jaundice, and biliary cirrhosis, which requires additional surgical procedures^[6].

Roux-en-Y hepaticojejunostomy is the most common method for repairing hilar biliary strictures. The

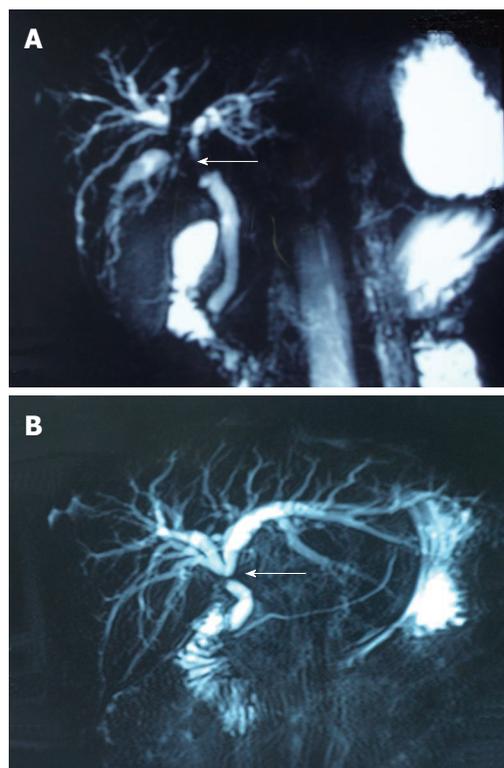


Figure 1 Magnetic resonance cholangiopancreatography images. A: Magnetic resonance cholangiopancreatography (MRCP) showing the hilar bile duct stricture (arrow) caused by cholangiocarcinoma; B: MRCP showing stricture at the hepatic duct confluence (arrow), due to post-laparoscopic cholecystectomy injury.

fundamental principle for repairing a biliary stricture at the hepatic hilum includes identification of healthy bile ducts proximal to the stricture, direct mucosa-to-mucosa anastomosis, a tension-free and wide anastomosis, and a 40-60 cm Roux-en-Y loop^[7]. However, in some circumstances, it is difficult and risky to perform a standard Roux-en-Y hepaticojejunostomy due to the presence of edematous and fragile biliary wall tissues, or the presence of more than one small and thin duct, which cannot be reconstructed to one anastomotic opening. The Kasai procedure has been used extensively for infants with congenital biliary atresia since 1968^[8]. However, there are few reports about the Kasai procedure performed in adults. Schlitt *et al.*^[9] performed the Kasai procedure for three adult patients with high ischemic-type biliary stricture after liver transplantation, but did not achieve satisfactory results. Pickleman *et al.*^[10] have reported five patients with bile duct injuries during laparoscopic cholecystectomy. These patients were managed by the Kasai procedure. All patients were symptom free and functioning normally for a follow-up period of 7-90 mo. In the present study, we applied the Kasai procedure to repair hilar biliary strictures in 10 patients. All patients had an uneventful recovery and have a good quality of life. Therefore, the Kasai procedure may be a good choice for the management of complex hilar biliary strictures that cannot be addressed by standard surgical methods.

Exposure of the proximal bile duct of hilar stricture remains the key to success in the repair of hilar biliary strictures. Some approaches are recommended to expose the hilar bile ducts, such as the hilar and transhepatic approaches^[11,12]. The hilar approach involves lowering the hilar plate to expose the bile duct confluence, to manage the lesion extrahepatically. It is very difficult to expose the second- and third-order branches of the intrahepatic bile duct. The transhepatic approach involves exposing the hilar bile ducts by transecting the liver parenchyma between the left and right lobes of the liver. Although this approach can provide excellent surgical visualization, it requires more elaborate and complex skills, and is high risk. In this study, we performed a concomitant quadrate lobectomy to expose the hilar bile duct. We first dissected the hilar plate and hepatoduodenal ligament to evaluate the lower margin of the lesion, and assessed the portal vein and hepatic artery for tumor invasion. We routinely resected the base of quadrate lobe to visualize the bile duct confluence. Lastly, adequate exposure of the bile ducts could not be obtained, therefore, we resected more of the quadrate lobe between the gallbladder bed and the round ligament, to improve exposure of the hilar ducts, including the second- and third-order branches. Adequate exposure of the hilar bile ducts was obtained in all patients.

In recent years, biliary stenting has become a new technique for the treatment of biliary strictures. The major advantages are that the procedure used to place them is minimally invasive and well tolerated. It was first applied as palliative treatment in patients with unresectable malignant strictures. Previous studies have shown that patients undergoing stent placement for malignant strictures have a significant improvement in abdominal comfort, jaundice, and quality of life. The application of biliary stents as palliative treatment of biliary malignancies is a widely accepted practice^[13,14]. With advances in stent material and the technical process of stent placement, many reports have described their use for treatment of benign biliary strictures^[15-17]. However, complications of stent placement, such as stent occlusion and cholangitis limit their use in benign strictures. Both the requirement for and duration of stenting for benign strictures have been controversial for many years. Siriwardana and Siriwardana have reported a systematic appraisal of the current status of the use of metallic endobiliary stents in the treatment of benign biliary strictures^[18]. They have demonstrated that, although stents can be deployed endoscopically or radiologically with relative ease and with a low procedure-related complication rate, there is a critical lack of data on long-term patency. Thus, currently, metallic endobiliary stents should not be used for benign strictures in patients with a predicted life expectancy of > 2 years. In our study, six patients with benign hilar strictures underwent the Kasai procedure. All patients had a good outcome during follow-up. Therefore, the Kasai procedure may be a good alternative for patients with benign hilar strictures.

The Kasai procedure is a portoenterostomy performed by suturing a jejunal loop to the hepatic parenchyma that surrounds the transected hepatic ducts. Direct mucosa-to-mucosa anastomosis is not required. Anastomotic leak and stricture are the most common postoperative complications. To prevent or lessen the probability of postoperative stricture and bile leak, we routinely placed transanastomotic catheters in the bile ducts, which were externalized through the intestinal Roux-en-Y loop. In our study, all 10 patients underwent the Kasai procedure with transanastomotic stents, and no anastomotic stricture was observed during follow-up. Transanastomotic catheters not only limit the tendency to stricture, but also serve to decompress the biliary system and provide access for radiographic imaging in the perioperative period^[19]. Innes *et al.*^[20] have suggested that a bilioenteric anastomosis to manage benign stenosis of the biliary tract might be undertaken without placing stents, which promises low postoperative morbidity and excellent obstruction-free long-term results. Although the use of postoperative transanastomotic stenting tubes is controversial, we recommend their use when the Kasai procedure is being performed.

In conclusion, the management of hilar biliary strictures is challenging. Surgical repair has been the preferred approach. A Roux-en-Y hepaticojejunostomy is a standard procedure to repair hilar stricture for most patients. The Kasai procedure may be a good choice for a small subset of patients who suffer from complex hilar biliary strictures that cannot be managed by standard surgical methods.

COMMENTS

Background

Surgical management of hilar bile duct stricture is a great challenge because of the complexity of the perihilar anatomy and the diversity of the lesions.

Research frontiers

The appropriate management of hilar bile duct strictures depends on the cause, type, and level of stricture. Roux-en-Y hepaticojejunostomy is a conventional procedure used to repair hilar bile duct stricture. However, in some circumstances, it is very difficult and high risk to perform a standard Roux-en-Y hepaticojejunostomy.

Innovations and breakthroughs

The authors investigated the role of the Kasai procedure in the surgical management of hilar bile duct strictures and demonstrated that the Kasai procedure is a successful method to treat complex hilar biliary strictures.

Applications

This study may help surgeons to choose the Kasai procedure as an appropriate procedure to deal with complicated hilar bile duct strictures.

Terminology

The Kasai procedure is a hepaticportoenterostomy, which is performed by suturing a jejunal loop to the hepatic parenchyma that surrounds the transected hepatic ducts.

Peer review

This is a small case series that describes the use of hepatic portoenterostomy in adults for the reconstitution of biliary-enteric continuity when mucosal to mucosal hepaticojejunostomy is not feasible. Although this is not a new concept, it is an important technique that is occasionally used by hepatobiliary surgeons. Because the literature describing this technique is scarce, this article should be published.

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S- Editor Tian L L- Editor Kerr C E- Editor Zhang DN

Three initial diets for management of mild acute pancreatitis: A meta-analysis

Wen-Bo Meng, Xun Li, Yu-Min Li, Wen-Ce Zhou, Xiao-Liang Zhu

Wen-Bo Meng, Xun Li, Yu-Min Li, Wen-Ce Zhou, Xiao-Liang Zhu, Key Laboratory of Digestive System Tumors, Gansu Province, Lanzhou 730000, China

Wen-Bo Meng, Xun Li, Wen-Ce Zhou, Xiao-Liang Zhu, The First Hospital of Lanzhou University, Lanzhou 730000, Gansu Province, China

Yu-Min Li, The Second Hospital of Lanzhou University, Lanzhou 730000, Gansu Province, China

Author contributions: Meng WB, Li YM and Li X contributed to the selection of studies and data extraction; All authors contributed to the study design, data analysis and interpretation of results, and reviewed the manuscript for important intellectual content and approved the final version.

Correspondence to: Yu-Min Li, Professor, The Second Hospital of Lanzhou University, Lanzhou 730000, Gansu Province, China. liym@lzu.edu.cn

Telephone: +86-931-8942744 Fax: +86-931-8942744

Received: May 9, 2011 Revised: August 1, 2011

Accepted: August 8, 2011

Published online: October 7, 2011

Abstract

AIM: To compare non-liquid and clear-liquid diets, and to assess whether the latter is the optimal treatment for mild acute pancreatitis.

METHODS: The Cochrane Library, PUBMED, EMBASE, EBM review databases, Science Citation Index Expanded, and several Chinese databases were searched up to March 2011. Randomized controlled trials (RCTs) that compared non-liquid with clear-liquid diets in patients with mild acute pancreatitis were included. A meta-analysis was performed using available evidence from RCTs.

RESULTS: Three RCTs of adequate quality involving a total of 362 participants were included in the final analysis. Compared to liquid diet, non-liquid diet significantly decreased the length of hospitalization [mean difference (MD): 1.18, 95% CI: 0.82-1.55; $P < 0.00001$] and total length of hospitalization (MD: 1.31, 95% CI: 0.45-2.17; $P = 0.003$). The subgroup analysis showed

solid diet was more favorable than clear liquid diet in the length of hospitalization, with a pooled MD being -1.05 (95% CI: -1.43 to -0.66; $P < 0.00001$). However, compared with clear liquid diet, both soft and solid diets did not show any significant differences for recurrence of pain after re-feeding, either alone [relative risk (RR): 0.95; 95% CI: 0.51-1.87; $P = 0.88$] and (RR: 1.22; 95% CI: 0.69-2.16; $P = 0.49$), respectively, or analyzed together as non-liquid diet (RR: 0.80; 95% CI: 0.47-1.36; $P = 0.41$).

CONCLUSION: The non-liquid soft or solid diet did not increase pain recurrence after re-feeding, compared with the clear-liquid diet. The non-liquid diet reduced hospitalization.

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Key words: Acute pancreatitis; Diet; Nutritious supplement; Meta-analysis; Length of stay

Peer reviewer: Shiu-Ming Kuo, MD, University at Buffalo, 15 Farber Hall, 3435 Main Street, Buffalo, New York, NY 14214, United States

Meng WB, Li X, Li YM, Zhou WC, Zhu XL. Three initial diets for management of mild acute pancreatitis: A meta-analysis. *World J Gastroenterol* 2011; 17(37): 4235-4241 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4235.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4235>

INTRODUCTION

Early enteral nutrition therapy is important for management of severe as well as mild acute pancreatitis^[1]. The initial part of the necessity is to prevent bacterial infection, as well as energy supplementation^[2]. This kind of nutrition is always preferable *via* the nasojejunal route with a nasal bowel nutrition tube^[3]. However, it is not necessary for mild acute pancreatitis patients because of

longer length of hospitalization and discomfort^[4,5].

In daily clinical care, 80%-90% of patients with acute pancreatitis demonstrate a mild clinical course of the disease^[6]. The traditional initial treatment in mild acute pancreatitis has included: (1) fasting for the first few days; and (2) administration of parenteral fluids followed by clear-liquid-diet intake, orally until the abdominal pain has resolved and the levels of pancreatic enzymes have decreased^[7-9]. It sounds reasonable that clear-liquid diet intake will shorten the presence of food in the duodenum, which reduces cholecystokinin release that stimulates pancreatic enzyme secretion^[10]. Hospital discharge is usually planned on the basis of the patient's tolerance to solid diet^[11].

Oral re-feeding has been recommended to start with small amounts of clear-liquid diet, rich in carbohydrates and proteins and low in fat, gradually increasing or shifting the intake to soft or solid diet during 3-7 d, to avoid abdominal pain and pancreatitis relapse^[12,13]. Unfortunately, to date, evidence is sparse concerning when one kind of diet should be shifted to another, and what kind of diet is definitely optimal re-feeding^[14].

Some recent studies have suggested that oral re-feeding with soft or solid diet instead of clear liquids can be considered safe for pain recurrence, and shorten the length of hospitalization. Some randomized trials in patients with mild acute pancreatitis have shown that non-liquid diets are feasible and safe^[15-17]. However, the results of these studies were inconclusive. The aim of the present study was to perform a meta-analysis of current randomized controlled trials (RCTs) to evaluate non-liquid diet (including soft and solid diets) as an initial treatment in mild acute pancreatitis.

MATERIALS AND METHODS

Study selection criteria

The titles and abstracts of all citations identified by the literature search were reviewed. Selection criteria were then applied to all potentially relevant studies. Editorials and expert opinions, reviews without original data, case reports and studies lacking control groups were excluded. The selection criteria for inclusion in the meta-analysis were as follows: (1) only RCTs that compared non-liquid diet, including soft and solid diet, with clear-liquid diet were included; (2) diagnosis of mild acute pancreatitis was confirmed according to computed tomography scores, APACHE II scores, and basic laboratory examination; (3) outcomes of length of hospitalization (LOH), total length of hospitalization (TLOH), and recurrence of pain after re-feeding were reported; and (4) no other nutritious supplement treatment was given to patients.

Search strategy for identification of studies

Trials were identified by searching the Cochrane Library (Issue 1 2011), PubMed (March 2011), EMBASE (March 2011), Science Citation Index Expanded, and CBM (Chinese Biomedical Literature Database). The query was constructed by using the combination of the following keywords: (mild pancreatitis or acute pancreatitis)

and (diet or nutritious supplement or nutrition). Articles published in any language were considered. Reference lists from the trials selected by electronic searching were hand-searched to identify further relevant trials. Abstracts of the articles selected from each of these multiple searches were reviewed and those meeting the criteria were recorded. In the case of duplicate reports, or studies obviously reporting results from the same study population, only the latest published results were used.

Assessment of study quality

The quality of included studies was assessed independently by two authors (Meng WB and Li YM) without blinding to authorship or journal. Discrepancies were resolved by involving the third author, Xun Li. The quality of the studies was assessed using the scores proposed by Cochrane handbook 5 standards: randomization, allocation, concealment, blinding (participants, investigators, outcomes assessors, and data analysis), and completeness of follow-up.

Data extraction

Two investigators (Meng WB and Li YM) extracted the data from the studies that met the selection criteria (Tables 1-3). The outcomes were totalled from the three studies. There was > 98% agreement for data extraction between the two investigators.

Statistical analysis

We analyzed the data using Review Manager (version 5.0)^[18] and pooled data for summary estimates. We expressed results for dichotomous outcomes as relative risk (RR), and mean difference (MD) with 95% CIs for continuous outcomes. We used the χ^2 test to assess heterogeneity between trials and the I^2 statistic to assess the extent of inconsistency. Statistical significance cut-off for the test of heterogeneity was set at 0.10. We used a fixed-effect model for calculations of summary estimates unless there was significant heterogeneity, in which case, results were confirmed using a random-effects statistical model.

RESULTS

Search results

The flowchart of reviews shows the detailed process of study selection (Figure 1). The comparison was made between non-liquid and clear-liquid diets^[15-17]. Three trials fulfilled the inclusion criteria.

Quality and characteristics of included studies

Data regarding characteristics of the studies, including patients, baseline characteristics and quality assessment of the studies are summarized in Tables 1-3, respectively.

Group and subgroup arrangement

Groups for non-liquid diet *vs* clear-liquid diet were established first. We deemed both solid and soft diets as non-liquid diets to perform the analysis. In the study of Moraes *et al*^[15] in Table 1, there were three arms with solid diet, soft diet, and clear-liquid diet, which were

Table 1 Baseline characteristics of the included studies

Study comparisons	Morales <i>et al.</i> ^[15]		Jacobson <i>et al.</i> ^[16]		Sathlaraj <i>et al.</i> ^[17]		
	Solid	Soft	Liquid	Soft	Liquid	Solid	Liquid
Type of diet	Solid	Soft	Liquid	Soft	Liquid	Solid	Liquid
No. of patients included	70	70	70	66	55	49	52
Mean age (yr)	53	49	51	51	47	37	39
Male/female	42/28	43/27	33/37	23/43	34/21	39/10	44/8
Mean body mass index (%)	ND	ND	ND	29	29	21.3	20.9
Cause							
Biliary system (<i>n</i>)	33	35	32	15	15	7	9
Alcohol (<i>n</i>)	17	14	16	14	19	26	25
Unknown and others (<i>n</i>)	20	21	22	26	32	16	18
Type of pain							
Acute (<i>n</i>)	ND	ND	ND	52	53	40	41
Acute or chronic (<i>n</i>)	ND	ND	ND	3	3	9	11
Time between admission and first meal (d)	3.4 ± 0.8	3.6 ± 1.0	3.5 ± 1.5	2	1	1.6	1.4
Total number of meals on study day 1 (<i>n</i>)	2	2	2	2	2	3	4
Calories in first meal on day 1 (kcal)	620	120	124	350	157	262	137
Fat in first meal on day 1 (g)	14	2	1	5	1	3	4
Total calories on first day (kcal)	1240	241	248	622	301	921	370
Total fat on first day (g)	28	4	2	13	2	15	8

ND: Not described.

Table 2 Quality assessment of randomized controlled trials included in the meta-analysis

Study	Methodological quality items			
	Randomization	Allocation concealment	Double blinding	ITT analysis
Morales <i>et al.</i> ^[15]	Yes	Yes	Yes	Yes
Jacobson <i>et al.</i> ^[16]	Yes	Yes	Yes	Yes
Sathlaraj <i>et al.</i> ^[17]	Yes	Yes	Yes	Yes

Based on Cochrane handbook 5. ITT: Intention-to-treat.

compared with each other simultaneously. We extracted the solid arm and placed it into the non-liquid diet group, and excluded the soft diet arm for good balance of the statistics. According to the type of control group compared, all the included studies were divided into two subgroups: subgroup A, soft diet *vs* liquid diet; and subgroup B, solid diet *vs* liquid diet.

Meta-analysis

Recurrence of pain: As shown in Figures 2-4, meta-analysis did not show any statistically significant difference between 174 patients in the non-liquid diet group and 188 in the clear-liquid diet group with regard to pain recurrence (RR: 0.80, 95% CI: 0.47-1.36; $P = 0.41$). There was no significant heterogeneity between them ($P = 0.74$, $I^2 = 0\%$). In the subcategory analysis, there was no difference between soft diet and clear-liquid diet (RR: 0.95, 95% CI: 0.51-1.87; $P = 0.88$), nor was heterogeneity ($P = 0.54$, $I^2 = 0\%$). Similarly, in subgroup B, RR was 1.22 (95% CI: 0.69-2.16, $P = 0.49$), and no significant heterogeneity was observed ($P = 0.46$, $I^2 = 0\%$).

LOH: Three trials comprising a total of 174 patients in the non-liquid diet group and 188 in the clear-liquid diet group reported LOH. There was a significant difference

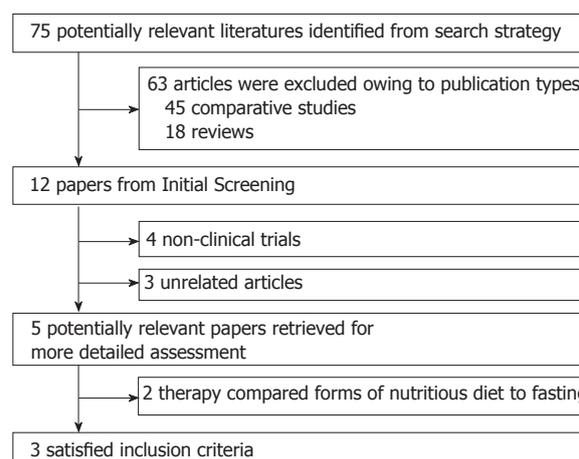


Figure 1 Flowchart of study selection.

between the non-liquid and clear-liquid diet groups (Figure 2), with a pooled MD of 1.18 (95% CI: 0.82-1.55; $P < 0.00001$). There was significant heterogeneity between them ($P = 0.0001$, $I^2 = 89\%$). In subgroup A, there was heterogeneity between two trials ($P < 0.0001$, $I^2 = 94\%$), although a pooled MD was -0.30 (95% CI: -0.78 to 0.17, $P = 0.21$). In subgroup B, MD was -1.05 (95% CI: -1.43 to -0.66; $P < 0.00001$); However, significant heterogeneity was observed ($P = 0.0003$, $I^2 = 92\%$).

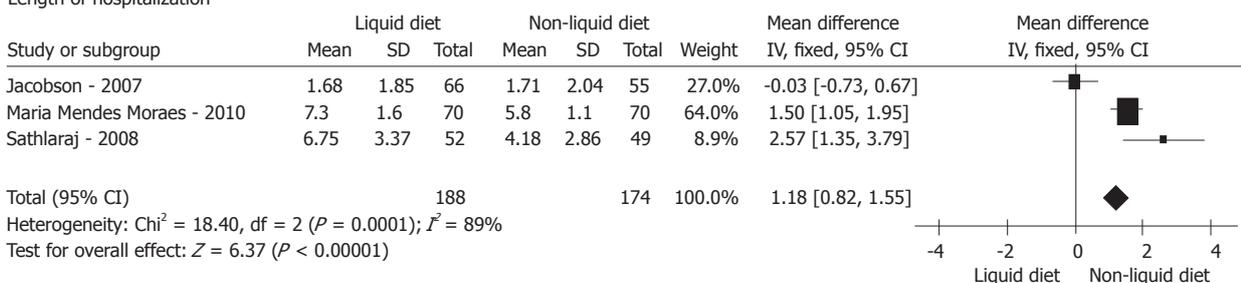
TLOH: TLOH was reported by three trials comprising a total of 119 patients on non-liquid diet and 122 on clear-liquid diet. There was a significant difference between the two groups (MD: 1.31, 95% CI: 0.45-2.17; $P = 0.003$) (Figure 2). The subgroup analyses showed no significant difference for subgroup A (MD: -0.59, 95% CI: -1.33 to 0.14; $P = 0.11$) and subgroup B (MD: -0.70, 95% CI: -1.71 to 0.31; $P = 0.17$). No significant heterogeneity was found between the non-liquid and clear-liquid diet groups

Table 3 Results on length of hospitalization, total length of hospitalization and recurrence of pain

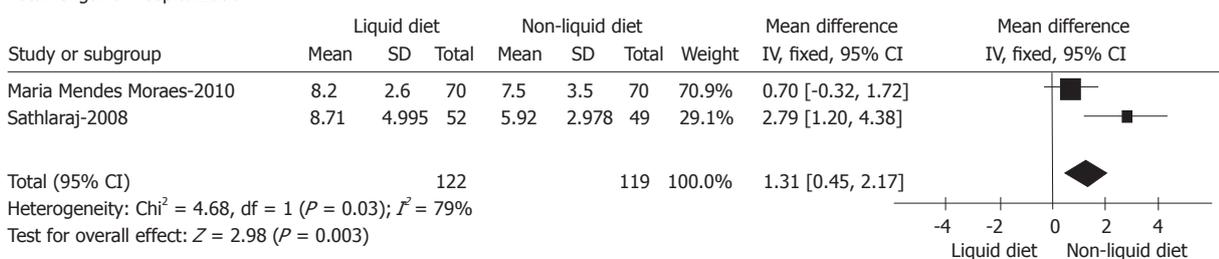
Study comparisons	Moraes <i>et al</i> ^[15]			Jacobson <i>et al</i> ^[16]		Sathlaraj <i>et al</i> ^[17]	
Type of diet	Solid	Soft	Liquid	Solid	Liquid	Soft	Liquid
LOH (d)	5.8 ± 1.1	7.4 ± 1.5	7.3 ± 1.6	1.71 ± 2.04	1.68 ± 1.85	4.18 ± 2.86	6.75 ± 3.37
TLOH (d)	7.5 ± 3.5	8.2 ± 2.4	8.2 ± 2.6	4 (3-6)	4 (3-5)	5.92 ± 2.978	8.71 ± 4.995
Recurrence of pain (n)	15	12	14	6	4	4	3

LOH: Length of hospitalization; TLOH: Total length of hospitalization.

Length of hospitalization



Total length of hospitalization



Recurrence of pain

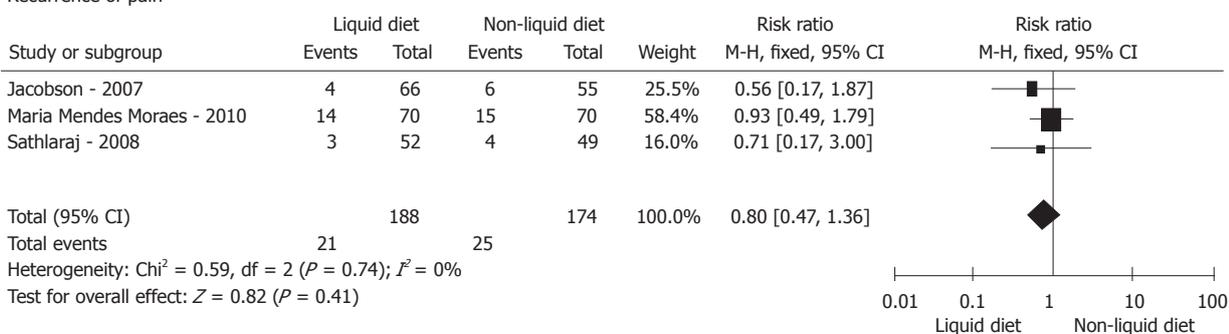


Figure 2 Outcomes in non-liquid diet vs liquid diet with length of hospitalization, total length of hospitalization and recurrence of pain. IV: Inverse-Variance; M-H: Mantel-Haenszel.

($P = 0.03$, $I^2 = 79\%$). However, significant heterogeneity was seen in subgroup A ($P = 0.002$, $I^2 = 89\%$).

DISCUSSION

The current meta-analysis demonstrated that, compared with clear-liquid diet, non-liquid diet did not increase the recurrence of pain after re-feeding in mild acute pancreatitis, and this finding was supported by the subgroup analyses. These outcomes totally challenged our belief that solid diet, even soft diet, would definitely induce the recurrence of abdominal pain and increase pancreatic enzyme secretion^[19-21]. Physicians had previously hypothesized that oral re-feeding could promote inflammatory

processes in the pancreas and increase production of enteral hormones (such as cholecystokinin, motilin and serotonin), which have a negative trophic effect on the pancreatic tissue, thus decreasing pancreatic blood flow and gastrointestinal motility^[22-25]. However, the meta-analysis did not show any significant difference between non-liquid and clear-liquid diets (RR: 1.22, 95% CI: 0.69-2.16; $P = 0.49$), as well as the two subgroups. There have only been a few studies on diet in acute pancreatitis thus far, therefore, it is possible to speculate that most of the patients could tolerate non-liquid diet successfully. Another key point was that our analysis only selected data from mild acute pancreatitis, with potentially severe types being excluded. The inclusion criteria of the three

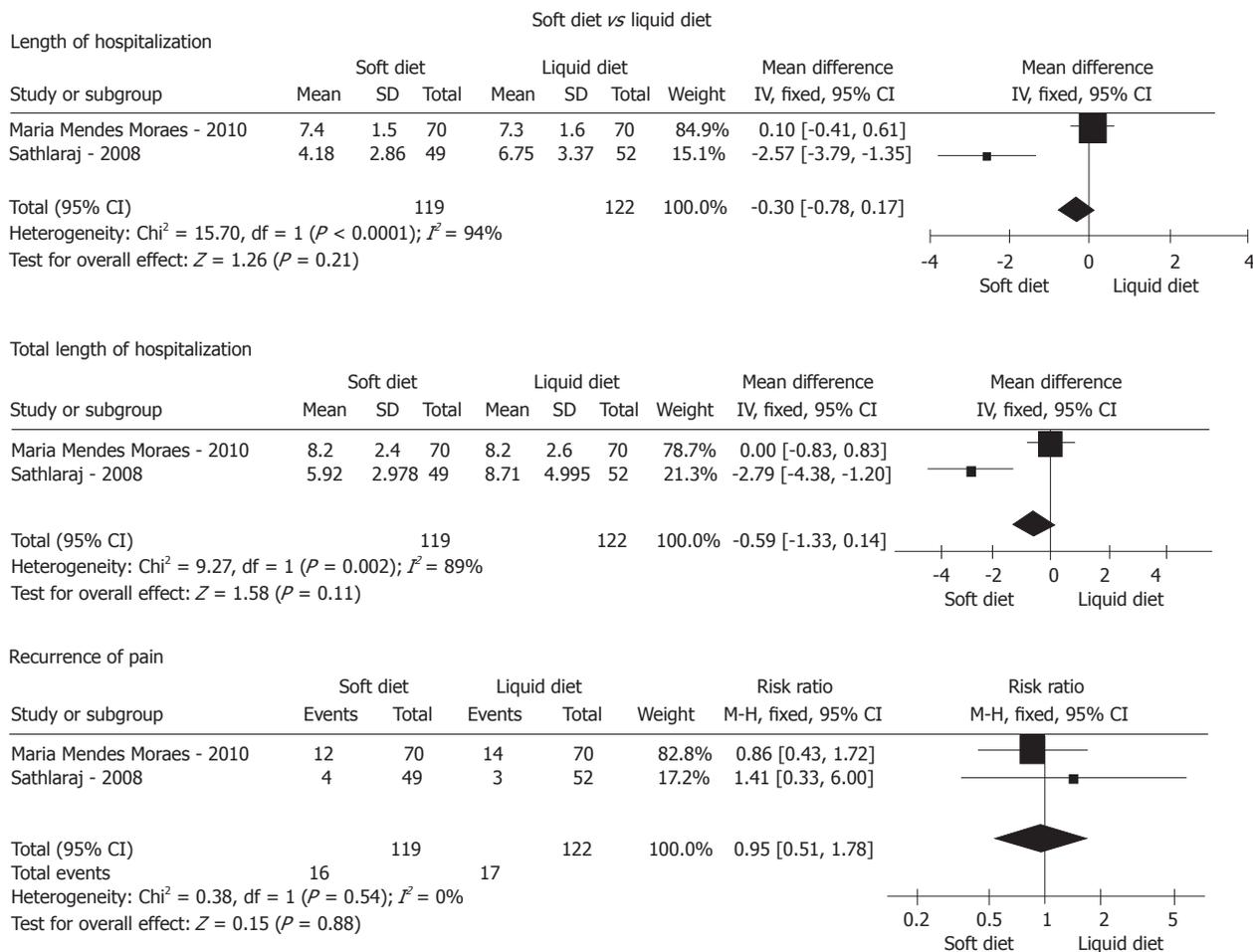


Figure 3 Outcomes in subgroup A: soft diet vs liquid diet with length of hospitalization, total length of hospitalization and recurrence of pain. IV: Inverse-Variance; M-H: Mantel-Haenszel.

RCTs were similar. There was no significant heterogeneity between any of the groups.

Non-liquid diet, especially solid diet, showed superiority over clear-liquid diet on the LOH in the meta-analysis. Consequently, as compared to soft diet, solid diet supplement showed significant beneficial effects on both LOH and TLOH in patients with mild acute pancreatitis. Significant heterogeneity was found in the non-liquid diet vs the clear-liquid diet groups ($P = 0.0001$, $I^2 = 89\%$) and in the comparison of solid diet vs clear-liquid diet ($P = 0.0003$, $I^2 = 92\%$). This heterogeneity could have originated from discrepancies in the criteria for hospital discharge and the limited number of RCTs. Although practice management guidelines have presented detailed information concerning the appropriate timing and form of nutrition in severe acute pancreatitis, little attention has been paid to optimizing the dietary management of mild pancreatitis^[4,14,26,27]. Earlier studies were not able to explain the benefits of soft or solid diet with fat re-feeding, or the patients' tolerance. There is a viewpoint that the pancreas may be less responsive to stimulation by nutrients in normal digestive tract than when patients are suffering from pancreatitis^[20,27].

With the respect to TLOH, a significant difference was only seen in the non-liquid diet vs the clear-liquid

diet group. In the subgroup study, although outcomes favored the soft and solid diet, there was no significant difference. Significant heterogeneity was found in the non-liquid diet vs clear-liquid diet groups ($P = 0.03$, $I^2 = 79\%$) and soft diet vs clear-liquid diet groups ($P = 0.002$, $I^2 = 89\%$). Due to the lack of exact data on TLOH, the heterogeneity analysis could not be performed. The heterogeneity would also have derived from discrepancies in the criteria of discharge and the time zone difference from being hospitalized to re-feeding.

One of the disadvantages of this meta-analysis was that only three RCTs were included. All three studies had high methodological quality and generalizability, nonetheless, there may still have been bias in the final results. There was one study from Brazil^[15] for which we could not obtain accurate data for TLOH. Additionally, another study^[16] that showed shorter LOH may have been related to the fast discharge protocol, which could have led to heterogeneity. Therefore, more multicenter cooperative studies with prospective design are needed.

To the best of our knowledge, many diseases can cause mild acute pancreatitis. The tolerant form of the different diets should be projected separately by disease. That is probably why there are always some patients who cannot tolerate re-feeding, hence prolonging LOH

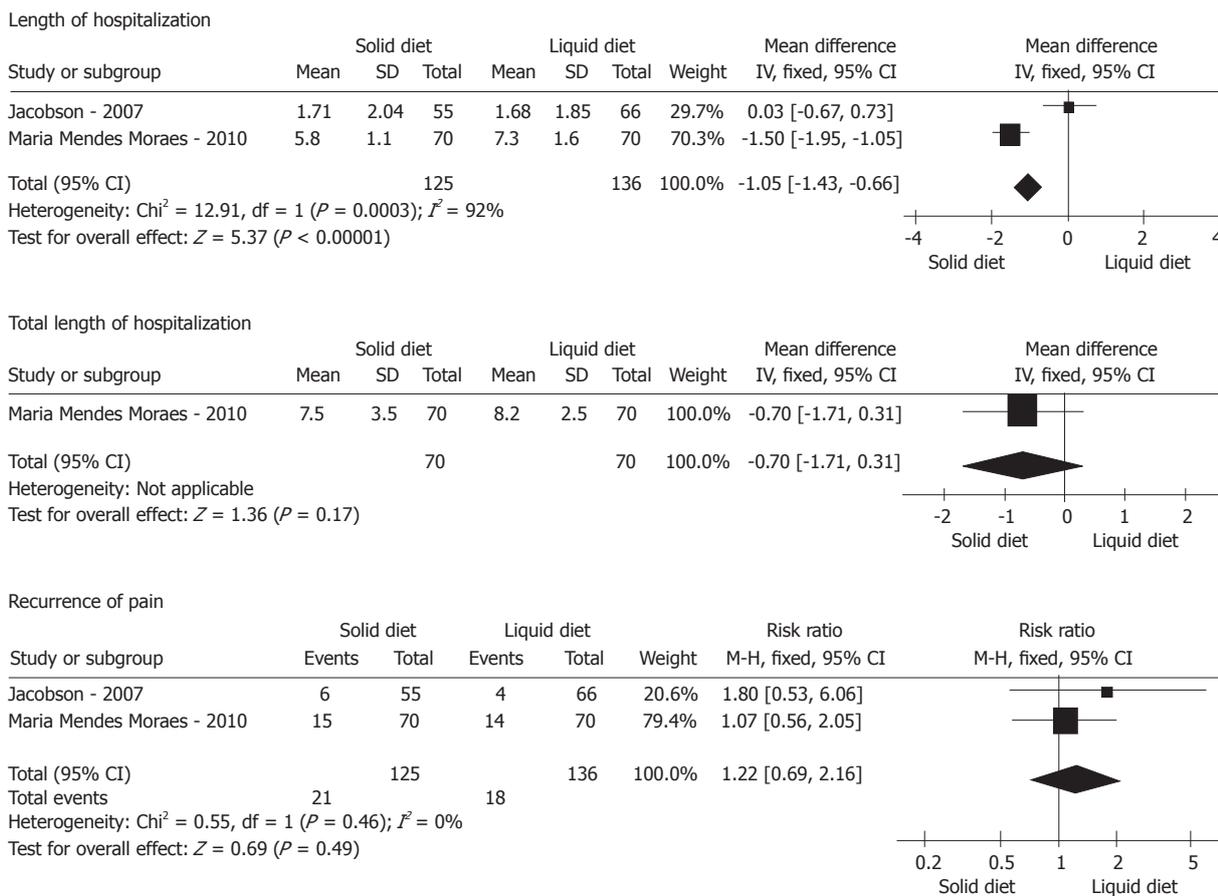


Figure 4 Outcomes in subgroup B: solid diet vs liquid diet with length of hospitalization, total length of hospitalization and recurrence of pain. IV: Inverse-Variance; M-H: Mantel-Haenszel.

in patients with mild pancreatitis^[28]. For example, if patients suffer from bile duct obstruction or infection, high pressure in the bile duct can cause deterioration of pancreatitis by increasing inflammation if the pressure is not released immediately^[29-31]. In that situation, even a small load with any kind of diet could lead to serious consequences^[32-34]. Therefore, treatment directed against the causes of pancreatitis is still an essential step. It is advisable to try and cure pancreatitis as soon as possible after the cause has been established, therefore, we should focus therapeutic options on the pathogenesis, in addition to the necessary supporting treatments; not only to ameliorate abdominal pain, but also to recover the whole function of the gastrointestinal tract. This will in turn improve the tolerance of the patients to earlier application of enteral nutritional therapy, thus reducing LOH. According to our meta-analysis, we obtained novel results that will encourage us to promote further new protocols with regard to dietary management of pancreatitis secondary to different protopathies. Also, more multicenter cooperative studies with prospective design are needed for ultimate conclusions about this issue.

In conclusion, the encouraging outcomes in this analysis may demonstrate a different notion from our previous experience in nutritional supplementation of the patients who are diagnosed with mild acute pancreatitis. None of the soft or solid non-liquid diets showed greater recur-

rence of pain after re-feeding, compared to the clear-liquid diet. Non-liquid diet nutritional supplementation, especially with solid diet, could reduce LOH and TLOH. At this point, we cannot explain our findings with previous pathophysiological experiments performed on acute pancreatitis. One possibility is that the upper digestive tract is less responsive to stimulation by nutrients than we assumed. However, new dietary experiments with animal models of acute pancreatitis and more RCTs comparing roles of different diet forms in the recovery of mild acute pancreatitis are expected to resolve these issues.

COMMENTS

Background

Early enteral nutrition therapy is important for management of mild acute pancreatitis. Initial treatment includes fasting in the first few days and administration of parenteral fluids, followed by gradual clear-liquid diet intake until abdominal pain has resolved and levels of pancreatic enzymes have decreased. Hospital discharge is usually planned on the basis of the patients' tolerance to solid diet.

Research frontiers

Some recent studies have suggested that oral re-feeding with soft or solid diet instead of clear liquids can be considered safe for recurrence of pain, but it inconsistently shortens length of hospitalization (LOH). Some randomized trials in mild acute pancreatitis have shown that non-liquid diets are both feasible and safe.

Innovations and breakthroughs

To review systematically the outcomes of non-liquid diet including soft and solid diet compared with clear-liquid diet in mild acute pancreatitis. The meta-analysis demonstrated that none of the non-liquid soft and solid diets increased

recurrence of pain after re-feeding, compared with clear-liquid diet. Surprisingly, non-liquid diet nutritious supplement re-feeding reduced LOH and total length of hospitalization (TLOH). Furthermore, solid diet decreased LOH.

Applications

With the encouraging outcomes, non-liquid diet nutritional supplementation, especially solid diet, could reduce LOH and TLOH. It might potentially improve the management of mild acute pancreatitis. However more randomized controlled studies on dietary experiments comparing roles of different diet forms in the recovery of mild acute pancreatitis are expected.

Terminology

LOH means the minimum number of days that patients stay in hospital. TLOH is the total length of hospitalization.

Peer review

Nutrition management of mild acute pancreatitis was the focus of the study. In the literature, comparison between total parenteral and enteral nutrition have been performed but not among different types of oral feeding. This study was unique in describing a carefully conducted meta-analysis comparing liquid, soft and solid diets. The conclusions are intriguing but sound. It potentially can improve the management of mild acute pancreatitis as well as further understanding of gastrointestinal physiology.

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S- Editor Tian L L- Editor Kerr C E- Editor Li JY

Integration of human papillomavirus 18 DNA in esophageal carcinoma 109 cells

Ke Zhang, Jin-Tao Li, Shu-Ying Li, Li-Hua Zhu, Ling Zhou, Yi Zeng

Ke Zhang, Shu-Ying Li, Li-Hua Zhu, College of Basic Medicine, Hebei United University, Tangshan 063000, Hebei Province, China

Jin-Tao Li, College of Life Science and Bio-engineering, Beijing University of Technology, Beijing 100124, China

Jin-Tao Li, Ling Zhou, Yi Zeng, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, and State Key Laboratory for Infectious Disease Prevention and Control, Beijing 100052, China

Author contributions: Zhang K and Li JT performed the majority of the experiments, and contributed equally to this work; Zhou L and Zeng Y were involved in editing the manuscript and provided financial support for this work; Li SY and Zhu LH designed the study and wrote the manuscript.

Supported by An independent research fund from the National Institute for Viral Disease Control and Prevention, the Chinese Center for Disease Control and Prevention; the State Key Laboratory for Infectious Disease Prevention and Control (Grant No. 2011SKLID103)

Correspondence to: Shu-Ying Li, PhD, Professor, Department of Pathogenic Biology, College of Basic Medicine, Hebei United University, Tangshan 063000, Hebei Province, China. lsy5001@sina.com

Telephone: +86-315-3725918 Fax: +86-315-3725171

Received: January 15, 2011 Revised: June 21, 2011

Accepted: June 28, 2011

Published online: October 7, 2011

Abstract

AIM: To detect human papillomavirus (HPV) DNA in esophageal carcinoma (EC) 109 cells and investigate the relationship between HPV and EC.

METHODS: Genomic DNA and total RNA from EC109 cells were isolated. HPV DNA was detected by polymerase chain reaction (PCR) with the general primer sets of My09/11 and GP5 +/6 + for the *HPV L1* gene and type-specific primer sets for HPV18 E6 and HPV18 E6-E7. Reverse transcription (RT) of mRNA isolated from EC109 cells was performed to produce a cDNA.

And then a PCR-based protocol for the amplification of papillomavirus oncogene transcripts was used to analyze HPV18 DNA and integrated transcripts of HPV18 in the chromosomes of EC109 cells. The final nested PCR products were cloned into a pMD-18T vector and sequenced to analyze the chromosomal location of HPV integration.

RESULTS: HPV18 DNA was detected in EC109 cells by PCR using the general primer sets of My09/11 and GP5 +/6 + for HPV L1 and the type-specific primer sets for HPV18 E6 and E6-E7 to generate products of 450 bp, 150 bp, 335 bp and 944 bp, respectively. Approximately 600 bp of integrated HPV18-specific transcript was identified. The final nested PCR product of integrated HPV18 DNA was cloned into a pMD-18T vector and sequenced to analyze the chromosomal location of HPV integration. Sequence alignment showed that the HPV18 sequence from EC109 cells was identical to that of the encoded early protein E7-E1 of the standard HPV18 strain X05015, and another partial gene sequence was identical to a partial sequence of human chromosome 8.

CONCLUSION: Integration of the HPV genome into the host cell chromosome suggests that persistent HPV infection is vital for malignant cell transformation and carcinogenesis.

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Key words: Esophageal carcinoma; Human papillomavirus; Integration; Infection; Genome

Peer reviewers: Piero Marco Fisichella, MD, Assistant Professor of Surgery, Medical Director, Swallowing Center, Loyola University Medical Center, Department of Surgery, Stritch School of Medicine, 2160 South First Avenue, Room 3226, Maywood, IL 60153, United States; Kenichi Goda, MD, PhD, Department of Endoscopy, The Jikei University School of Medicine, 3-25-8 Nishi-shimbashi, Minato-ku, Tokyo

105-8461, Japan

Zhang K, Li JT, Li SY, Zhu LH, Zhou L, Zeng Y. Integration of human papillomavirus 18 DNA in esophageal carcinoma 109 cells. *World J Gastroenterol* 2011; 17(37): 4242-4246 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4242.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4242>

INTRODUCTION

The esophageal carcinoma (EC) cell line EC109 was established in 1976 by the Cell Biology Research Group at the Chinese Academy of Medical Sciences Institute of Cancer Research. The cell line was derived from esophageal cells that were surgically removed from a patient with a pathological diagnosis of EC^[1]. The cells were determined to be positive for human papillomavirus (HPV) type 18^[2].

EC is one of the major cancers in China. The etiology of EC has yet to be established despite extensive investigation of the contribution of environmental factors, lifestyle, and low levels of chemical elements. In 1982, Syrjanen formulated a hypothesis on the relationship between HPV infection and the development of EC; the hypothesis was based on the presence of papilloma-like tissues in EC specimens and other molecular evidence^[3]. Thereafter, numerous clinical studies have supported this hypothesis^[4-13]. However, the link between HPV infection and the etiology of EC remains inconclusive.

A link between HPV infection and squamous cell cancer of the cervix has been identified^[14]. Currently, several oncogenic types of HPV are regarded as the etiological agents responsible for the development of cervical squamous cell carcinoma^[15,16].

We further studied the association between HPV infection and carcinogenesis using HPV18 E6-E7-transfected stable cell lines derived from fetal esophageal epithelial tissue. Our results strongly supported the conclusion that the expression of HPV18 proteins E6 and E7 induced the transformation of the esophageal cells^[17]. HPV18 was also detected in EC109 cells^[2]. These results support a link between HPV infection and esophageal carcinogenesis.

Persistent infection with high-risk HPV and the integration of viral genomes into the host genome have been implicated in the etiology of malignant and pre-malignant disease of the female lower genital tract^[18,19]. HPV is divided into low-risk (LR) and high-risk (HR) types according to the presumed degree of risk for the development of cancer. HR HPV types such as HPV16, HPV18, and HPV31 are associated with cancer, while LR HPV types such as HPV6, HPV11, and HPV40 are the causative agents of benign warts^[20]. Episomal and integrated HPV can be distinguished using a polymerase chain reaction (PCR)-based protocol for the amplification of papillomavirus oncogene transcripts (APOT), developed by Klaes and his colleagues^[21]. The same

group hypothesized that HPV transcripts derived from the integrated HPV genome represent suitable molecular markers for a pre-neoplastic lesion at risk for progression to carcinoma. For EC, however, few data are available concerning HPV status and integration patterns. The aim of the present study was to assess high-risk HPV infection and HPV18 DNA integration into the host cell genome in EC.

MATERIALS AND METHODS

Cell lines

EC109 cells, the human embryonic kidney (HEK) 293 cell line, and the HeLa cell line were maintained by our laboratory. HeLa cells served as the HPV18-positive control, and HEK293 cells served as the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-positive control. The cell lines were cultivated in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin, the concentration of each antibiotic was 100 U/mL) under standard conditions. Cells were maintained as a subconfluent monolayer at 37 °C in a humidified atmosphere of 5% CO₂/95% air. Exponentially proliferating cells were harvested with 0.25% trypsin and 0.02% EDTA, resuspended in fresh medium, and seeded in new flasks. The cells (2 × 10⁶) were collected and washed with phosphate-buffered saline. Then, the cells were separated into two cryotubes, immediately frozen in liquid nitrogen, or stored at -70 °C until further use.

Detection of HPV DNA

Genomic DNA was isolated from each cell line using the Easy-DNA kit ("BioTake", China) according to the supplier's instructions. HPV DNA was detected by PCR with the general primer sets of My09/11 (My09: 5'-CGTCCMARRGGAWACTGATC-3', MY11: 5'-GC-MCAGGGWCATAAAYAATGG-3', amplicon size 450 bp) and GP5 +/6 + (GP5 +: 5'-TTTGTACTGTG-GTAGATACTAC-3', GP6 +: 5'-GAAAAATAAACT-GTAAATCATATTC-3', amplicon size 150 bp) for the *HPV L1* gene^[22] and type-specific primer sets for HPV18 E6 (forward: 5'-GCGCTTTGAGGATCCAACAC-3', reverse: 5'-ATTCAACGGTTTCTGGCAC-3', amplicon size 335 bp) and HPV18 E6-E7 (forward: 5'-AACACAC-CACAATACTATGGCGCG-3', reverse: 5'-GCATTTTC-GTCCCTCGTCATCTG-3', amplicon size 944 bp). The type-specific primer sets for HPV18 E6 and HPV18 E6-E7 were designed according to the GenBank-provided *HPV18* gene sequences of X05015 (<http://www.ncbi.nlm.nih.gov/nuccore/X05015>).

PCR was conducted in a final volume of 25 µL containing 1× PCR buffer ("BioTake", China), 0.2 mmol/L dNTPs, 1 µL complexed recombinant Taq DNA polymerase, 0.2 mmol/L of each primer, and 100 ng DNA. An initial 5-min denaturation step at 95 °C was followed by 30 amplification cycles of 30 s at 95 °C, 30 s at 55 °C,

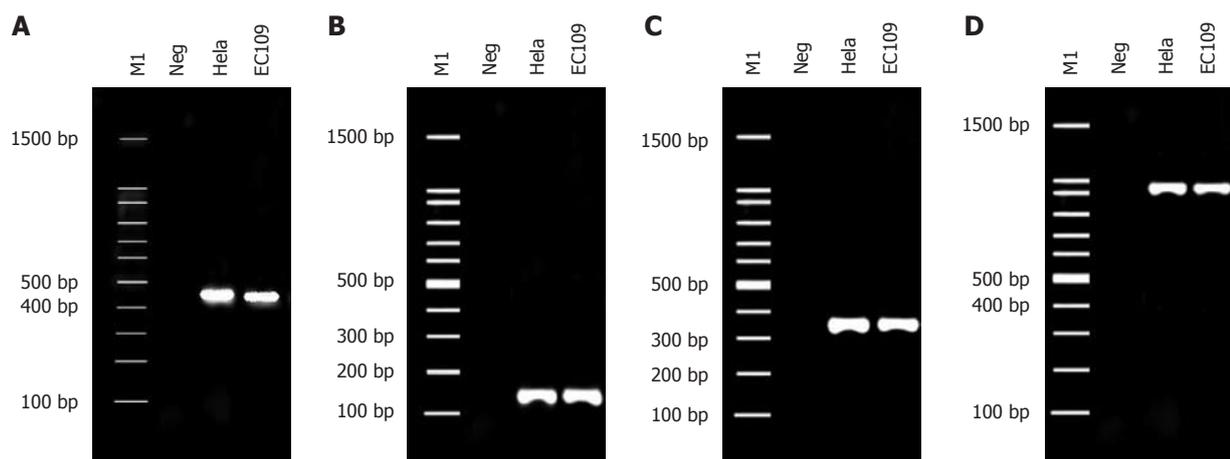


Figure 1 Detection of HPV18 DNA in EC109 cells. A-D: HPV18 DNA detection in EC109 cells using the primer pairs Y09/11, GP5 +/6 +, HPV18E6, and HPV18E6-E7, respectively. M1: 100 bp DNA ladder; Neg: Negative control (template of HEK 293 cell DNA); HeLa: Positive control (template of HPV18-infected cervical cancer cells); EC109: DNA from EC109 cells. HPV: Human papillomavirus; EC: Esophageal carcinoma.

and 1 min at 72 °C, with a final extension step of 5 min at 72 °C using a block thermocycler (PeQLab Biotechnology, Germany). A negative control (HEK293 cell DNA template) was included in each amplification step. DNA from HeLa cells was included as an HPV18-positive control. The PCR products were resolved on a 1.0% agarose gel.

Reverse transcription

Total RNA from the cell lines was isolated using the Micro-to-Midi Total RNA Purification System kit (“Bio-Take”, China) according to the manufacturer’s instructions. The RNA was quantified by spectrophotometry. Reverse transcription (RT) of mRNA isolated from EC109 cells was performed to produce a double-stranded DNA product that was then amplified by PCR. The final concentrations for the RT reaction were RNase-free H₂O (9.2 μL), 0.2 mmol/L dNTP mixture (1 μL), 10 pmol (dT)17-p3 (oligonucleotide primer: GACTCGAGTC-GACATCGATTTTTTTTTTTTTTTTTTTTTT; 1 μL), 5 × first-strand buffer (“BioTake”, China; 4 μL), 200 U SuperScript reverse transcriptase (“BioTake”, China; 1 μL), 40 U RNase inhibitor (1 μL), and total RNA (1-2 μg) in a total volume of 20 μL. The RNA in each reaction was reverse transcribed by heating at 42 °C for 50 min and inactivated by heating at 70 °C for 15 min. Samples were stored at 4 °C.

To confirm that EC109 cells with detectable HPV18 E7 mRNA were indeed harboring HPV, mRNA for a human housekeeping gene was amplified by RT-PCR to ensure that mRNA isolated from EC109 cells was of sufficient integrity to be amplified by PCR. mRNA encoding human GAPDH was used as a target for the RT-PCR. PCR was carried out as described previously^[19], using 10 pmol/L of each GAPDH primer (forward 5’-CATCACCATCTTCCAGGA-3’; reverse 5’-GTCTAC-CACCCTATTGCA-3’) and 2 μL cDNA at a 52 °C annealing temperature for 30 s to generate a GAPDH product of 500 bp.

Detection of viral-cell fusion transcripts by nested PCR

HPV18 PCR reactions were prepared as described by Klaes *et al.*^[21] using forward primer p1-18 specific for HPV18 E7 (5’-TAGAAAGCTCAGCAGACGACC-3’) and p3 (5’-GACTCGAGTCGACATCG-3’) as reverse primer, 1 × buffer, 2.5 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 10 pmol/L primers, 1 U Ex Taq DNA polymerase, 3 μL cDNA product in a total volume of 25 μL. PCR was conducted as follows: 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 3 min. The last cycle was followed by a final extension step at 72 °C for 5 min.

The amplification product (5 μL) was used for nested PCR under identical conditions using forward primers p2-18 specific for HPV18 E7 (5’-ACGACCTTCGAG-CATTCCAGCAG-3’) and (dT)₁₇-p3 as reverse primer, except that the annealing temperature was 67 °C. The positions of the two primers were 814-835 for p1-18 and 830-853 for p2-18. To control for false-positives, a negative control (HEK293 cell DNA template) was included in each amplification. Electrophoresis was performed using a 1.2% agarose gel.

Cloning and sequence analysis

To confirm specific HPV18 oncogene transcription in EC109 cells, the final nested PCR products were cloned into a pMD-18T vector and sequenced to analyze the chromosomal location of HPV integration.

RESULTS

Detection of HPV DNA in EC109 cells

HPV18 DNA was detected in EC109 cells by PCR using the general primer sets of My09/11 and GP5 +/6 + for HPV L1 and the type-specific primer sets for HPV18 E6 and E6-E7 to generate products of 450 bp, 150 bp, 335 bp and 944 bp respectively (Figure 1).

HPV infection and the development of EC. However, the role of HPV in the carcinogenesis of EC remains unclear. In this study, the authors demonstrate the integration of HPV DNA into host chromosomes, which could be a potential mechanism where by HPV infection leads to the development of EC.

Innovations and breakthroughs

Recent reports have highlighted the importance of HPV infection in EC. This paper is the first study to report that HPV18 integrated into one part of chromosome 8 in a cell line, EC109, derived from human EC cells. This study further suggests that HPV infection may be the cause of EC.

Applications

By understanding how EC is induced after HPV infection, this study may provide a future strategy for the diagnosis and prevention of EC.

Peer review

The authors examined HPV18 integration into one part of chromosome 8 in EC109 cells. Integration of the HPV genome into the host cell chromosome suggests that persistent HPV infection is a key factor in malignant cell transformation and carcinogenesis. The results may represent a molecular mechanism for the development of EC.

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S- Editor Sun H L- Editor Cant MR E- Editor Zhang DN

Johanson-Blizzard syndrome

Nabeel Almashraki, Mukarram Zainuddin Abdulnabee, Maja Sukalo, Abdullah Alrajoudi, Iman Sharafadeen, Martin Zenker

Nabeel Almashraki, Mukarram Zainuddin Abdulnabee, Abdullah Alrajoudi, Iman Sharafadeen, Department of Pediatric, Al-Thawra Teaching Hospital, 25122 Sana'a, Yemen
Martin Zenker, Maja Sukalo, Institute of Human Genetics, University Hospital, Magdeburg, Leipziger Str. 44, 39120 Magdeburg, Germany

Author contributions: Almashraki N, Abdulnabee MZ, Alrajoudi A, Sharafadeen I designed the study and collected clinical data; Sukalo M and Zenker M performed the molecular genetic analysis; Almashraki N, Abdulnabee MZ and Zenker M wrote the paper.

Correspondence to: Nabeel Almashraki, MD, Department of Pediatric, Al-Thawra Teaching Hospital, PO Box, 25122 Sana'a, Yemen. al_mashraki@yahoo.com

Telephone: +967-01-711731632 Fax: +967-01-486642

Received: January 22, 2011 Revised: March 2, 2011

Accepted: March 9, 2011

Published online: October 7, 2011

Abstract

Johanson-Blizzard syndrome (JBS) is a rare autosomal recessive disease characterized by exocrine pancreatic insufficiency, hypoplastic or aplastic nasal alae, cutis aplasia on the scalp, and other features including developmental delay, failure to thrive, hearing loss, mental retardation, hypothyroidism, dental abnormalities, and anomalies in cardiac and genitourinary systems. More than 60 cases of this syndrome have been reported to date. We describe the case of a male infant with typical symptoms of JBS. In addition, a new clinical feature which has not previously been documented, that is anemia requiring frequent blood transfusions and mild to moderate thrombocytopenia was observed. A molecular study was performed which revealed a novel homozygous UBR1 mutation. Possible explanations for this new association are discussed.

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Key words: Alae nasi aplasia; Anemia; Cutis aplasia; Exocrine pancreatic insufficiency; Johanson-Blizzard

syndrome

Peer reviewer: Dr. Paul Sharp, BSc (Hons), PhD, Nutritional Sciences Division, King's College London, Franklin Wilkins Building, 150 Stamford Street, London, SE19NH, United Kingdom

Almashraki N, Abdulnabee MZ, Sukalo M, Alrajoudi A, Sharafadeen I, Zenker M. Johanson-Blizzard syndrome. *World J Gastroenterol* 2011; 17(37): 4247-4250 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i37/4247.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i37.4247>

INTRODUCTION

Johanson-Blizzard syndrome (JBS) is a rare autosomal recessive disorder, first described in 1971 by Johanson and Blizzard^[1]. The genetic defect causing the disease was unknown until 2005, when it was shown to result from mutations of the *UBR1* gene located on chromosome 15q15-21. *UBR1* encodes one of at least four functionally overlapping E3 ubiquitin ligases of the N-end rule pathway, a conserved proteolytic system whose substrates include proteins with destabilizing N-terminal residues 20^[2]. The precise pathophysiological link between altered protein degradation and the clinical anomalies observed in JBS remains to be determined.

The reported cases of JBS showed no difference in gender. Parental consanguinity is frequently observed. The typical clinical features of JBS are the following (with decreasing frequency): exocrine pancreatic insufficiency, hypoplasia/aplasia of the alae nasi, dental anomalies, congenital scalp defects, sensorineural hearing loss, growth retardation, psychomotor retardation, hypothyroidism, imperforate anus and genitourinary anomalies. A detailed list of observed clinical features is given in Table 1.

CASE REPORT

A 5-mo-old male infant of consanguineous Yemeni par-

Table 1 Clinical features of Johanson-Blizzard syndrome

<i>Exocrine pancreatic insufficiency</i> ^[1,3-5]
<i>Hypoplasia/aplasia of alae nasi</i> ^[1,4,6-8]
<i>Scalp defect/aplasia cutis</i> ^[1,6,8]
<i>Sensory neural hearing loss</i> ^[1,3,8,9]
<i>Bilateral cystic dilation of cochlea, low set ears, and temporal bone defect</i> ^[10]
<i>Growth retardation, short stature</i> ^[1,11]
<i>Dental anomalies: oligodontia and absence of permanent teeth</i> ^[1,6,7,11]
<i>Anorectal anomalies: imperforate anus</i> ^[4,11,12]
<i>Hypotonia, microcephaly, and mental retardation sometimes normal intelligence</i> ^[3,7,11]
<i>lacrima duct anomalies, coloboma of the lids, superior puncta absence, lacrima cutaneous fistula, and congenital cataract</i> ^[13]
<i>Abnormal frontal hair pattern (upsweep)</i> ^[7]
<i>Vesicoureteric reflux, hypospadias, and duplex of uterine and vagina</i> ^[8]
<i>Congenital heart diseases such as myxomatous mitral valve, PDA, VSD, ASD, dextrocardia, complex congenital heart disease, and cardiomyopathy</i> ^[13,14]
<i>Cholestatic liver disease (one case)</i> ^[15]
<i>Café au lait spots</i> ^[16]
<i>Hypothyroidism</i> ^[1]
<i>Growth hormone deficiency</i> ^[5]
<i>Hypopituitarism</i> ^[17]
<i>Impaired glucagon secretion response to insulin induced hypoglycemia</i> ^[18]
<i>Diabetes mellitus</i> ^[19,20]

Italic letters show common features. PDA: Patent ductus arteriosus; VSD: Ventricular septal defect; ASD: Atrial septal defect.



Figure 1 Typical facial appearance of this patient with Johanson-Blizzard syndrome, showing aplasia of alae nasi, scalp defect, and sparse hair.

ents was referred because of poor feeding. The history started when he was 2-mo old with recurrent attacks of pallor and edema in the feet and hands. In addition, the infant showed failure to thrive and greasy stools. He received 3 blood transfusions. There was a family history of two previous male siblings with the same facial features as the index case, who also received several blood transfusions and expired at 4 and 4 ½ mo, respectively

On physical examination, the patient was lethargic, hypotonic, and pale. His body weight was 3.3 kg (< 5% percentile), and body length was 52 cm (< 5% percentile). Head circumference was 37 cm (microcephaly), and the anterior fontanel was wide (6 cm × 3 cm). There was aplasia of the alae nasi, midline cutis aplasia and a small scalp defect on the occiput, the scalp hair was sparse with areas of alopecia (Figure 1), and eye lashes and eyebrows were sparse. Hypospadias was detected, and the anus was narrow and displaced anteriorly. There was also pitting edema on the feet and hands.

Routine laboratory tests revealed the following re-

sults: hemoglobin (Hb) was 4 g/dL, with reticulocytes 7%, mean corpuscular volume 85 fl and mildly decreased platelets (75 000/μL). Erythrocyte morphology showed anisocytosis and normochromia. Hb electrophoresis and bone marrow aspiration were normal (Table 2).

Serum pancreatic enzymes (amylase, lipase) were low. Total protein and albumin were low, and other liver function tests, renal function tests, serum electrolytes and blood sugar were within the normal range (Table 2).

Thyroid function tests revealed low free T4 and slightly increased thyroid-stimulating hormone. These results indicated hypothyroidism.

Echocardiography showed a small atrial septal defect. Whole body X-ray, abdominal ultrasound, brain computed tomography (CT) scan, and temporal bone CT scan were normal.

The patient received oral thyroxine, pancreatic enzyme replacement, multivitamins and strict monitoring to avoid complications. When the patient was seen last time at the age of 9 mo, his overall condition had significantly improved. He has gained weight, although still below the 3rd centile, and blood cell counts had normalized (Table 2). The child still showed muscular hypotonia and delay in motor milestones.

Genetic studies

After obtaining informed written consent from the parents for the genetic investigation, venous blood samples were taken from the index patient and his parents. DNA was extracted from blood leukocytes according to standard procedures. All 47 exons of the *UBR1* gene including the flanking intronic regions were analyzed by direct bidirectional sequencing as described previously^[19]. Sequencing in the index patient revealed a homozygous mutation in exon 19. The nucleotide substitution c.2089 C > T predicts a missense change (p.S700P) affecting an

	At presentation (5 mo)	At 9 mo	Normal range
Hemoglobin	4 g/dL Post transfusion: 12 g/dL,	11.7g/dL	10.5-12
Retics count	7%	1.20%	0.2-2%
MCV	85	85	70-86 fl
Leukocyte count	5600	16 000	6000-17 500/mm ³
Platelets	192 000, 79 000, 100 000	390 000	150 000-400 000/mm
RBC blood morphology	Anisocytosis and normochromia	Anisocytosis and normochromia	
Total bilirubin	3.5	3	0-24 mmol/L
Direct bilirubin	1.5	1.13	0-5.1 mmol/L
ALT	44	39	0-41 U/L
AST	45	55	0-35 U/L
ALP	235	410	180-1200 U/L
Total protien	35	56	60-87 g/L
Albumin	17.3	38.8	34-48 g/dL
Urea	1	1.7	3.3-6.4 mmol/L
Creatinine	13	19	62-106 mmol/L
Pancreatic amylase	2.96	15	13-53 U/L
Pancreatic lipase	10	20	13-60 U/L
FT4	9.28	11.55	13.9-26.1 pmol/L
TSH	17.8	6.43	1.4-8.8 uIU/mL
Serum iron	88.39	157	60-170 mg L/dL
Total iron binding capacity	120	106	100-400 mg/dL
Serum ferritin		1260 (repeated BT)	7-140 ng/mL
Serum folate level		20	15-55 ng/mL
Serum vitamin B12 level		252	197-866 pg/mL
Insulin		0.2	2.6-24.9 Uu/mL
Bone marrow results (normal)	Erythropoiesis, granulopoiesis, and lymphopoiesis are normal cellularity and maturity and megakaryocytes are present	The same result	
Hemoglobin electrophoresis	Normal		

MCV: Mean corpuscular volume; RBC: Red blood cell; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; FT4: Free Thyroxine; TSH: Thyroid-stimulating hormone; BT: Blood transfusion.

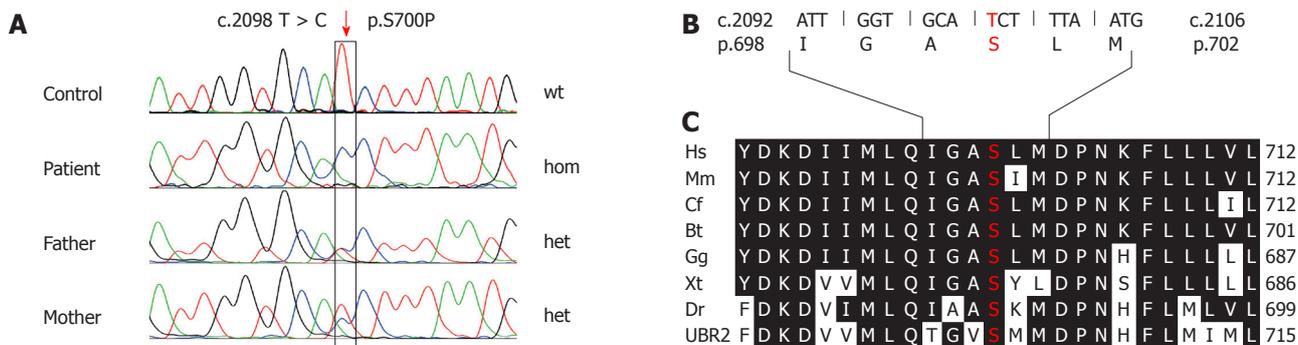


Figure 2 Demonstration and characterization of the familial mutation. A: Comparison of electropherograms around the T > C transition at position c.2098; B: Nucleotide and amino acid sequence; C: Multiple protein alignment (<http://www.ebi.ac.uk/Tools/msa/clustalw2>) of vertebrate UBR1. Black shading indicates identical residues. wt: Wild-type; Hom: Homozygous; Het: heterozygous; Hs: Homo sapiens; Mm: Mus musculus; Cf: Canis familiaris; Bt: Bos taurus; Gg: Gallus gallus; Xt: Xenopus tropicalis; Dr: Danio rerio; UBR2: Human UBR2 protein.

amino acid residue that is 100% conserved throughout vertebrate UBR1 and UBR2 proteins (Figure 2). To date, this change has not been known as a mutation or polymorphism. PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) predicts that this mutation is probably damaging with a score of 0.992 (sensitivity: 0.59; specificity: 0.96). Both parents were found to be heterozygous for the mutation. Based on this evidence we regarded p.S700P as the disease-causing mutation in this family.

DISCUSSION

JBS is a rare autosomal recessive disorder that affects many systems with a wide range of congenital abnormalities. A small beak-like nose (due to aplasia or hypoplasia of the alae nasi), and exocrine pancreatic insufficiency are considered the most consistent manifestations, while others features (Table 1) occur at varying frequencies in the affected patients. The patient presented here had typ-

ical facial features and exocrine pancreatic insufficiency, this combination is pathognomonic for JBS. Our patient also presented with scalp defects, developmental delay, and generalized hypotonia, which have been described in reported cases of JBS. Remarkably, our patient presented with an additional phenotypic feature, namely significant anemia, and required frequent blood transfusions from the age of 2 mo. The hematologic abnormalities also included thrombocytopenia and mild leukopenia. No definite etiology could be established. This feature has not been described in previous reports of JBS. Remarkably, two previous male siblings, who were assumed to have the same disease based on the report of similar facial features, also had significant anemia (Hb: 4 g/dL) and received frequent blood transfusions.

In addition, there was also mild to moderate thrombocytopenia in the other affected children of this family. The unusual and consistent association of JBS with a hematologic phenotype in this family may raise different speculations, such as a second autosomal recessive condition that might segregate JBS in this family or a specific function of the UBR1 protein carrying the novel missense mutation.

In infants, anemia caused by iron, vitamins and trace element deficiencies are unusual before the age of 6 mo, but in this patient the nutritional consequences of malabsorption might have appeared earlier due to many factors such as low birth weight, malnutrition in the mother and hypothyroidism in which normochromic, normocytic anemia may be secondary to reduced red blood cell production and reduced red cell survival.

Although we cannot exclude these possibilities, the fact that the hematologic disease resolved after efficient pancreatic enzyme and vitamin supplementation suggests a major contribution of malnutrition.

ACKNOWLEDGMENTS

We thank the family for their kind cooperation.

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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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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

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Islam Khan, PhD, Professor, Department of Biochemistry, Faculty of Medicine, Kuwait University, PO box 24923, Safat 13110, Kuwait

Takumi Kawaguchi, MD, PhD, Department of Digestive Disease Information & Research, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan

Yuyuan Li, Professor, Department of Gastroenterology, First Municipal People's Hospital of Guangzhou, 1 Panfu Road,

Guangzhou 510180, Guangdong Province, China

Dr. Oliver Mann, MD, Senior Attending Physician and Deputy Director, Department of General, Visceral and Thoracic Surgery, University of Hamburg, Martini Str. 52, D-20246 Hamburg, Germany

Ole Haagen Nielsen, MD, DMSc, Professor, Department of Gastroenterology, D112M, Herlev Hospital, University of Copenhagen, Herlev Ringvej 75, DK-2730 Herlev, Denmark

Shotaro Nakamura, MD, Department of Medicine and Clinical Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

Dr. Richard Parker, MB, ChB, MRCP (London), Department of Gastroenterology, University Hospital of North Staffordshire, Newcastle Road, Stoke on Trent, North Staffordshire, ST4 6QG, United Kingdom

Dr. Zoltan Rakonczay, MD, PhD, First Department of Medicine, University of Szeged, P.O. Box: 427, H-6701 Szeged, Hungary

Eduardo de Santibañes, MD, PhD, Professor, Department of Surgery, Hospital Italiano de Buenos Aires, Gascón 450, Buenos Aires 1181, Argentina

Joerg F Schlaak, MD, Professor of Medicine, Department of Gastroenterology and Hepatology, University Hospital of Essen, Hufelandstr. 55, 45122 Essen, Germany

Dr. Rupjyoti Talukdar, MD, Department of Gastroenterology and Hepatology, Mayo Clinic, 200 1st Street SW, Rochester, MN 55905, United States

Yoshitaka Takuma, MD, PhD, Department of Gastroenterology, Kurashiki Central Hospital, 1-1-1 Miwa, Kurashiki, Okayama 710-8602 Japan

Eddie Wisse, Professor, Cell Biology and Histology of the Faculty of Medicine and Pharmacy, Free University of Brussels (VUB), Laarbeeklaan 103, B 1090 Brussels-Jette, Belgium

Events Calendar 2011

- January 14-15, 2011
 AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States
- January 20-22, 2011
 Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States
- January 27-28, 2011
 Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany
- January 28-29, 2011
 9. Gastro Forum München, Munich, Germany
- February 4-5, 2011
 13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany
- February 13-27, 2011
 Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia
- February 17-20, 2011
 APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver Bangkok, Thailand
- February 22, 2011-March 04, 2011
 Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada
- February 24-26, 2011
 Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland
- February 24-26, 2011
 2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil
- February 24-26, 2011
 International Colorectal Disease Symposium 2011, Hong Kong, China
- February 26-March 1, 2011
 Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada
- February 28-March 1, 2011
 Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates
- March 3-5, 2011
 42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States
- March 7-11, 2011
 Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States
- March 14-17, 2011
 British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom
- March 17-19, 2011
 41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany
- March 17-20, 2011
 Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States
- March 18, 2011
 UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States
- March 25-27, 2011
 MedicRes IC 2011 Good Medical Research, Istanbul, Turkey
- March 26-27, 2011
 26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States
- April 6-7, 2011
 IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States
- April 7-9, 2011
 International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy
- April 15-16, 2011
 Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany
- April 18-22, 2011
 Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States
- April 20-23, 2011
 9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea
- April 25-27, 2011
 The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia
- April 25-29, 2011
 Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States
- April 28-30, 2011
 4th Central European Congress of Surgery, Budapest, Hungary
- May 7-10, 2011
 Digestive Disease Week, Chicago, IL 60446, United States
- May 12-13, 2011
 2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom
- May 19-22, 2011
 1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain
- May 21-24, 2011
 22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venice, Italy
- May 25-28, 2011
 4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina
- June 11-12, 2011
 The International Digestive Disease Forum 2011, Hong Kong, China
- June 13-16, 2011
 Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy
- June 14-16, 2011
 International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia
- June 22-25, 2011
 ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain
- June 29-2, 2011
 XI Congreso Interamericano de Pediatría "Monterrey 2011", Monterrey, Mexico
- September 2-3, 2011
 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany
- September 10-11, 2011
 New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States
- September 10-14, 2011
 ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States
- September 30-October 1, 2011
 Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium
- October 19-29, 2011
 Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia
- October 22-26, 2011
 19th United European Gastroenterology Week, Stockholm, Sweden
- October 28-November 2, 2011
 ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States
- November 11-12, 2011
 Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan
- December 1-4, 2011
 2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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Name of journal

World Journal of Gastroenterology

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 Identifier, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 Gastroenterology and Hepatology).

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World Journal of Gastroenterology®

Volume 17 Number 38
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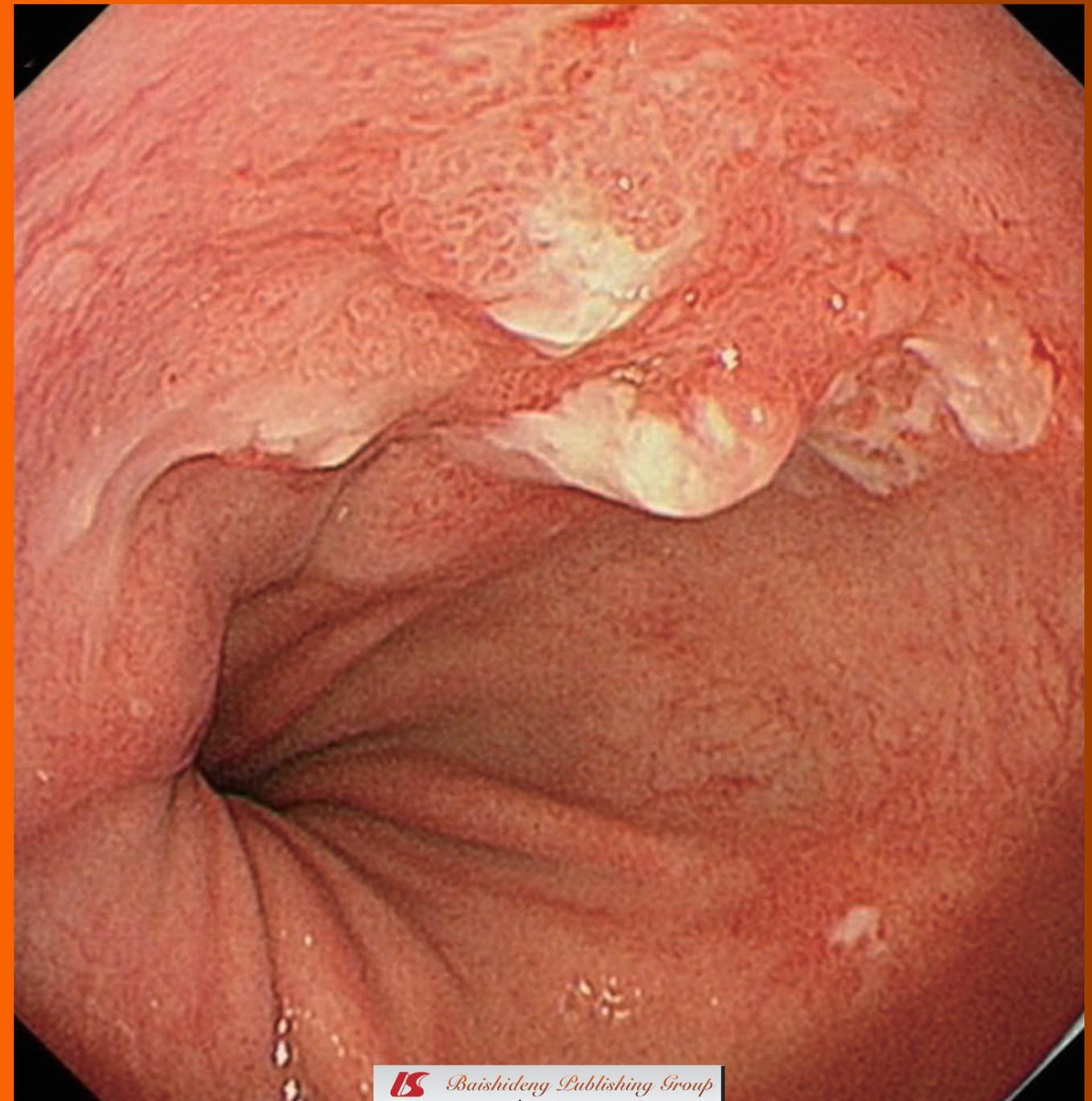
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ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

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

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

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Systematic review of modulators of benzodiazepine receptors in irritable bowel syndrome: Is there hope?

Pooneh Salari, Mohammad Abdollahi

Pooneh Salari, Medical Ethics and History of Medicine Research Center, and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran
Mohammad Abdollahi, Faculty of Pharmacy, and Pharmaceutical Sciences Research Center, and Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences, Tehran 1417614411, Iran

Author contributions: Salari P collected the data and drafted the manuscript; Abdollahi M conceived the study, reviewed the data, and edited the manuscript.

Correspondence to: Mohammad Abdollahi, Professor, Faculty of Pharmacy, and Pharmaceutical Sciences Research Center, and Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences, Tehran 1417614411, Iran. mohammad.abdollahi@utoronto.ca

Telephone: +98-21-66959104 Fax: +98-21-66959104

Received: February 23, 2011 Revised: May 20, 2011

Accepted: May 27, 2011

Published online: October 14, 2011

of BZDs in IBS, but bearing in mind the concentration-dependent effect of BZDs on cytokines and cell proliferation, future studies using pharmacodynamic and pharmacokinetic approaches are highly recommended.

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Key words: Benzodiazepines; Benzodiazepine receptor modulators; Dextofisopam; Irritable bowel syndrome; Systematic review

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Salari P, Abdollahi M. Systematic review of modulators of benzodiazepine receptors in irritable bowel syndrome: Is there hope? *World J Gastroenterol* 2011; 17(38): 4251-4257 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4251.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4251>

Abstract

Several drugs are used in the treatment of irritable bowel syndrome (IBS) but all have side effects and variable efficacy. Considering the role of the gut-brain axis, immune, neural, and endocrine pathways in the pathogenesis of IBS and possible beneficial effects of benzodiazepines (BZD) in this axis, the present systematic review focuses on the efficacy of BZD receptor modulators in human IBS. For the years 1966 to February 2011, all literature was searched for any articles on the use of BZD receptor modulators and IBS. After thorough evaluation and omission of duplicate data, 10 out of 69 articles were included. BZD receptor modulators can be helpful, especially in the diarrhea-dominant form of IBS, by affecting the inflammatory, neural, and psychologic pathways, however, controversies still exist. Recently, a new BZD receptor modulator, dextofisopam was synthesized and studied in human subjects, but the studies are limited to phase II b clinical trials. None of the existing trials considered the neuroimmunomodulatory effect

PRESENT KNOWLEDGE ON IRRITABLE BOWEL SYNDROME

Irritable bowel syndrome (IBS) is well-defined as a functional abdominal disorder categorized by intestinal pain or distress, and changes in bowel habits without recognizable malefactor^[1]. The symptoms are either constant or intermittent and vary from diarrhea or constipation, hypersecretion of colonic mucus, flatulence, to the sensation of weakness or depression^[2]. The pathophysiology of IBS is in some ways multifaceted, yet no consistent biomarker, anatomic, or biochemical alteration has been found^[3]. Several pathophysiologic paths have been deliberated for elucidation leading symptoms such as motor and sensory dysfunction, neuroimmune mechanisms, dysregulation of mucus secretion, emotions, colonic motility, enteric nervous system, changes in intraluminal situation and visceral feeling^[4]. Gut motility turbulence creates

different bowel behaviors and IBS subtypes, according to these outlines they are presented as IBS-D (diarrhea-predominant), IBS-C (constipation-predominant) and IBS-M (mixed)^[5].

Earlier, the role of parasympathetic and hormonal provocations in motor activity of some portions of the gut was demonstrated^[2]. The most important observation is well-thought-out visceral hypersensitivity as a substantial element in combination with fluctuations in gastrointestinal (GI) motility and secretory activity^[6]. Recent epidemiological findings further underline the central and peripheral disease activators or exacerbations as psychosomatic contributions^[7-9] and gastroenteric infections^[10]. In addition, the brain-gut axis endorses the strong relationship between the brain and the gut *via* neural, immune and endocrine paths which is affected by neuroimmunological or neuroendocrinological stressors^[11,12]. A summary of the pathophysiological mechanisms involved in IBS are presented in Table 1.

Contemporary treatment approaches depend on patients' signs and symptoms as well as comorbid conditions. Other than lifestyle and dietary changes, psychotherapy and psychopharmacological treatment, prokinetics (dopamine antagonists, 5-HT₃ antagonists and/or 5-HT₄ agonists), antispasmodics, sedatives and tranquilizers, antibiotics^[13], probiotics^[14], fecal bulking agents and complementary and alternative therapies are now considered as symptomatic treatment^[2,15]. Prokinetics and antispasmodics^[16] are usually used in IBS-C type, while patients with IBS-D benefit from opioid agonists, anticholinergics, and 5-HT₃ antagonists. Treatment options are summarized in Table 2.

Despite the wide range of medications and the high prevalence of the disease, to date no completely effective remedy is available. Therefore, investigations in this area should be continued. Pain relief is one of the challenges in the management of IBS as existing visceral analgesics have significant adverse effects and there is a balance to be struck between their usage and side effect profiles. Although various classes of drugs are used for visceral analgesia or other symptoms of IBS such as 5-HT₃ antagonists^[17], tricyclic antidepressants (TCAs)^[18], selective serotonin reuptake inhibitors (SSRIs)^[19,20], gabapentinoids, corticotrophin-releasing factor receptor-1 antagonists^[21], β_3 adrenoceptor agonists^[21], somatostatin, and N-methyl d-aspartate receptor antagonists, melatonin^[22], and sildenafil^[23], there are hopes for new drug investigations. In accordance with this idea, the efficacy of benzodiazepines (BZD) receptor modulators is being determined in ongoing phase III clinical trials^[24].

Bearing in mind the new advances in drug classes, and the special attention paid to new BZDs, we intend to study the promising advantageous effects of BZDs from diverse themes including neuroimmunology, anxiolytic, and visceral pain.

In the present review, the most relevant articles on the subject were searched using PubMed, Scopus, Web of Science, and Google Scholar databases up to February 2011. MeSH terms including irritable bowel syndrome,

Table 1 A summary of the pathophysiological mechanisms of irritable bowel syndrome

Mechanism	Description
Visceral hypersensitivity	↓ Threshold of visceral perception, hyperalgesia; ↑ Viscerosomatic referral areas
Modulation of CNS	Altered activation in reaction to rectosigmoid stimuli; fail to inhibit pain perception; activation of pain facilitatory pathways
Stress	Changes in visceral perception and neuro-endocrine responses to stressor
Infection	↑ Incidence after bacterial, viral, parasitic infections
Inflammation	Increased inflammatory cytokines; decreased anti-inflammatory cytokines
Serotonin	Influencing gut motility, sensation and secretion; altered serotonin signaling in IBS
Genetic factors	Familial clustering

CNS: Central nervous system; IBS: Irritable bowel syndrome.

benzodiazepines, benzodiazepine receptor modulators, and gabamimetics were used as search terms. The search was limited to English articles only. All non-clinical and clinical studies were included. The search resulted in 69 papers on the role of benzodiazepines in IBS; of these, after thorough evaluation and the omission of duplicate or non-relevant articles, 10 papers were included and evaluated in detail.

FINDINGS

Visceral pain

Visceral sensitivity and abdominal pain are dual warning signs of IBS but are not present in all patients^[25,26]. These patients show diminished visceral perceptual edges, augmented viscerosomatic referral area and larger sensory scores^[26]. The regulation of sensory neurotransmission in the gut is indicative of a satisfactory goal in the treatment of IBS. Incidentally, sensory afferents from the intestine have been examined in preclinical and clinical models.

New approaches in brain imaging provided new understandings of the likeness between IBS symptoms and different non-gastrointestinal disorders which pointed to the reformed sense of visceral drive in the central nervous system (CNS) in IBS^[27-29]. There is the possibility that the CNS fails to excite pain inhibitory pathways or activates pain facilitatory trails in patients with IBS^[30]. As a consequence of the convenient connection between the brain and gut *via* neural, immune, and endocrine pathways (Figure 1), the involvement of the CNS in the pathophysiology of IBS is prominent^[8,11,31-36]. Several parts of the CNS including cerebral regions, dorsal vagal nuclei, as well as the enteric nervous system contain γ -amino butyric acid (GABA) receptors^[37,38]. Vagal fibers influence migrating motor complex activity *via* the enteric nervous system^[38]. With the intention of reducing visceral hypersensitivity and the consequential pain, different pathological and pharmacological tactics have been used, for instance motility modulators (SSRIs), and special receptors or ion channels on visceral afferent pathways.

Table 2 Common therapeutic modalities

Therapeutic approach	Mechanism	Example of drugs	Therapeutic issues
Cholinergic system	Muscarinic receptor antagonists	Hyoscine, cimetropium, zamifenacin, solifenacin	Limited value, limited side effects, no interaction
Serotonin system	5-HT receptor antagonists	Alosetron, tegaserod, renzapride	Safe on cardiovascular system, anxiolytic, psychological side effects, adverse effects
Antidepressants (SSRIs, TCAs, SNRIs)	Neurotransmitter reuptake inhibitor	Paroxetine, desipramine, venlafaxine	Limited use, serious side effects, limited efficacy
Adrenergic agents	α , β adrenergic agents	Clonidine, solabegron	Limited use, side effects, limited efficacy
Corticoid system	CRF antagonists	A-Helical CRH9-41	Limited use, under investigation
Cholecystokinin		Loxiglumide, dexloxiglumide	Limited use, under investigation
Neurotrophins	Enhance neuron survival and development	NT3	Expensive, under investigation, limited use
Sleeping process	Sleep regulator	Melatonin	Limited use
BZD receptor modulators		Gabapentin, dextofisopam, pregabalin	Side effects, limited use, under investigation
Guanylate cyclase-c agonists	Activates guanylate cyclase-c receptor in enterocytes	Linaclotide	Limited use, well toleration, minimal side effects, under investigation
Opioid system	Modulating visceral nociception	Asimadoline, naloxone, naltrexone	Limited central side effects, good efficacy
Neurokinin antagonists	Affect colonic motility	Ezlopitant, nepadutant	Under investigation

SSRIs: Selective serotonin reuptake inhibitors; TCAs: Tricyclic antidepressants; SNRIs: Serotonin norepinephrine reuptake inhibitors; NT3: Neurotrophin-3; BZD: Benzodiazepine; 5-HT: 5-hydroxytryptamine; CRF: Corticotrophin-releasing factor.

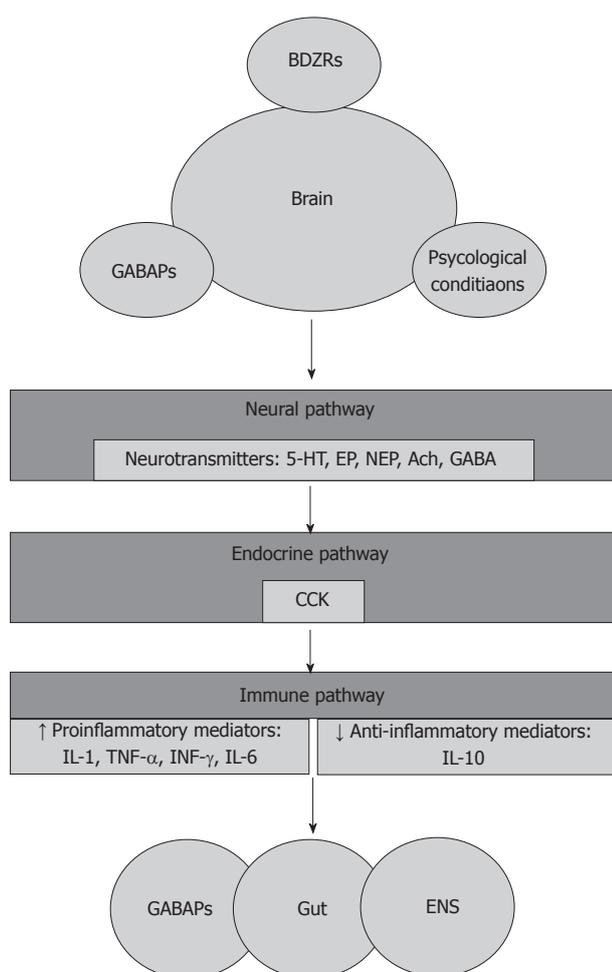


Figure 1 The brain-gut axis. BDZRs: Benzodiazepine receptors; GABARs: γ -aminobutyric acid receptors; 5-HT: 5-hydroxytryptamine; EP: Epinephrine; NEP: Norepinephrine; GABA: γ -aminobutyric acid; Ach: Acetylcholine; CCK: Cholecystokinin; IL-1: Interleukin-1; IL-6: Interleukin-6; TNF- α : Tumor necrosis factor- α ; IFN- γ : Interferon- γ ; IL-10: Interleukin-10; ENS: Enteric nervous system.

One of the newly targeted classes of drugs for the treatment of visceral pain, BZD receptor modulators, reduce sensitivity and ache perception. Consistent with the promising effects of these modulators, dextofisopam the R enantiomer of tofisopam was developed for the management of IBS-D^[39].

BZDs interact with GABA receptors which exist in the CNS and influence the autonomic nervous system, dorsal vagal nuclei, and the enteric nervous system. Vagal fibers affect migrating motor complex movement by the enteric nervous system^[40].

BZD receptors were identified in subcortical and hypothalamic regions and appear important in controlling autonomic function^[41], such as motor and sensory activity of the gut^[42]; nevertheless they do not exist in the gut^[43]. Animal studies on the R-enantiomer of tofisopam (the non-sedating anxiolytic), dextofisopam, showed encouraging results in reducing colonic motility and visceral sensitivity with little effect beneath basal conditions^[44]. Leventer *et al*^[45] in a phase II b study of dextofisopam for 12 wk in 140 patients with IBS observed overall symptom relief (primary end point) in 57% of patients as compared with placebo (43% of patients). Although dextofisopam improved stool consistency in men and women, the recurrence rate was only decreased in females. This occurred within one week. The most common side effects were headache and abdominal pain (in 12% of patients in comparison with 4% in the placebo group) which was comparable to placebo. No benefit on bloating, partial defecation, or hospital anxiety and depression scale scores was observed^[45]. Interestingly, dextofisopam showed a slight influence on basal GI movement in animals, while after induction of hypermotility, it showed more efficacy^[46].

There are a few studies on other BZDs in IBS patients. Castedal *et al*^[40] showed a small effect of midazol-

am on small bowel motility using manometry, however, phase III related retroperistalsis did not work.

Other than the anxiolytic effect of BZDs, their effect on GABA may be constructive. Two antiepileptic drugs, gabapentin and pregabalin are effective in neuropathic pain. They equally affect GABA receptors in the CNS and increase their binding affinity for endogenous GABA ligand and elevate chloride ion efflux. In this regard, numerous studies assessed the beneficial influence of pregabalin and gabapentin on visceral pain.

Gabapentin, an amplifier of GABA transmission, prevents central neurotransmitter release by impeding $\alpha_2\delta$ subunits of voltage-dependent calcium channels^[47,48]. Gabapentin has favorable effects on neuropathic pain and hyperalgesia^[49,50]. Lee *et al*^[51] demonstrated the effect of gabapentin in reducing human experimental hyperalgesia. They randomized 40 IBS-D patients to receive gabapentin 300 mg/d and then 600 mg/d for 5 d. Gabapentin reduced rectal sensory thresholds by decreasing rectal sensitivity to expansion and improving rectal compliance.

Although the structure of pregabalin is related to GABA, it is inactive at GABA and BZD receptors. It strongly attaches to the $\alpha_2\delta$ subunit of voltage-dependent calcium channels and reduces calcium arrival at nerve endings^[52] and results in the release of excitatory neurotransmitters (noradrenaline, glutamate, substance P, and calcitonin gene-related peptide) decreasing their involvement in pain pathogenesis^[53]. Accordingly, pregabalin reduces normal colonic pain responses and colonic hyperalgesia in a dose-dependent manner in animal studies^[54,55]. In animal studies, pregabalin reduced viscerosomatic and autonomic responses caused by colorectal distension resulting in visceral pain relief^[56]. In addition, the effect of GABA_B receptors in visceral pain was confirmed^[57]. In another preclinical study, pregabalin reduced both visceral allodynia and hyperalgesia with no change in basal sensitivity. Houghton *et al*^[58] randomized 26 hypersensitive IBS patients to increasing doses of pregabalin for 3 wk or placebo. They reported significant increases in sensory thresholds from baseline for first defecation and pain, and rectal compliance. In IBS patients, pregabalin restored sensory thresholds to normal levels^[58].

Neuroimmunology

Recent approaches to the pathophysiology of IBS have changed from spastic colitis to mucosal immune activation^[59,60] and inflammation^[61] which is supported by animal studies^[62,63]. Generally, in 7%-30% of patients, there is a history of recent bacterial gastroenteritis^[64]. Failure to reduce the inflammatory reaction to infection may increase cytokines or special inflammatory cells^[65]. There is a discrepancy between pro-inflammatory and anti-inflammatory cytokines in IBS. The influence of the neuroimmune system in the pathogenesis can be elucidated by an augmented number of activated mast cells in the vicinity of colonic nerves, decreased interleukin-10/interleukin-12 (IL10/IL12) ratio and changes in local defense mechanisms in the sigmoid and colonic mucosa

in IBS^[65-67]. In fact, a number of investigators have proposed low-grade inflammation in IBS which is defined as infiltration of T lymphocytes, mast cells, and enteroendocrine cells into colonic mucosa with mast cells priority^[68]. Excessive production of tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ), a trend toward excessive production of interleukin-6 (IL-6), and a defect in the production of interleukin-10 (IL-10) were reported in IBS^[15,69]. In accordance with cellular infiltrations, increased levels of interleukin-1 β (IL-1 β), IL-6, INF- γ and TNF- α , and an abnormal ratio of IL-10/IL-12 in IBS patients were observed^[68].

There is a counteraction between the endocrine, central and autonomic nervous and immune systems which is mediated by signal transduction, cytokines and mediators^[70]. BZD receptors in both central and peripheral forms and their ligands create a regulatory network between anxiety and the immune system^[71]. Central BZD receptors located in the CNS and their activation affects GABA binding to GABA_A receptors which regulates chloride flux^[72]. The peripheral receptors are located in peripheral tissues and are involved in cell proliferation, heme biosynthesis, cholesterol transport and immunomodulation^[73]. Peripheral BZD receptors **have been identified on immune cells such as macrophages, neutrophils, leukocytes, and lymphocytes** and may have a crucial role in the relationship between the CNS, behavior and immunity^[74-78]. Activation of cell growth and DNA synthesis requires nanomolar concentrations of BZDs, thus inhibition of cell proliferation is subject to micromolar concentrations of these compounds^[79]. Of BZDs, diazepam and tofizopam bind to both types of receptors^[80,81]. Kalashnikov *et al*^[70] in an *in vitro* study confirmed the inhibitory effect of diazepam on cell proliferation at high doses, while tofizopam enhanced cell proliferation at low and moderate doses. In addition, they found dose-dependent suppression of TNF- α production with both diazepam and tofizopam and a wide range of effects of tofizopam on IL-2 production (enhancement to suppression), **while diazepam suppressed IL-2 production**^[70].

Psychotherapy

A history of psychiatric complaints or mental instabilities in patients is a key factor in IBS and some studies have indicated extensive occurrence of psychiatric disorders in IBS^[82,83]. Similarly, the mental state of the patient affects bowel symptoms and may relieve symptoms^[84]. The most dominant psychological features of IBS patients are hypochondriasis, depression, anxiety, obsession, and neuroticism^[84]. Whitehead *et al*^[85] demonstrated higher scores of psychopathology in IBS patients. High comorbidity exists between functional bowel and stress-related psychiatric disorders^[86,87]. **Stress precipitates or exacerbates IBS**^[88].

Since the possibility of comorbidity with mood disturbances such as depression and anxiety is high in IBS, almost all patients with IBS may benefit from TCAs or BZDs. These agents may also decrease pain perception.

Guidelines of the Britain Society of Gastroenterol-

ogy and the American Gastroenterology Association endorse psychotherapeutic interventions for severe forms of IBS^[89,90] to relieve psychological, visceral, and somatic symptoms. Overall benefit was found in IBS patients following psychological treatments in a meta-analysis^[91]. In these conditions, combining psychotherapy with psychopharmacological treatment is effective^[92]. Studies show that the two most common antidepressant and anxiolytic classes of drugs, TCAs and SSRIs, are effective in symptom relief^[93]. Compared to TCAs, SSRIs have fewer side effects but do not improve bloating or visceral pain^[93,94]. BZDs are used routinely in anxiety disorders but their efficacy in symptom relief of IBS is under debate^[90].

Clouse *et al.*^[95] studied the effect of antidepressants in a retrospective study in 138 IBS patients. They evaluated patients' response to a wide range of antidepressants such as TCA (amitriptyline, doxepin, *etc.*), trazodone, amoxapine, alprazolam and thioridazine at lower doses than used for affective disorders. They reported significant responses regardless of the presence or absence of psychiatric illness.

CONCLUSION

In the present work, all possible beneficial effects of BZD receptor modulators in IBS from the view points of visceral pain, psychopharmacologic effects, and immunologic properties were reviewed. It seems that these BZDs influence IBS symptoms *via* different mechanisms, and these mechanisms are under investigation. There are a small number of studies examining the effects of dexetofisopam on patient's symptoms, however, we are still waiting for the results of phase III trials. In addition, none of these trials have considered the neuroimmunomodulatory effect of BZDs in IBS. Moreover, bearing in mind the concentration-dependent effect of BZDs on cytokines and cell proliferation, future studies using pharmacodynamic and pharmacokinetic approaches are highly recommended.

ACKNOWLEDGMENTS

This paper is the outcome of an in-house non-financially supported study. The paper has been written upon invitation of Professor Mohammad Abdollahi by EIC of *WJG*.

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S- Editor Tian L L- Editor Webster JR E- Editor Xiong L

Factors predicting occurrence and prognosis of hepatitis-B-virus-related hepatocellular carcinoma

Yi-Fang Han, Jun Zhao, Li-Ye Ma, Jian-Hua Yin, Wen-Jun Chang, Hong-Wei Zhang, Guang-Wen Cao

Yi-Fang Han, Jian-Hua Yin, Wen-Jun Chang, Hong-Wei Zhang, Guang-Wen Cao, Department of Epidemiology, Second Military Medical University, Shanghai 200433, China
Jun Zhao, Department of Hepatobiliary Surgery, the 3rd Affiliated Hospital, Second Military Medical University, Shanghai 200433, China

Li-Ye Ma, Department of General Surgery, the 1st Affiliated Hospital, Second Military Medical University, Shanghai 200433, China

Author contributions: Han YF and Zhao J collected data, provided interpretation and contributed equally to this work; Ma LY, Yin JH, Chang WJ and Zhang HW critically read the paper; Cao GW wrote the paper.

Supported by National Natural Science Foundation of China, No. 81025015 and No. 30921006

Correspondence to: Guang-Wen Cao, MD, PhD, Professor of Medicine, Chairman, Department of Epidemiology, Second Military Medical University, 800 Xiangyin Rd., Shanghai 200433, China. gcao@smmu.edu.cn

Telephone: +86-21-81871060 Fax: +86-21-81871060

Received: May 12, 2011 Revised: July 12, 2011

Accepted: July 19, 2011

Published online: October 14, 2011

Abstract

Primary liver cancer is an important cause of cancer death, and hepatocellular carcinoma (HCC) accounts for 70%-85% of total liver cancer worldwide. Chronic hepatitis B virus (HBV) infection contributes to > 75% of HCC cases. High serum viral load is the most reliable indicator of viral replication in predicting development of HCC. HBV genotype C is closely associated with HCC in cirrhotic patients aged > 50 years, whereas genotype B is associated with development of HCC in non-cirrhotic young patients and postoperative relapse of HCC. Different HBV subgenotypes have distinct patterns of mutations, which are clearly associated with increased risk of HCC. Mutations accumulate during chronic HBV infection and predict occurrence of HCC. Chronic inflammation leads to increased frequency of viral mutation *via* cellular cytidine deaminase induction.

Mutations are negatively selected by host immunity, whereas some immuno-escaped HBV mutants are active in hepatocarcinogenesis. Inflammatory pathways contribute to the inflammation-necrosis-regeneration process, ultimately HCC. Their hallmark molecules can predict malignancy in HBV-infected subjects. Continuing inflammation is involved in hepatocarcinogenesis and closely related to recurrence and metastasis. HBV load, genotype C, viral mutations and expression of inflammatory molecules in HBV-related HCC tissues are significantly associated with poor prognosis. Imbalance between intratumoral CD8⁺ T cells and regulatory T cells or Th1 and Th2 cytokines in peritumoral tissues can predict prognosis of HBV-related HCC. These factors are important for developing active prevention and surveillance of HBV-infected subjects who are more likely to develop HCC, or for tailoring suitable treatment to improve survival or postpone postoperative recurrence of HCC.

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Key words: Hepatitis B virus; Hepatocellular carcinoma; Viral load; Genotype; Mutation; Immune cells; Signaling pathway; Cytokine; Prognosis

Peer reviewers: Gloria González Aseguinolaza, BSc, MSc, PhD, Department of Gene Therapy in Hepatology, FIMA, CIMA University of Navarra, Navarra, Spain

Han YF, Zhao J, Ma LY, Yin JH, Chang WJ, Zhang HW, Cao GW. Factors predicting occurrence and prognosis of hepatitis-B-virus-related hepatocellular carcinoma. *World J Gastroenterol* 2011; 17(38): 4258-4270 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4258.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4258>

INTRODUCTION

Liver cancer in men is the fifth most frequently diagnosed

cancer worldwide but the second most frequent cause of cancer death. It is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer death in women. Globally, liver cancer rates are more than twice as high in men as in women. An estimated 748 300 new liver cancer cases and 695 900 cancer deaths occurred worldwide in 2008^[1]. The highest liver cancer rates are found in East and South-East Asia and in Central and Western Africa. Developing countries contribute more than 80% of cases. About half of these cases and deaths occur in China, due to the endemicity of hepatitis B virus (HBV) infection. Chronic HBV infection accounts for about 60% of the total liver cancer in developing countries and for about 23% of the cancer in developed countries, while hepatitis C virus (HCV) infection accounts for about 33% of the total liver cancer in developing countries and for about 20% in developed countries^[2]. Recently, the incidence of liver cancer has been increasing in some western countries due to the increasing prevalence of HCV infection and immigration of people from countries with high endemicity for HBV infection^[3]. Among primary liver cancers, hepatocellular carcinoma (HCC) accounts for 70%-85% of the total liver cancer burden worldwide^[1]. Chronic HBV infection results in approximately one-third of all cases of liver cirrhosis and more than three-quarters of all cases of HCC worldwide^[4]. The relative risks of HCC among people infected with HBV ranges from 5 to 49 in case-control studies and from 7 to 98 in cohort studies, and the incidence of HCC (per 100 000 person/years) among people with chronic HBV infection ranges from 400 to 800 in men and from 120 to 180 in women^[5]. Standard HBV vaccination dramatically decreases HCC prevalence among vaccinees aged 6-19 years in Taiwan^[6]. In Mainland China, the prevalence of hepatitis B surface antigen (HBsAg) in people born after 1992 has dramatically decreased, possibly due to expanded program of immunization of HBV vaccination, however, the rate in those born before 1992 is around 8%-9%^[7,8]. Thus, HCC will remain one of the major public health problems in Mainland China in next 40-50 years.

The patients with HCC usually present late and have mostly developed serious liver cirrhosis. The life expectancy of patients with newly diagnosed HCC is measured in terms of weeks to months with a mortality to incidence ratio close to 1^[3]. Therefore, it is critical to clarify some risk factors that can be clinically applied for the prediction of this malignancy in the HBV-infected population. Viral characteristics of HBV and hepatic inflammation status are not only associated with the occurrence, but also with the recurrence and metastasis of HCC after surgical treatment. Prognostic factors for predicting survival probability are invaluable to tailor suitable treatment options.

In this paper, we characterize viral properties of HBV and inflammatory factors that have been recently proven to be significantly associated with the occurrence and the prognosis of HCC, and summarize a group of viral

and inflammatory factors (Table 1) that can be of significance for active prevention and surveillance of the HBV-infected subjects who are more likely to develop HCC, and for tailoring suitable treatment options like antiviral treatment to improve the survival or postpone the recurrence of HBV-related HCC.

ROLES OF VIRAL AND INFLAMMATORY FACTORS IN PREDICTING OCCURRENCE OF HBV-ASSOCIATED HCC

HBV replication status and viral variations

Association of viral replication status with HCC: HBV precore region encodes the precore protein, which is processed in the endoplasmic reticulum to produce secreted hepatitis B e antigen (HBeAg). HBeAg expression indicates active viral replication. In community-based HBV-infected subjects, HBeAg-positive rates and viral loads are high in the young, and decrease with age^[8]. Continuing high viral load and/or HBeAg expression have been significantly associated with increased risk of HCC in prospective studies^[9-11]. With reference to the low serum HBV DNA level ($\leq 10^{4.5}$ copies/mL), the hazard ratio (HR) for HCC of the intermediate HBV DNA level ($10^{4.5-6.5}$ copies/mL) is 1.62 (95% CI: 1.05-2.48) and that of the high HBV DNA level ($> 10^{6.5}$ copies/mL) is 2.73 (95% CI: 1.76-4.25)^[12], indicating viral load predicts the risk of developing HCC dose-dependently. In another group of prospective studies, HBV viral load rather than HBeAg has been documented to be independently associated with an increased risk of HCC^[13,14]. In cross-sectional case-control studies, high viral load ($\geq 10^4$ copies/mL) is independently associated with an increased risk of HCC^[15-17]. However, HBeAg expression is not usually high in patients with HCC, and is not significantly associated with an increased risk of HCC in some case-control studies^[16,17]. Ongoing high levels of HBV replication in patients seronegative for HBeAg are strongly associated with poor prognosis of patients with chronic hepatitis B (CHB)^[18]. Thus, serum viral load is the most reliable indicator in predicating the development of HCC. HBeAg seroconversion reflects viral mutations in the HBV genome, especially in the precore and core promoter regions^[19], and this usually happen during the natural course of chronic HBV infection, possibly caused by cytotoxic T-lymphocyte (CTL)-mediated clearance.

Association of HBV genotypes/subgenotypes with HCC: HBV genotypes and subgenotypes have distinct geographical distributions and have been shown to differ with regard to HBeAg seroconversion, clinical outcome, prognosis, and response to antiviral treatment^[20-22]. HBV genotypes A, B, D and F are associated with earlier spontaneous seroconversion of HBeAg than genotype C. HBV subgenotype B2 is more likely to cause acute hepatitis B, while subgenotype C2 is more prone to caus-

Table 1 Viral and inflammatory factors associated with the occurrence and prognosis of hepatitis-B-virus-related hepatocellular carcinoma

Factor	Occurrence	Ref.	Prognosis	Ref.
Viral load				
Sera	≥ 10 ⁴ copies/mL	[9,11,12,15-17]	> 10 ⁴ copies/mL (poor survival)	[99,100]
Non-cancerous liver tissues	-	-	> 3 × 10 ⁷ copies/g (poor survival)	[101]
HBV genotype/subgenotype	C2 > B2 C2: cirrhotic patients ≥ 50 yr B2: non-cirrhotic ones < 50 yr	[15,24-26]	C > B (poor survival) B2 in the young (recurrence)	[15,104]
HBV mutation				
Individual	C1653T, T1753V, T1674C/G A1762T/G1764A, C1766T, T1768A; G1899A, C2002T, A2159G, A2189C, G2203A/T; T53C, preS2 start codon mutation, preS1 deletion, C2964A, A2962G, C3116T, C7A	[17,27-29]	A1762T/G1764A and short stretch (< 100 bp) pre-S deletions in non-cancerous liver tissue of the HCC patients (poor survival)	[101]
Combined	2964C-3116T-preS2 start codon wildtype-7A; 2964C-3116T-7A-76C; 2964A-3116T-7C-76A/T; 2962G-preS2 start codon wildtype-105C-1762T/1764A	[30,31]	-	-
Intratumoral immune cells	M2 macrophage (risk), CD8 + T cells (protective); Imbalance between CD8 + T cells and Treg or between Th1 and Th2	[52-58]	NK and CD8 + T cell (good prognosis); Treg cells (poor prognosis)	[106,107]
Inflammatory pathways and cytokine	Pathway: NF-κB, STAT3, Wnt/β-catenin, IGF-α1, RAF/MEK/ERK, PI3K/AKT/mTOR, p53, VEGF Cytokine: IL-6, TNF-α	[60-81]	IL-2 and IL-15 (good prognosis); G-CSF (poor prognosis); Wnt-1 protein (poor prognosis); LI-cadherin (short survival); ErbB-2 (short survival); Cyclooxygenase-2 (recurrence); Low miR-199-3p (poor survival)	[108-113,116]
Genetic polymorphism of inflammatory molecules	HLA, TGF-β1, IL-β1, IL-18, IL-12, KIF1B-, UBE4B- or PGD-related pathways, NF-κB1, IκBα	[82-88]	VEGF	
ALT	> 45 U/L	[17,28,31]	> 50 U/L (poor survival)	[104]

HBV: Hepatitis-B-virus; HCC: Hepatocellular carcinoma; NF: Nuclear factor; TNF: Tumor necrosis factor; IL: Interleukin; STAT: Signal transducer and activator of transcription; TGF: Transforming growth factor; RAF: Raf protein kinases; PI3K: Phosphatidylinositol-3 kinase; MEK: Mitogen-activated protein kinase; ERK: Extracellular-signal-regulated kinase; mTOR: Mammalian target of rapamycin; AKT: Protein kinase B; HLA: Human leukocyte antigen; ALT: Alanine aminotransferase; VEGF: Vascular endothelial growth factor; G-CSF: Granulocyte-colony stimulating factor; LI: Liver intestine; NK: Natural killer.

ing chronic infection than is subgenotype B2 following an acute course^[23]. Chronic infection with genotype C is more likely to cause liver cirrhosis and HCC, as compared with chronic infection with genotype B^[24,25]. Chronic infection with genotype C (C2) is closely associated with an increased risk of HCC in the HBV-infected subjects, especially in cirrhotic patients aged > 50 years, whereas infection with genotype B (B2) has been found to be associated with development of HCC in non-cirrhotic young patients and relapse of HCC after surgical treatment^[15,26].

Association of HBV mutations with HCC: Different HBV subgenotypes have distinct patterns of mutations. We and others have found that HBV mutations including C1653T, T1753V, A1762T/G1764A, T1674C/G and C1766T/T1768A in the enhancer II/basal core promoter (Enh II/BCP) regions; G1899A, C2002T, A2159G, A2189C, and G2203A/T in the precore/core gene; and T53C, preS2 start codon mutation, preS1 deletion, C2964A, A2962G, C3116T, and C7A in the preS region are significantly associated with an increased risk of HCC^[17,27-29]. Of the HCC-associated mutations, combined rather than single mutations are significantly associated with the risk of HCC. In the preS region, the frequencies of combined mutations (haplotypic carriage) including 2964C-3116T-preS2 start codon wildtype-7A, 2964C-3116T-7A-76C, and 2964A-3116T-7C-76A/T are sig-

nificantly higher in the patients with HCC than in those without HCC, whereas the haplotypic carriage with single mutation and other three wild types are inversely associated with HCC^[30]. In the preS and Enh II/BCP regions, a haplotypic carriage with 105C and 2962G is significantly more frequent in the patients with HCC than in those without HCC, and the frequency of 2962G-preS2 start codon wild type-105C-1762T/1764A is 47.9% in the patients with HCC and 4.3% in those without HCC^[31]. Interestingly, the HBV mutations, either in the preS or in the core promoter region, are significantly associated with HCC, whereas the wild-type nucleotides in these regions are mostly associated with liver cirrhosis^[17,28]. HBV mutations can be used as indicators for the prediction of end-stage liver diseases including HCC. Although these mutations and the combinations are specific for HCC to some extent, it will be more practicable if they can predict this malignancy in the HBV-infected subjects before the occurrence of HCC.

Role of viral mutations in prediction of HCC: The majority of patients with chronic HBV infection are believed to be infected *via* perinatal transmission, especially in HBV-endemic areas. The HCC-associated HBV mutants may not transmit *via* mother-to-child transmission because the children whose mothers carry both HBV mutants and wild-type virus are mostly found to be in-

ected with wild-type virus alone^[32]. In the early stage of HBV infection, serum viral load is high and HBeAg is frequently positive because immune selection is weak. Immune selection may increase during HBeAg seroconversion. HBV mutations tend to be gradually generated during a chronic immunopathological course after infection. Recent studies have shown that accumulation of HBV mutations in the core region in children with HCC differ from those in HBV-infected children without HCC, and the frequencies of the HCC-associated mutations do not increase with increasing age of HCC patients, in contrast to patients without HCC^[17,33]. These data support that the frequencies of HBV mutations might increase to high levels during malignant transformation, no matter how old the HBV-infected subjects are. However, HCC mostly occurs in HBV-infected subjects between 45 and 65 years of age. The HBV mutations mostly experience long-term processes of immune selection. A1762T/G1764A occurs up to 10 years before the onset of HCC and is a valuable indicator of HBV-infected men who are more likely to develop HCC^[13,34,35]. The mutations in the BCP region accumulate sequentially during the development of HCC^[36]. Some of the mutations including the preS deletion, C1653T, T1753V and A1762T/G1764A accumulate during the course of HBV infection from asymptomatic HBsAg carrier state to cirrhosis or HCC^[27]. Thus, these viral mutations might accumulate before the diagnosis of HCC and predict the occurrence of HCC.

Possible mechanisms of viral mutation/selection: The HCC-associated HBV mutations are probably generated *via* an evolutionary process on two aspects: increased frequencies of the viral mutations and directional selection of the mutations by the host immune system. As a result of the spontaneous error rate of viral reverse transcriptase, HBV genome might exhibit higher frequencies of mutations than other DNA viruses. This kind of HBV mutation may be considered as a random mutation. In addition, HBV is highly vulnerable to the editing activity of an endogenous human cytidine deaminase activated by proinflammatory cytokines such as tumor necrosis factor (TNF)- α *via* nuclear factor (NF)- κ B activation, especially in late-stage liver diseases in which up to 35% of genomes can be edited^[37,38]. The majority of the newly synthesized HBV DNA genomes displayed extensive G-to-A mutations in the presence of APOBEC3C, an important member of cellular cytidine deaminases, contributing to innate anti-HBV host responses^[39]. Some of the cytidine deaminases play a role in the carcinogenesis of HCC through the generation of HBVx mutants, providing preneoplastic and neoplastic hepatocytes with a selective clonal growth advantage^[40]. The cytidine-deaminase-driven HBV mutations may be classified as “semi-directional mutations”. Either random or semi-directional mutations might be responsible for HBV mutagenesis during viral replication and inflammatory processes. Moreover, the direction of the mutations should be negatively selected

by host immune responses. Reduction of CD8⁺ T cell epitopes in HBV is one of the common means to evade immune clearance. HBV accumulates escape mutations *via* reduction in the epitopes number, eventually leading to the removal of epitopes in HBVx and surface proteins^[41,42]. The preS1 and preS2 play an essential role in the interaction with host immune responses because they contain several epitopes for T and/or B cells^[43]. The preS deletion is one of the most frequent HCC-associated HBV mutations, even in children with HCC^[27,28,30,44]. These mutations might facilitate immune escape of HBV.

Potential function of HBV mutations: Currently, the HCC-associated HBV mutations are mainly found in the preS and Enh II /BCP/precure regions. Two functional viral proteins coded by these regions are preS2 and truncated HBVx. HBVx protein may increase the expression of telomerase reverse transcriptase (TERT) and telomerase activity, prolonging the lifespan of hepatocytes and contributing to malignant transformation. A1762T has introduced a translation initiation site ATG at the C-terminal of HBVx protein. HBVx mutations, especially the C-terminal deletions, have been frequently found in tumor tissues of HCC^[45]. Most of the C-terminally truncated HBVx proteins lose their inhibitory effects on cell proliferation and transformation, retain their ability to bind to p53, and attenuate DNA repair and p53-mediated apoptosis, which may provide a selective clonal advantage for preneoplastic or neoplastic hepatocytes and contribute to hepatocarcinogenesis^[46,47]. HBVx and the preS2 activators exert a tumor-promoter-like function, resulting in positive selection of cells that produce functional regulatory proteins^[48]. HBV preS2 also promotes HCC development *via* activation of human TERT^[49]. However, the role of the mutated preS2 on hepatocarcinogenesis remains unknown. HBV mutations in the BCP region may alter viral replication ability. *In vitro* transfection of HBV mutants has indicated that high-replication clones with 1762/1764/1766 or 1753/1762/1764/1766 mutations expressed very low levels of HBeAg, whereas high-replication clones with 1753/1762/1764 triple mutations expressed high levels of HBeAg, and both 1762/1764/1766 and 1753/1762/1764/1766 mutations conferred significantly higher viral replication and lower HBeAg expression than 1762/1764 mutation alone^[50]. In addition, HBV mutations usually accompany high viral load in patients with HCC^[17]. These results indicate that some HBV mutations might promote hepatocarcinogenesis.

Taken together, HBV mutagenesis might be augmented in hepatic inflammatory microenvironment due to induction of activated cytidine deaminases. Host immune selection might differ in determining the distinctive outcomes of chronic HBV infection. Upon negative and incomplete immune selection, the mutations facilitate viral replication in the hepatocytes. The escaped HBV accumulates mutations that might predict

the occurrence of HCC and in turn play an active role in hepatocarcinogenesis.

Inflammatory signaling pathways and hallmark cytokines

The liver is the primary organ in which mammals metabolize nutrients, environmental toxins, and drugs. The liver also comprises enrichment of innate immune cells (such as macrophages, natural killer, natural killer T, and $\gamma\delta$ T cells), $CD8^+$ cytotoxic T cells, $CD4^+$ T helper cells (such as Th1, Th2, Th17 and Treg) and B cells, playing an important role not only in host defenses against invading microorganisms and tumor transformation, but also in liver injury and repair^[51]. Liver injuries caused by HBV are traditionally considered to be immuno-mediated and are mainly due to the activity of HBV-specific T cells. Liver-infiltrating neutrophils, natural killer cells and activated bystander lymphocytes also play important roles in causing HBV-related liver damage. These inflammatory cells release cytokines and chemokines which may favor cancer growth.

Liver immune cells: Macrophages can be divided schematically into two main classes in line with the Th1/Th2 dichotomy: M1 and M2. M1 macrophages (classically activated) originate upon encounter with interferon (IFN)- γ and microbial stimuli and are characterized by interleukin (IL)-12 and IL-23 production and consequent activation of polarized type I T cell response, defending the host from viral infections, fighting against tumors, producing high amounts of inflammatory cytokines, and activating the immune response. Monocytes may differentiate into M2 macrophages upon stimulation with IL-4, IL-10 and IL-13. M2 macrophages are responsible for scavenging debris, angiogenesis, remodeling and repair of wounded/damaged tissues, and promote carcinogenesis and downregulate M1 function and adoptive immunity. Tumor-associated macrophages (TAMs) resemble M2-polarized macrophages^[52]. $CD8^+$ T cells recognize viral peptides derived from phagocytosed and proteolytically cleaved HBV proteins, activate and differentiate B cells, and secrete IFN- γ and TNF- α , which inhibit the replication and gene expression of HBV. As discussed before, HBV mutations within T-cell epitopes have been documented during chronic HBV infection. These viral mutations may downregulate T-cell functions, such as proliferation or cytokine secretion, and completely or partially inhibit the immune response against the original epitope. Effective $CD8^+$ T-cell-mediated cytotoxic killing may play a crucial role in the control of cancer development. Although tumor-reactive CTL responses are evident in HCC patients, tumor regression is rarely seen, implying that tumor microenvironment inactivates antitumor effector cells, or induces immune tolerance. Macrophages in liver (also called Kupffer cells) function as M2 and suppress $CD8^+$ T cells in human HCC *via* B7-H1/programmed death-1 interactions and favor HCC growth^[53]. Depletion of TAMs in HCC enhances the therapeutic

effect of sorafenib on HCC^[54]. Treg cells serve as critical gatekeepers in immune homeostasis. Increased Treg cells ($CD4^+CD25^+FoxP3^+$) in HCC may impair the effector function of $CD8^+$ T cells and promote HCC progression^[55]. Th17 cells elicit a highly inflammatory immune response. The frequency of IL-17⁺ cells is significantly elevated in the patients with chronic liver diseases including viral hepatitis and HCC, and the tumor-derived Th17 cells may promote tumor growth and inflammation, and tumor-activated monocytes secrete a set of key proinflammatory cytokines that trigger proliferation of functional Th17 cells^[56,57]. Th17 cells promote angiogenesis, tumor growth and inflammation, while Treg cells appear to have counter-regulatory effects on Th17 cells and can inhibit their function. Therefore, an imbalance between Th17 and Treg cell function may be central in some inflammation-associated malignancies, possibly including HBV-associated HCC. The same is true for the imbalance between Th1 and Th2 functions in the microenvironment of HBV-related HCC^[58]. These immune imbalances caused by chronic HBV infection contribute to chronic inflammation, hepatic necrosis and subsequent regeneration, accumulated mutagenesis in hepatocytes and ultimately HCC. Multiple signaling pathways are involved in this inflammation-necrosis-regeneration process and in human HCC development. Hallmark cytokines and related molecules in these pathways might be candidate biomarkers for the prediction of the occurrence of HCC in the HBV-infected population.

NF- κ B pathway and hallmark cytokines: NF- κ B, a collection of dimeric transcription factors including NF- κ B1 (p105 and p50), NF- κ B2 (p100 and p52), RelA (p65), RelB and c-Rel, is present in all cells in inactive form. In non-stimulated cells, most NF- κ B dimers are retained in the cytoplasm by binding to inhibitory I κ B proteins. In response to proinflammatory stimuli, such as TNF- α or IL-1 β , the I κ B kinase (IKK) complex, composed of the IKK α and IKK β catalytic subunits and the IKK γ regulatory subunit, is activated, resulting in I κ B phosphorylation and eventual ubiquitin-mediated degradation^[59], leading to the nuclear entry of freed NF- κ B dimers. Of the two catalytic subunits, IKK β is the most critical for I κ B degradation, forming the core of what is known as the classical NF- κ B activation pathway^[60]. The classical IKK β -dependent NF- κ B signaling pathway promotes hepatocyte survival in both developing and adult livers. NF- κ B activation is often observed in human HCC, particularly following hepatitis. It plays a crucial role in liver inflammatory responses by controlling the expression of an array of growth factors and cytokines. One of the most important NF- κ B-dependent cytokines that is produced by activated Kupffer cells is IL-6. IL-6 released by Kupffer cells after NF- κ B activation also controls HBV gene expression and replication in hepatocytes at the level of transcription shortly after infection^[60]. HBVx protein also stimulates IL-6 expression in hepatocytes *via* a MyD88-dependent pathway^[61]. Hepatocyte IKK/NF- κ B promotes

HCC development by maintaining liver inflammatory responses^[62]. The inflammatory process triggers hepatocyte NF- κ B through upregulation of TNF- α in adjacent endothelial and inflammatory cells. NF- κ B inhibition through anti-TNF- α treatment or induction of I κ B super repressor in later stages of tumor development results in apoptosis of transformed hepatocytes and failure to progress to HCC^[63]. Serum levels of IL-6 and TNF- α have been found to be significantly higher in HBV-infected patients with liver cirrhosis and HCC than those without or in accordance with the progress of the disease phases^[64,65]. In this pathway, IL-6 and TNF- α are hallmark cytokines whose expression might indicate the risk of HCC in HBV-infected population.

Signal transducer and activator of transcription 3 pathway and hallmark cytokines: Signal transducer and activator of transcription (STAT)3 is inactive in non-stimulated cells, but is rapidly activated by various cytokines and growth factors, such as IL-6 and epithelial growth factor family members, as well as hepatocyte growth factor (HGF)^[61]. STAT3 activation requires phosphorylation of a critical tyrosine residue, Tyr705, which mediates its dimerization that is a prerequisite for nucleus entry and DNA binding. The phosphorylation of STAT3 at Tyr705 is most commonly mediated by Janus kinases (JAKs), especially JAK2. C-Jun N-terminal kinase (JNK) plays a dual role in the development of HCC. JNK promotes an inflammatory hepatic environment that supports tumor development, but also functions in hepatocytes to reduce tumor development^[66]. Activation of STAT3 also turns on strong negative feedback loops involving suppressor of cytokine signaling 3 (SOCS3). STAT3 is activated in the majority of HCCs with poor prognosis and not in surrounding non-tumor tissue or in normal liver. HBVx expression *in vitro* has a significant inverse correlation with the expression of the highly expressed members of the let-7 miRNA family in HCC patients, while the most highly expressed let-7 family member, let-7a, negatively regulates cellular proliferation partly through targeting STAT3, indicating that HBVx upregulates STAT3^[67]. HBVx mutant proteins express an atypical nuclear and perinuclear localization in HCC samples, and the effect of HBVx mutants on STAT/SOCS signaling demonstrates a significant upregulation of STAT3 activation in comparison to wild-type HBVx^[68]. These data indicate an active role of HBVx mutants in hepatocarcinogenesis that involves dysregulation of STAT/SOCS signaling. Hepatocyte-specific STAT3 ablation prevents HCC development^[69]. Other reasons like obesity-promoted HCC development is dependent on enhanced production of the tumor-promoting cytokines IL-6 and TNF- α , which cause hepatic inflammation and activation of STAT3^[70]. The main cause of STAT3 activation in human HCC could simply be the elevated expression of IL-6 and related cytokines, such as TNF- α , IL-11 and IL-23. One of the most critical tumor-promoting cytokines in HCC is IL-6. High serum level of IL-6 predicts future occur-

rence of HCC in patients with CHB^[65].

Wnt/ β -catenin signaling pathway: A hallmark of Wnt signaling is the stabilization of cytoplasmic β -catenin. Wnt/ β -catenin signaling regulates cytokine-induced human inducible nitric oxide synthase expression through interaction with NF- κ B, and plays an important role in the pathophysiology of inflammation-associated carcinogenesis^[71]. β -catenin was first identified on the basis of its association with cadherin adhesion molecules, and is widely recognized as a key molecule of the Wnt signaling cascade. Mutations of β -catenin, specifically stabilizing mutations in exon 3, are detected in approximately 30% of primary HCCs, raising the possibility that activation of Wnt/ β -catenin signaling contributes to hepatocarcinogenesis^[72]. Aberrant activation of the Wnt signaling pathway together with transforming growth factor (TGF)- β has been used for gene expression profiling-based classification of HCC^[73]. Wnt/ β -catenin signaling is activated relatively early during hepatocyte regeneration, mostly through post-translational modifications. Once activated, β -catenin signaling drives the expression of target genes that are critical for cell cycle progression and contribute to initiation of the regeneration process. Wnt-1 is a survival factor for HCC cells. The blockade of Wnt-1-mediated signaling may offer a potential pathway-specific therapeutic strategy for the treatment of a subgroup of HCC that over-expresses Wnt-1^[74]. Wnt/ β -catenin signaling is particularly activated by ectopic expression of Wnt-1 in HBV-infected HCC cells. Wnt-1 is necessary but insufficient to activate Wnt/ β -catenin signaling in HCC. The enhanced stabilization of β -catenin by HBVx, in addition to Wnt-1, is essential for the activation of Wnt/ β -catenin signaling in HCC^[75]. However, it should be clarified if HBVx mutant and other HBV mutants play a distinct role in activating this pathway.

TGF- β 1 pathway: The balance between death and survival is dysregulated in HCC mainly due to overactivation of antiapoptotic pathways^[76]. TGF- β signaling involves both tumor suppression and oncogenesis. TGF- β 1 is an important regulatory suppressor factor in hepatocytes, inhibiting proliferation and inducing cell death, however, it may also modulate other pro-tumorigenic processes, such as cell invasion^[77]. Elevated TGF- β 1 may accelerate hepatic fibrosis through increased TGF- β 1-induced proinflammatory signaling pathways in hepatic stellate cells^[78]. TGF- β activates TGF- β type I receptor (T β R I) and JNK, which differentially phosphorylate the mediator Smad3 to become C-terminally phosphorylated Smad3 (pSmad3C) and linker-phosphorylated Smad3 (pSmad3L). Reversible shifting of Smad3-mediated signaling between tumor suppression and oncogenesis in HBVx-expressing hepatocytes indicates that T β R I-dependent pSmad3C transmits a tumor-suppressive TGF- β signal, while JNK-dependent pSmad3L promotes cell growth. HBVx shifts hepatocytic TGF- β signaling from

the tumor-suppressive pSmad3C pathway to the oncogenic pSmad3L pathway in early carcinogenesis^[79]. Hepatocytic pSmad3L and pSmad3C assessment in HBV-infected liver specimens should prove clinically useful for predicting risk of HCC.

Other inflammatory pathways: Apart from above described pathways, raf protein kinases (RAF)/mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK) pathway, phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, insulin-like growth factor pathway, HGF/c-Met pathway, the p53 pathway, and growth factor-regulated angiogenic signaling are implicated in hepatocarcinogenesis^[76]. These pathways are of interest for a therapeutic perspective, because targeting them may help to reverse, delay or prevent hepatocarcinogenesis^[80]. For example, the antitumor effect of sorafenib on HCC can be improved by vertical blockade of RAF/MEK/ERK signaling with CI-1040^[81].

Genetic susceptibility of inflammatory factors

The imbalances in inflammation reactions play an important role in HBV-induced hepatocarcinogenesis, therefore, it is necessary to clarify the role of key inflammatory molecule germline mutations, which influence the expression and function of those molecules, on the susceptibility of HCC. Human leukocyte antigen-DR13 polymorphism has been shown to be strongly associated with the clearance of HBV^[82]. A study in Japan has evaluated the association of genetic polymorphism in the cytokines TNF- α , IFN- γ , TGF- β 1, IL-6 and IL-10 with the risk of HCC in HBV-infected subjects and has found that the risk of HCC was significantly lower in HBV carriers with C/C genotype than in those with T/C or T/T genotype in position +29 of the TGF- β 1 gene, but was not associated with genetic polymorphism of other molecules^[83]. It has been demonstrated that in the presence of the IL-1 receptor antagonist *2 allele, the IL- β 1-31 polymorphism T/C and T/T genotypes in Taiwan are significantly associated with HBV-related HCC, with adjusted odds ratios (ORs) of 2.93 (95% CI: 1.07-8.07) and 5.76 (95% CI: 1.79-18.53), respectively^[84]. A study in Korea has documented that the -148C, +8925G, and +13925C alleles of the IL-18 gene are associated with HBV-related HCC and the 148G > C single nucleotide polymorphism is functionally important in determining disease outcome^[85]. IL-12 polymorphisms have been recently associated with HBV-related HCC in the Chinese population^[86]. A recent genome-wide association study (GWAS) in Mainland China has shown that the 1p36.22 locus confers susceptibility to HBV-related HCC, and suggests that KIF1B-, UBE4B- or PGD-related pathways might be involved in the pathogenesis of this malignancy^[87]. Our recent data have demonstrated that NF- κ B1 gene promoter NFKB1-94ATTG2 allelic carriage and IKK β gene promoter NFKBIA-826T and NFKBIA-881AG allelic carriage, which are related to upregulation

of NF- κ B function, are significantly associated with an increased risk of HCC in patients infected with genotype C HBV, while the estimated haplotype frequency of NFKBIA promoter -881G-826T-519C is significantly higher in patients with HCC than in HBV-infected subjects without HCC^[88]. Problems in current studies on genetic susceptibility of HBV-associated HCC are: (1) GWAS with enough samples has limited coverage, resulting in loss of data; (2) individual case-control studies lack sufficient samples and strict controls; and (3) significant polymorphisms found by GWAS and individual case-control studies have few loci in common. Nevertheless, the contribution of genetic polymorphisms to the occurrence of HCC may be limited because of low ORs obtained both in current GWAS and in case-control studies.

Other parameters

Family history is one of the major risk factors for HCC^[89,90]. First-degree relatives of patients with HCC are associated with an increased risk of HBV-related HCC in Taiwan and with HCC in United States independently of HBV and HCV infection. In HBV-endemic regions, family clustering of HCC is, to some extent, related to family clustering of HBV infection *via* perinatal transmission. Apart from HBV and HCV infection, exposure to aflatoxin B1, alcoholic abuse, and diabetes are well-established risk factors for HCC. Regular consumption of coffee or green tea is significantly associated with a decreased risk of HCC in subjects with or without HBV and/or HCV infection^[91,92]. Old age, male sex and poor socioeconomic status are also related to the occurrence of HCC in the HBV-infected subjects.

Poor liver function, as indicated by low serum level of albumin and high level of bilirubin, favor HBV-induced hepatocarcinogenesis. Persistent or intermittent increase in serum alanine aminotransferase (ALT) level reflects persistent liver damage. CHB patients with ALT levels 0.5-1 times the upper limit of normal (ULN) and 1-2 \times ULN have an increased risk for the development of complications compared with patients with ALT levels < 0.5 \times ULN ($P < 0.0001$ for both)^[93]. In HBeAg-negative subjects, high viral load is frequently associated with abnormal ALT level, while ALT abnormality is more frequent in those with liver cirrhosis than those without (19.5% *vs* 7.8%, $P = 0.001$)^[24]. Abnormal ALT level (> 45 U/L) has been frequently shown to be an independent risk factor for HCC in HBV-infected subjects^[17,28,31]. Liver cirrhosis is a well-established important risk factor of HCC^[94]. Older age, higher total bilirubin, ALT, and HBV DNA levels, and HBV preS deletion, A1846T and/or T1768A mutations are major independent determinants of progression to cirrhosis in HBeAg-negative patients^[17,24,28,95].

Clinical scoring system for prediction of HCC occurrence

A research group in Hong Kong prospectively evaluated 1005 HBV carriers and found that age, albumin, bilirubin,

bin, HBV DNA, and cirrhosis independently predicted the development of HCC. They used these variables to construct a prediction score ranging from 0 to 44.5 and then validated the score in a different cohort of 424 HBV carriers. In the training cohort, they found cutoff values of 5 and 20 best discriminated HCC risk. In the validation cohort, the 5-year HCC-free survival rates were 98.3%, 90.5% and 78.9% in the low-, medium- and high-risk groups, respectively. HR for HCC in the medium- and high-risk groups was 12.8 and 14.6, respectively. This simple prediction score constructed from routine clinical and laboratory parameters is accurate in predicting HCC development in HBV carriers^[96]. To set up a scoring system for the prediction of HCC from CHB patients, 820 CHB patients have been followed up for a mean duration of 76.8 mo in a prospective study. It has been found that male [relative risk (RR) = 2.98], increasing age (RR = 1.07), higher HBV DNA levels (RR = 1.28), core promoter mutations (RR = 3.66), and presence of cirrhosis (RR = 7.31) are independent risks for the development of HCC. A risk score is derived and validated with sensitivity > 84% and specificity > 76% to predict the 5- and 10-year risks for the development of HCC^[97]. The two scoring systems can accurately predict HBV carriers and CHB patients who will more likely develop HCC in the near future, and have important implications for treatment allocation and strategic screening for HCC in CHB patients and HBV carriers.

ROLE OF VIRAL AND INFLAMMATORY FACTORS IN PREDICTING PROGNOSIS OF HBV-ASSOCIATED HCC

Viral load

In a study carried out in Japan, a total of 74 patients with HBV-associated HCC who had received either conventional treatment or not were followed up for survival analysis. In multivariate regression analysis, it has been found that serum level of HBV DNA and tumor size at diagnosis are independent and significant prognostic factors ($P = 0.0022$ and $P = 0.0106$, respectively), and a low level of viremia is associated with longer survival ($P = 0.0057$), even in patients seronegative for HBeAg^[98]. In another study, a total of 62 HBV-related HCC patients who had achieved complete necrosis with transarterial chemolipiodolization were followed up for analysis of recurrence. Multivariate analysis has established that high viral load ($> 10^5$ copies/mL) at complete necrosis is among the most important risk factors for post-treatment recurrence^[99]. Seventy-two patients who underwent liver resection for HBV-related HCC were followed up. By multivariate analysis, high viral load ($> 10^4$ copies/mL) (OR = 22.3, $P = 0.001$), α -fetoprotein > 1 mg/mL (OR = 7.4, $P = 0.02$), tumor size > 5 cm (OR = 5.1, $P = 0.02$), and age > 60 years (OR = 4, $P = 0.01$) at the time of tumor resection are independently associated with HCC recurrence after resection. Of those,

high viral load ($> 10^4$ copies/mL) is the most important correctable risk factor^[100]. In a Taiwan study, HBV DNA $> 3 \times 10^7$ copies/g in the non-cancerous liver tissues of patients with HCC was found to be independently associated with shorter overall survival^[101]. Lamivudine therapy is beneficial for patients after initial treatment for HBV-related HCC because it contributes to improving remnant liver function, decreasing the risk of liver failure, and increasing the chances of receiving available treatment modalities for recurrent HCC^[102]. Treatment of the HBV-infected HCC patients with IFN- α after curative resection efficiently prevents early recurrence and improves overall survival^[103]. These data indicate that high viral load is the most reliable factor in predicting poor prognosis of the patients with HBV-related HCC, therefore, antiviral treatment after surgical resection is highly recommended.

HBV genotype and viral mutations

A Taiwan study enrolled 64 patients who underwent liver resection for HBV-related HCC. During a mean follow-up of 26.6 ± 13.2 mo, patients with genotype C had worse disease-free survival rate ($P = 0.028$) than those with genotype B. By univariate analysis, genotype C, ALT > 50 U/L, tumor size ≥ 5 cm, and microvascular invasion were associated with tumor recurrence. Multivariate analysis indicated that genotype C was a risk factor independently associated with poor prognosis ($P = 0.034$)^[104]. Recently, the core gene of HBV isolated from HCC tissues has been found to have fewer mutations compared with those isolated from adjacent non-tumor tissues from the same patients ($P < 0.05$)^[105], implying that active immune selection of viral mutation most likely happens in the peritumoral liver tissues. In a study carried out in Taiwan, the association of virological characteristics with the prognosis of the patients was investigated by using the non-cancerous part of surgically removed HBV-associated HCC tissues from 185 patients. All virological and clinicopathological factors were subjected to Cox proportional hazard model analysis to estimate postoperative survival. After adjusting for other confounding factors, multivariate analysis revealed that age older than 50 years, bilirubin > 1.4 mg/dL, and A1762T/G1764A mutation were independently associated with shorter overall survival. Kaplan-Meier survival analysis indicated that in-frame, short stretch (< 100 bp) preS deletions, but not large fragment (> 100 bp) preS deletions, were significantly associated with poorer disease-free ($P = 0.005$) and overall ($P = 0.020$) survival. A hot deletion region located between codons 107 and 141 of the preS sequence was identified for the short stretch preS deletion mutants^[101]. The result of viral load in the inflammatory liver tissues seems to be consistent with that of serum viral load. It remains unknown if the mutations of HBV in the tissues are consistent with those in the sera.

Inflammatory cells and molecules

Inflammatory cells: Immune cells infiltrated into HCC

tissues and adjacent non-cancerous tissues have various roles in conducting inflammatory responses. NK and T cells are present in tumors of HCC patients with longer survival, and exclusively in areas devoid of proliferating tumor cells. NK and CD8⁺ T cell densities are correlated positively with tumor apoptosis, and negatively with tumor proliferation^[106]. On the other hand, higher density of intratumoral Treg cells and lower density of intratumoral CD8⁺ T cells are independently associated with poor prognosis of HCC. In addition, high Treg cell density is associated with both absence of tumor encapsulation and presence of tumor vascular invasion^[107]. Intratumoral balance of regulatory and cytotoxic T cells is a promising independent predictor for recurrence and survival in the HBV-infected patients with HCC.

Th1/Th2-like cytokines: Peritumoral hepatic tissues of patients with HCC have been used for investigating the association of inflammatory cytokines with prognosis in Mainland China. Higher levels of IL-2 and IL-15 in peritumoral liver tissues, but not in tumor tissues, are significantly associated with decreased incidence of recurrence of intrahepatic tumor and prolonged overall survival, whereas peritumoral expression of granulocyte-colony stimulating factor, a Th2-like cytokine, is significantly associated with poor prognosis^[108,109]. This reflects that the imbalance of Th1/Th2-like cytokines affects inflammation status, which later determines the malignant phenotype of HCC. Peritumoral levels of Th1/Th2-like cytokines are useful for stratifying patients, even those with early-stage HCC, into subgroups with different prognoses after curative resection. Treatment with Th1 cytokine IFN- α benefits HBV-related HCC patients after curative resection^[103], possibly *via* correcting the imbalances.

Other inflammatory molecules: Up-regulation of Wnt-1 protein has been reported in HBV-related HCC tissues. High Wnt-1 expression in HBV-related HCC tissues correlates with enhanced nuclear β -catenin accumulation, diminished membranous E-cadherin expression, and increased HCC recurrence after curative resection^[110]. Liver-intestine cadherin (LI-cadherin; CDH-17) is a new member of the cadherin superfamily with distinct structural and functional features. Overexpression of LI-cadherin is well correlated with microvascular invasion in HBV-positive HCC tissues and strongly associated with shorter overall survival as well as higher incidence of tumor recurrence^[111]. Thus, LI-cadherin should be a candidate target for HCC intervention. ErbB-2 is strongly upregulated in HBV-infected liver and correlated with HBVx expression. ErbB-2 contributes to the stabilization of β -catenin. Strong ErbB-2 staining in liver tissues of patients with HCC is associated with dysplasia and a shorter survival after tumor diagnosis^[112]. Cyclooxygenase-2 is a proinflammatory factor whose expression in non-cancerous liver tissue increases the postoperative recurrence of HCC in patients with HBV-related cirrhosis^[113]. These inflammatory molecules may serve as

prognostic markers and/or candidate therapeutic targets of HBV-related HCC after surgical treatment.

Signaling-associated miRNAs: miRNAs are a class of small non-coding RNAs that modulate gene function at the post-transcriptional level and act as fine tuners of various processes including cell signaling and apoptosis. miRNAs are associated with different types and stages of cancer, and are involved in liver diseases caused by various factors, including HBV^[114]. Some miRNAs are specifically found in HCC tumors and sera of patients. Serum miRNAs including miR-25, miR-375 and let-7f have been recently identified as biomarkers and clearly separate HCC cases from controls, and miR-375 and miR-92a have been identified as HBV-specific^[115]. miR-199a/b-3p exhibits its biological activity *via* inhibiting the Raf/MEK/ERK pathway, and is the most consistently decreased miRNA in HCC. Low miR-199-3p expression correlates with poor survival of HCC patients^[116]. Current problems of developing miRNAs as diagnostic and prognostic markers are their stability in sera and selection of internal controls. It will be a great challenge to search for miRNA markers, especially for those that target some existing inflammatory signaling pathways involved in the recurrence and metastasis of HBV-associated HCC.

Clinical scoring systems for prediction of HCC prognosis

Clinical scoring systems have been developed not only for the prediction of HCC occurrence, but also for its prognosis. Nathan *et al.*^[117] have prospectively evaluated survival of HCC patients with small tumors, and have found that tumor size > 2 cm (HR = 1.51), multifocal tumors (HR = 1.51), and vascular invasion (HR = 1.44) remained independent predictors of poor survival (all $P < 0.05$) after adjusting for demographic factors and histological grade. Based on these findings, they developed a prognostic scoring system by allotting 1 point each for these factors. Patients with early HCC could be stratified into three distinct prognostic groups (median and 5-year survival, respectively): 0 point (70 mo, 55%), 1 point (52 mo, 42%), and ≥ 2 points (24 mo, 29%) ($P < 0.001$). However, this scoring system has not been validated using an independent cohort. Hsu *et al.*^[118] have prospectively investigated the prognostic ability of the five currently used staging systems with 1713 enrolled HCC patients, and have concluded that the CLIP staging system is the best long-term prognostic model for HCC in a cohort of patients with early to advanced stage HCC. Current scoring systems for the prediction of HCC prognosis lack useful viral and inflammatory markers like serum HBV load and expression of inflammatory molecules in tumors and/or non-cancerous liver tissues. Inclusion of viral and inflammatory factors that are closely related to malignant phenotype and poor prognosis will make current scoring systems more accurate in predicting the prognosis of HBV-related HCC after surgical resection.

In summary, continuing inflammation caused by chronic HBV infection is not only involved in hepatocarcinogenesis, but also plays critical roles in the recurrence and metastasis of HCC after surgical treatment. Viral load, genotype/subgenotype, and a subset of viral mutations are major viral factors that may predict the occurrence of HCC. Inflammatory microenvironment may increase the frequency of viral mutation *via* induction of cellular cytidine deaminases, whereas HBV mutations selected by inflammation reaction might in turn promote hepatocarcinogenesis. Imbalance either between peritumoral Th1 and Th2 cytokines or between intratumoral CD8+ T cells and Treg cells contributes, at least partially, to inflammation-necrosis-regeneration response, ultimately HCC. Inflammatory pathways including NF- κ B, STAT3, Wnt/ β -catenin, and TGF- β 1 signaling pathways contribute to the development of HCC, and their hallmark molecules including IL-6, TNF- α and Wnt-1 can predict the occurrence or recurrence of this malignancy. HBV load, viral mutations and imbalance between infiltrating immune cells or between Th1 and Th2 cytokines can predict the prognosis of HBV-related HCC. These factors are of significance for developing active prevention and surveillance of the HBV-infected subjects who are more likely to develop HCC, or tailoring suitable treatment options for HBV-related HCC patients to improve the survival or postpone recurrence after surgical resection.

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S- Editor Tian L L- Editor Kerr C E- Editor Li JY

Dr. Rajvinder Singh, Series Editor

Advanced endoscopic imaging in Barrett's oesophagus: A review on current practice

Rajvinder Singh, SweeLin Chen Yi Mei, Sandeep Sethi

Rajvinder Singh, SweeLin Chen Yi Mei, Sandeep Sethi, Gastroenterology Unit, Department of Medicine, Lyell McEwin Hospital/University of Adelaide, Elizabeth Vale 5112 SA, Australia
Author contributions: Singh R, Chen Yi Mei S and Sethi S wrote and edited the paper.

Correspondence to: Dr. Rajvinder Singh, MBBS, MRCP, MPhil, FRACP, AM, FRCP, Consultant Gastroenterologist, Gastroenterology Unit, Department of Medicine, Lyell McEwin Hospital, Haydown Road, Elizabeth Vale 5112 SA, Australia. rajvindarsingh2003@yahoo.com

Telephone: +61-8-81829909 Fax: +61-8-81829837

Received: March 17, 2011 Revised: May 30, 2011

Accepted: June 6, 2011

Published online: October 14, 2011

Abstract

Over the last few years, improvements in endoscopic imaging technology have enabled identification of dysplasia and early cancer in Barrett's oesophagus. New techniques should exhibit high sensitivities and specificities and have good interobserver agreement. They should also be affordable and easily applicable to the community gastroenterologist. Ideally, these modalities must exhibit the capability of imaging wide areas in real time whilst enabling the endoscopist to specifically target abnormal areas. This review will specifically focus on some of the novel endoscopic imaging modalities currently available in routine practice which includes chromoendoscopy, autofluorescence imaging and narrow band imaging.

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Key words: Autofluorescence imaging; Barrett's oesophagus; Chromoendoscopy; High magnification endoscopy; Narrow band imaging

Peer reviewer: Ian D Wallace, MD, Shakespeare Specialist Group, 181 Shakespeare Rd, Milford, Auckland 1309, New Zealand

Singh R, Chen Yi Mei S, Sethi S. Advanced endoscopic imaging in Barrett's oesophagus: A review on current practice. *World J Gastroenterol* 2011; 17(38): 4271-4276 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4271.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4271>

CHROMOENDOSCOPY

Chromoendoscopy involves topical application of various dyes during endoscopy which improves the visualisation of mucosal surfaces. The stains can be divided into three main classes: contrast, absorptive and reactive. Contrast stains, for example indigo carmine (IC), accumulate in the mucosal fissures thereby accentuating surface topology. In contrast, absorptive stains such as Lugol's iodine (LI), crystal violet and methylene blue (MB) are absorbed into components of the cellular structure in the mucosa. Differences in the uptake of these stains can therefore be used to elucidate different types of mucosa. Reactive stains such as Congo red and Phenol red are pH-dependent. Congo red turns dark blue or black in acidic conditions, while phenol is yellow in an acidic environment and turns red in the presence of alkaline substances. These stains are, however, not used routinely in the oesophagus. There are two essential steps in chromoendoscopy - firstly, removal of mucous which is then followed by dye application. The former is achieved by using water, or occasionally some centres have advocated the use of a mucolytic agent; N-Acetylcysteine^[1,2]. This can be achieved by flushing the agent through the working channel, using a spray catheter or even administering it as an oral solution before the endoscopic procedure^[3]. Once the mucous is cleared, the dye can then be applied. The volume, concentration and the dye contact time varies considerably. Canto's group^[4,5] used 10-20 mL of 0.5% MB for every 5 cm of Barrett's mucosa, while Raganath *et al*^[6] used 4 mL of 0.5% MB for every 1 cm.

LUGOL'S IODINE

LI is a compound iodine solution that is absorbed by the glycogen containing squamous epithelium and stains it brown. The demarcation between squamous epithelium of the oesophagus and columnar epithelium of the stomach can therefore be clearly delineated. Damaged mucosa due to oesophagitis or malignant infiltration does not stain as well as normal mucosa. In BE, specialised intestinal metaplasia does not stain after application of LI. Woolf *et al*^[7] used LI to improve demarcation of squamous from columnar mucosa in patients with BE.

Toluidine blue

This is an absorptive stain taken up by the nuclei of columnar cells. Chobanian and colleagues used 1% toluidine blue to aid in the endoscopic detection of BE and reported improved sensitivity compared to standard endoscopy alone^[8]. The main limitation of this technique is that the dye stains all columnar cells, hence distinguishing between the intestinal and non intestinal epithelial subtypes of BE is not feasible especially since intestinal metaplasia can be patchy in BE.

Methylene blue

MB, an absorptive dye, is probably the most investigated stain for evaluation of BE and also the most controversial. It is a vital stain taken up by actively absorbing epithelial cells after topical application at a concentration of 0.5%-1.0%^[9,10]. The dye is absorbed by goblet cells present in specialised intestinal metaplastic epithelium. Initial work done by Canto's group revealed that MB can distinguish IM and dysplasia in BE with high precision. However, these results were not reproducible. Various other subsequent studies have revealed mixed findings. The main contention with MB in BE is that dysplastic areas do not stain, but the problem with that is that even areas which do not harbour IM do not absorb the dye. This makes it difficult for the endoscopist to decide on which areas to target the biopsies during the procedure. There were also some issues with the uniformity of the dye and recently even toxicity with MB. It has been examined in both long and short segment BE^[11,12,13]. Two patterns of staining have been documented - diffuse and focal. Canto *et al*^[5,11] found that most patients with long segment BE exhibited diffuse staining, whereas Wo *et al*^[14] observed focal staining in their cohort of patients with long segment BE. Similar discrepancies have been reported in short segment BE. Sharma *et al*^[13] found that the majority of their patients with short segment BE stained diffusely. In contrast, in 30 patients with short segment BE assessed by Kiesslich's group^[12], 80% demonstrated staining in a focal pattern.

The published data for biopsy related sensitivity of MB in detecting specialised intestinal metaplasia (SIM) vary considerably. Some studies reported high sensitivities ranging from 81% to 98%^[3,11,12], while others show markedly less favourable sensitivities ranging from 37% to 61%^[6,13-16]. The reasons for this variation in results are

not clear. Differences in stain concentration^[4,11,12] and the volume used^[4,6,14] may have influenced results. The biopsy protocol used in specific studies has also varied. Some investigators performed random 4 quadrant biopsies irrespective of the staining pattern^[4,6,15], while others obtained equal numbers of stained and unstained mucosa^[11] or biopsied stained mucosa only^[3]. Another possible explanation for the inconsistent published data is the discrepancy in operator skill and experience. Most procedures were performed by a single expert endoscopist in a "tertiary centre"^[4,5,11,15], hence the generalisability of the procedure itself has to be questioned. The role of MB in the detection of foci of dysplasia in BE is even more unclear. Early studies by Canto *et al*^[4,11] showed that dysplastic tissue did stain with MB, although histology revealed predominantly low grade dysplasia. Subsequent work suggested that lack of staining was more predictive of dysplasia, attributed to the loss of goblet cells with progression of dysplasia^[5]. A focal non-staining area in a sea of blue has been found to be highly predictive of dysplastic change. However, investigators continue to report dysplasia and carcinoma occurring in stained biopsies^[12,14]. The inter-observer variability in differentiating between shades of blue has not been determined, and the interpretation of deeply *vs* lightly stained mucosa is largely subjective. As a result of all these controversies and confusion, MB has hence not really gained widespread acceptance in the gastrointestinal fraternity.

A recent meta-analysis assessing the diagnostic yield of MB in detecting SIM and dysplasia in BE looked at 9 published studies that included 450 patients. Despite controlling for differences in technique and quality of published data, the meta-analysis showed no significant benefit of MB chromoendoscopy compared with random biopsies in detecting SIM, dysplasia or early oesophageal cancer^[17].

Crystal violet

Crystal violet has been used as an absorptive stain to evaluate colonic polyps since it is preferentially taken up by the crypts of Lieberkuhn^[18,19]. Its role in the assessment of BE is less clear. A case report using a combination of crystal violet and Methylene blue has been described to be useful for the detection of a minute focus of adenocarcinoma in BE^[20]. These investigators found that 0.05% crystal violet directly dyes the surface of BE, thereby enhancing MB stained mucosa.

Indigo carmine

This dye is not absorbed when applied topically to the mucosa. Instead it augments mucosal details and is therefore used as a contrast stain to delineate irregularities of the mucosal surface. Since it provides a clearer definition of the mucosal pattern in BE, evaluation of IC is best considered in conjunction with magnification endoscopy. Sharma *et al*^[21] showed that IC magnification endoscopy may improve mucosal imaging and the detection of dysplasia in BE. However, Kara *et al*^[22] showed that when a

high resolution endoscope is used, the adjunctive use of IC chromoendoscopy is of limited use for the primary detection of lesions.

High resolution magnification endoscopy

Standard video endoscopes are tailored to view the mucosa from a focal distance of 1-2 cm from the endoscope tip. With a pixel density of 200 000, detailed inspection is limited especially if the tip of the scope is advanced closer to the area of interest. The focused area tends to exhibit a blurred view. Coupled with low resolution monitors, the quality of images obtained in real time can be compromised. As technology improves, the pixel density and resolution of monitors has increased tremendously, and this has resulted in improved image quality with high resolution (> 850 000 pixel density) and high magnification (115X) systems. This phenomenon is especially crucial in BE surveillance as early, subtle lesions harbouring dysplasia or cancer should not be missed.

Recent advancements in endoscopic technology have produced high magnification endoscopes with electronically moveable lenses which allow real time visualisation of mucosal morphology in greater detail. Magnification enlarges the endoscopic image, while better resolution improves the ability to discriminate detail by enabling two closely approximated points to be better appreciated. The clinical utility of this modality had been limited by the size of the endoscope in the past. However, improvement in the design of the charged-couple device, an electronic light sensing apparatus located at the tip of the endoscope, has given rise to less bulky and more manageable instruments. High resolution magnification endoscopy (HRME) has been evaluated in coeliac disease where it was found to be valuable in assessing the degree of villous atrophy^[23]. Inoue's group used HRME to characterise the blood vessel morphology, hence facilitate the diagnosis of superficial oesophageal cancer^[24]. The morphology of intrapapillary loops became progressively more tortuous and disorganised with the evolution of dysplasia to cancer. HRME has also been assessed in the stomach, where the authors have shown that it can reliably identify normal gastric mucosa, *Helicobacter pylori*-associated gastritis and gastric atrophy^[25]. In the colon, magnification endoscopy has been used to assess colonic polyps^[26,27] and colon cancer^[28,29].

Magnification endoscopy has been proposed as a diagnostic tool to improve the sensitivity of standard endoscopy in the detection of specialised intestinal metaplasia and dysplasia. Stevens *et al*^[30] used IC as a contrast stain to assess BE using magnification endoscopy and noted a villiform appearance correlated with the histological finding of specialised intestinal metaplasia. Endo and colleagues^[31] characterised the pit pattern of BE using magnification endoscopy and MB staining and found that specialised intestinal metaplasia was detected in patients who exhibited a tubular/villous pattern in their BE segment. Similarly, Sharma's group found that 97% of their cohort of patients with a ridged/villous pattern on magnification chromoendoscopy using IC had specialised intestinal

metaplasia, and 100% with an irregular and distorted pattern exhibited high grade dysplasia^[21]. Fortun and colleagues reported that enhanced magnification endoscopy with acetic acid (Figure 1A) allows clear visualisation of the epithelial pit patterns within BE, and targeted biopsy resulted in a high yield of specialised intestinal metaplasia and dysplasia^[32]. However, despite the increasing availability of high resolution magnification endoscopes, there is a lack of diagnostic criteria for magnified endoscopic images.

Autofluorescence imaging

When tissues are exposed to short wave length light, endogenous biological substances (i.e., fluorophores) are excited, leading to emission of fluorescent light of a longer wavelength. This phenomenon is known as autofluorescence^[33]. Autofluorescence imaging (AFI) is a technique that can potentially differentiate tissue types based on their differences in fluorescence emission. Normal and neoplastic tissue have different autofluorescence spectra which may enable their distinction. This is due to the various different compositions of the endogenous fluorophores which includes collagen, NADH, aromatic amino acids and porphyrins in these tissues. Until recently, AFI has been restricted to either autofluorescence spectroscopy or autofluorescence endoscopy using the older generation fibre optic endoscopes^[34-36]. The main limitation of AFI using this modality is that the quality of the images produced was inferior. Recently, video AFI which incorporates high resolution endoscopy has been evaluated^[37]. In an uncontrolled feasibility study, AFI led to the detection of a significant number of patients with high grade dysplasia/early cancer in Barrett's oesophagus (BE). There was, however, a very high false positive rate (51%) using this modality.

Narrow band imaging

The quest for a simpler technique which would obviate the complexity of chromoendoscopy led to the development of narrow band imaging (NBI) (Figure 1B-G: HRME and corresponding images on NBI). Termed "electronic chromoendoscopy" by some quarters, this unique technology was first described by Gono *et al*^[38]. Standard white light endoscopy consists of 3 light waves: blue, green and red. The principles behind NBI technology are that the bandwidths of blue (440-460 nm) and green (540-560 nm) wave light are narrowed whilst the contribution of red wave light is totally negated out of the emitted light. This is achieved by a special filter which is electronically activated once the endoscopist presses a switch on the endoscope. The whole process takes less than 1 s and is practical during any endoscopy procedure provided the system is equipped with NBI. The narrowed bandwidths of green and blue light lead to superficial penetration of the mucosa accentuating the microvasculature pattern as haemoglobin has a peak absorption spectrum towards both these wave lengths. The quality of the surface pit pattern morphology is also clearly enhanced by this technology. It enables the endoscopist to

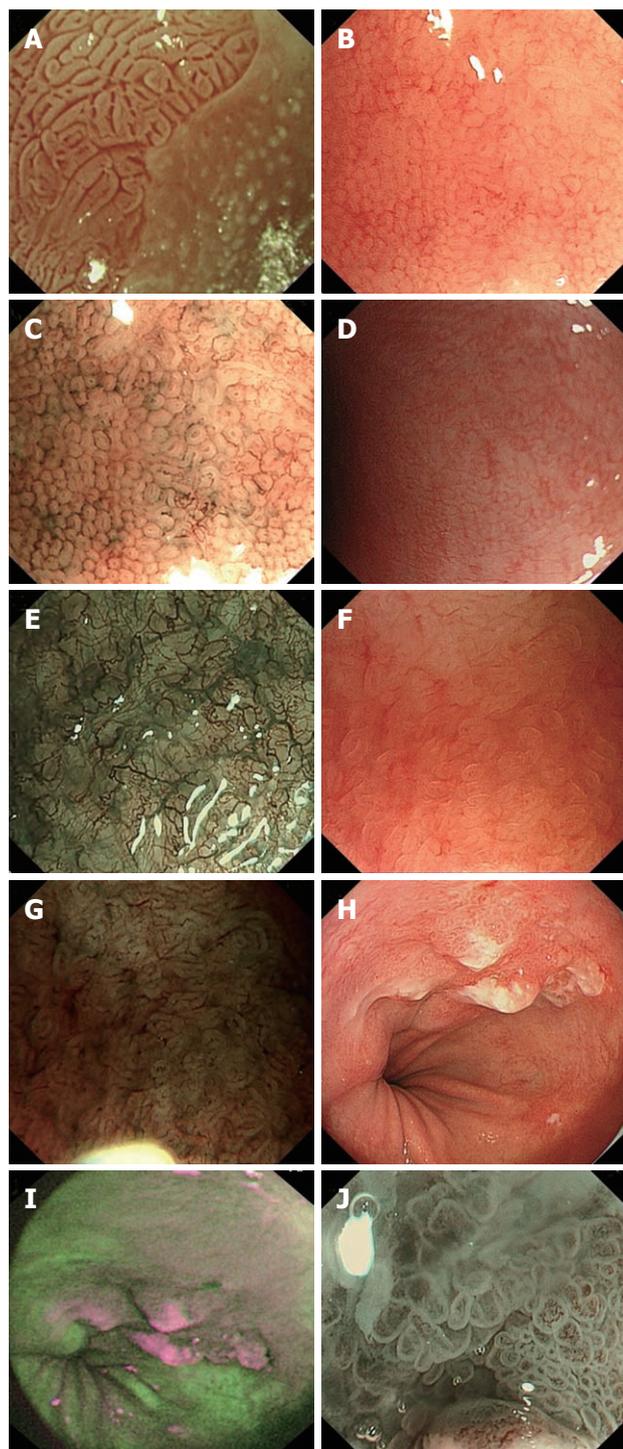


Figure 1 Images of various advanced imaging modalities in Barrett's oesophagus. A: Acetic acid used to visualise Barrett's oesophagus, ridge pattern signifying Intestinal metaplasia; B: High magnification white light endoscopy-round pits in keeping with columnar mucosa without intestinal metaplasia; C: Corresponding area on image B seen with narrow band imaging (NBI) and magnification; D: High magnification white light endoscopy - absent pits in keeping with columnar mucosa with intestinal metaplasia; E: Corresponding area on image D seen with NBI and magnification; F: High magnification white light endoscopy - villous/ridge pits in keeping with columnar mucosa with intestinal metaplasia; G: Corresponding area on image F seen with NBI and magnification; H: White light endoscopy of Barrett's cancer; I: Corresponding area on autofluorescence imaging; J: Abnormal area on NBI with magnification showing total distortion of the pit pattern.

switch between conventional white light and NBI views easily and quickly during the procedure, thus making the procedure itself less messy and cumbersome compared to chromoendoscopy. By depressing a lever on the endoscope, the focal distance of the lens at the tip of the endoscope can be adjusted electronically thus enabling the endoscopist to achieve a maximal magnification of 115X in real time. NBI has been evaluated in BE with very promising results^[39-43]. A recent meta-analysis of 8 published studies which included 446 patients with 2194 lesions showed that NBI-Z has high diagnostic precision in detecting high grade dysplasia with a sensitivity and specificity of 96% and 94%, respectively^[44]. However, the results of NBI-Z in characterising SIM were inferior with a sensitivity of 95% and a specificity of 65%.

Trimodal imaging

With various new technologies available, it was inevitable that combining them into a single system was the next step forward, hence the introduction of the novel concept of trimodal imaging. This modality incorporates three advanced endoscopy imaging techniques into a single endoscope: HRME (Figure 1H), AFI (Figure 1I) and NBI (Figure 1J), thereby enabling the endoscopist to use all 3 modalities during a single procedure. Promising early results have been reported in a multicentre feasibility study^[45] and more recently in a multicentre randomised cross-over study^[46].

CONCLUSION

Although chromoendoscopy has been available for more than 20 years, the lack of standardisation of the technique is one major reason for the indifference towards it. The dearth of well controlled studies that determine its clinical utility, cost efficacy, patient acceptance and tolerability in terms of the additional time needed are amongst the other reasons why chromoendoscopy has not truly caught on. With the rapid development of various novel technologies, it seems that the ideal endoscopy system could very well be on the horizon. It would incorporate a "red flag" technique similar to the AFI system but with hopefully a lower rate of false positives followed on by further detailed interrogation of the suspicious area detected by the technique with either NBI or a confocal probe to obtain "optical biopsies". This may enable the endoscopist to ascertain the histopathological diagnosis in real time. There are, however, numerous issues which would need to be overcome. Standardisation of the various classification systems as well as incorporation of all these techniques into a single easily managed, less bulky unit which is financially viable and less time consuming could eventually lead to widespread availability of a technique in the community.

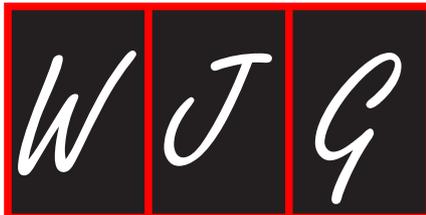
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S- Editor Tian L L- Editor Webster JR E- Editor Xiong L



Functional imaging and endoscopy

Jian-Guo Zhang, Hai-Feng Liu

Jian-Guo Zhang, Hai-Feng Liu, Department of Gastroenterology, General Hospital of Chinese Armed Police Forces, Beijing 100039, China

Author contributions: Zhang JG and Liu HF contributed equally to this paper; Zhang JG wrote the paper and Liu HF revised the paper.

Correspondence to: Hai-Feng Liu, MD, Professor of Medicine, Chief, Department of Gastroenterology, General Hospital of Chinese Armed Police Forces, 69 Yongding Road, Haidian District, Beijing 100039, China. haifengliu333@163.com

Telephone: +86-10-57976547 Fax: +86-10-57976549

Received: March 28, 2011 Revised: May 20, 2011

Accepted: May 27, 2011

Published online: October 14, 2011

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Key words: Endoscopy; Functional imaging; Multi-modal imaging; Optical coherence tomography; Fluorescence molecular imaging; Photoacoustic tomography; Cerenkov luminescence tomography

Peer reviewer: Jae J Kim, MD, PhD, Associate Professor, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

Zhang JG, Liu HF. Functional imaging and endoscopy. *World J Gastroenterol* 2011; 17(38): 4277-4282 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4277.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4277>

Abstract

The emergence of endoscopy for the diagnosis of gastrointestinal diseases and the treatment of gastrointestinal diseases has brought great changes. The mere observation of anatomy with the imaging mode using modern endoscopy has played a significant role in this regard. However, increasing numbers of endoscopies have exposed additional deficiencies and defects such as anatomically similar diseases. Endoscopy can be used to examine lesions that are difficult to identify and diagnose. Early disease detection requires that substantive changes in biological function should be observed, but in the absence of marked morphological changes, endoscopic detection and diagnosis are difficult. Disease detection requires not only anatomic but also functional imaging to achieve a comprehensive interpretation and understanding. Therefore, we must ask if endoscopic examination can be integrated with both anatomic imaging and functional imaging. In recent years, as molecular biology and medical imaging technology have further developed, more functional imaging methods have emerged. This paper is a review of the literature related to endoscopic optical imaging methods in the hopes of initiating integration of functional imaging and anatomical imaging to yield a new and more effective type of endoscopy.

INTRODUCTION

Traditional endoscopic imaging of anatomical lesions has mainly been used for disease diagnosis. This imaging modality for diagnosing gastrointestinal diseases has been very successful, but the use of only anatomic imaging as an endoscopic imaging modality has exposed a number of shortcomings. Many diseases show not only anatomical abnormalities, but also dysfunction, which can be difficult to diagnose based solely on anatomical observations and differential diagnosis. Thus, it seems important to combine endoscopy with anatomical and functional imaging. In recent years, as molecular biology and medical imaging have rapidly developed, more functional imaging methods have emerged and may be the future of endoscopy. This paper will focus on the latest imaging methods, especially those closely related to endoscopic methods for optical imaging. We hope a combination of functional imaging and anatomical imaging will be developed for endoscopy.

OPTICAL COHERENCE TOMOGRAPHY

In 1990, the Austrian scientist Fercher first reported the

use of low-time coherent optical interferometric techniques (low time_coherence interferometry) to observe the topology of the human retina^[1]. The following year, Huang *et al.*^[1] at the Massachusetts Institute of Technology used optical coherence tomography (OCT) technology to image microstructure of the coronary artery. OCT technology has since developed rapidly^[2,3]. OCT technology as a low-coherence interferometer has been used together with confocal scanning microscopy and heterodyne detection techniques to non-invasively obtain internal information on living structures and physiological functions. The imaging depth is in the millimeter range, and the spatial resolution is in the micrometer range. Thus, OCT technology has quickly become a focus in biomedical imaging research. It is now considered a promising, non-destructive, high-resolution, real-time imaging technique and is the next most promising technique for optical imaging^[4,5].

OCT technology provides micrometer-scale images of opaque or translucent tissue superficial cross-sectional imaging. OCT imaging and ultrasound imaging are similar, except that OCT uses near-infrared light instead of ultrasound, and is aptly called “light ultrasound imaging”^[2]. OCT is generally composed of five parameters: the light source, beam splitter, reference mirror, detector and image sample. First, given the low temporal coherence light source, exposure to the beam splitter causes part of the sample to be exposed to light through the spectroscope. Another portion of light is reflected to the reference beam splitter mirror and generates a Doppler shift effect. Then, the beam from the reference mirror and different depths of the sample are reflected back together and are received by the detector. When the optical path between the two beams is less than the low-coherent light coherence length of time, it will produce more obvious interference, which is called the “coherence gate”. With the use of coherent gate technology, OCT can differentiate sample depths due to separation of the reflected light to reveal structural information and thus the direction of imaging^[6].

Currently, OCT technology has developed into a new, cutting-edge diagnostic technique and plays an important role in examining the eye, heart, gastrointestinal tract and skin and in diagnosing cancer and other diseases. In 2005, Evans *et al.*^[7] described technical monitoring and diagnosis using OCT to examine Barrett’s esophagus and reported that this technology can be used to view a certain depth of the digestive tract with cross-sectional imaging and can reliably identify high-level changes and intestinal tumors of Barrett’s esophagus. In 2010, Woitkowski^[8] reported basic and applied research reports describing high-speed OCT imaging. OCT technology has the potential to distinguish between metabolism and function to achieve functional imaging^[9-13]. In the same year, Fercher^[4] published reports showing that endoscopic OCT technology can not overcome the traditional shortcomings of the depth of imaging. However it is very good for examining the mu-

cosa, lamina propria, mucosal primary and submucosa and can accurately assess the esophagus, stomach, duodenum, pancreas and bile duct, and diagnose colorectal and other diseases, especially atypical hyperplasia, intestinal metaplasia, Barrett’s esophagus and pancreatic duct diseases. In 2011, Srinivasan *et al.*^[14] reported the use of OCT technology to examine cerebral blood flow and removed the quantitative determination of hydrogen ions. This study showed the huge potential of using cerebral vascular imaging to examine physiological functions, thus confirming OCT technology as a non-invasive method for quantitative determination of cerebral blood flow and metabolism.

Currently, OCT technology is carried out first for digestive diseases and reports of functional imaging studies are increasing. Given the non-invasive, high resolution, multi-level, real-time imaging and functional imaging features, as well as many other advantages, OCT technology will likely play an increasingly important role in the diagnosis of gastrointestinal diseases

FLUORESCENCE MOLECULAR IMAGING

Fluorescence molecular imaging (FMI) is an important branch of optical molecular imaging. FMI is non-invasive, uses non-ionizing radiation, has high resolution and sensitivity, is quick, easy and inexpensive, has relatively high access, and has many other advantages, which have developed rapidly in recent years^[15]. The 2008 Nobel Prize in Chemistry was awarded for discovering uses for green fluorescent protein, which is widely used in the scientific community. Green fluorescent protein is a molecular probe in FMI technology, and the clinical application of imaging methods has great potential in the field of optical imaging.

Molecules in different states and at different energy levels absorb photons of different wavelengths. Molecular absorption of light involves upward transitions from ground state molecules to the excited state, called the excitation light. When molecules are excited, they transition from the excited state to the ground state and emit light. When a molecule absorbs a photon of energy and transitions from one electronic state to another low-energy electronic state, the luminescence is called fluorescence^[15]. In short, the production of fluorescent molecular probes involves the process of absorbing fluorescent energy to the excited state after the transition, which occurs after a short stay and returns to the ground state emitting fluorescence^[16]. According to different fluorescent substances, FMI can be divided into two broad categories: direct fluorescence imaging and indirect fluorescence imaging. In the direct fluorescence imaging mode, a fluorescent substance is injected. Exogenous dyes or fluorescent probes then target specific molecules. For example, such probes are currently used for fluorescence imaging of human breast tissue. In the indirect fluorescence imaging mode, the fluorescent material is fluorescent protein. In this imaging mode,

no fluorescent substances are injected to find the target. Due to the need for genetic modification for indirect fluorescence imaging, this technology can not be applied to the human body. Currently, FMI technology can be divided into a two-dimensional technique and three-dimensional space-oriented fluorescence molecular tomography (FMT) technology^[13-18]. Charge-coupled device (CCD) cameras are used in the two-dimensional FMI system by directly inducing the tissue imaging surface to fluoresce. The fluorescent image is added to the white image, which shows the general distribution of fluorescence in the body. However, because of high scattering in biological tissue, fluorescence images obtained this way do not accurately reflect the organization or spatial distribution of fluorescent material. Furthermore, FMI is difficult to use for quantitative analysis, and therefore, its application in some studies is limited. Three-dimensional FMT utilizes optical imaging to analyze absorption and scattering in the sample and the receiving surface of the light intensity. Mathematical methods are used to reconstruct the distribution in body tissue and the concentration of fluorescent material. Thus, three-dimensional FMT provides relatively accurate quantitative analysis.

In 2007, Montet^[19] used FMT in experimental animals to examine tumor blood vessels and thus demonstrated the success of functional imaging. In the same year, Corlu *et al.*^[20] reported the use of FMT technology in human breast cancer and showed clear imaging. Using indocyanine green (ICG) as a fluorescent dye and magnetic resonance imaging, diffuse optical tomography images were compared showing the accuracy of FMT imaging, the optical FMT image and the high contrast ratio of diffuse optical tomography^[20]. In 2008, Willmann^[21] and others used FMI imaging technology in the field of drug discovery. Currently, reports of the use of FMI technology for human body imaging are few, partly because only ICG is approved for use in humans and because fluorescence spreads a short distance, limiting its application in the human body. AS FMI technology continues to improve, its application will be further expanded.

PHOTOACOUSTIC TOMOGRAPHY

As early as 1880, workers at Bell Labs discovered the photoacoustic phenomenon. Over the last century, combinations of the photoacoustic effect, modern laser technology and weak signal monitoring technology have developed rapidly. In the 1970s, the photoacoustic effect was used to develop photoacoustic spectroscopy. In the 1980s, photoacoustic imaging of biological tissues was introduced. Currently, photoacoustic tomography (PAT) technology represents a new generation of biomedical imaging technology. Combined with the optical advantages of imaging and ultrasound imaging, PAT can provide high resolution and high contrast imaging and can provide structural and functional imaging of biological tissues to study tissue morphology, physiological characteristics, pathological characteristics and metabolic

functions^[22-24].

Beam irradiation occurs with a varying absorber that cause thermal expansion of ultrasound, a phenomenon known as the photoacoustic effect, which is the "light" produced by the ultrasonic acoustic signal^[25]. PAT imaging involves a beam of pulsed light that shines on a sample. Multiple ultrasonic detectors detect the light emitted by the acoustic signal, and then mathematical methods are used to reconstruct the photoacoustic signals to produce a three-dimensional image. An advantage of traditional optical imaging is that the image is better. However, a significant limitation involves the depth and spatial resolution. Thus, the light diffusion caused by the strong high spatial resolution is accompanied by a sharp drop in the imaging depth, and vice versa. Ultrasound imaging increases the depth of tissue that can be examined and has the advantages of larger, higher spatial resolution, but has the disadvantage of an image with poor contrast between the different types of tissues. PAT is a hybrid type of imaging technology, which combines optical imaging and ultrasound imaging with the advantages of both, utilizing the absorption properties of biological tissues to obtain an image with higher image contrast and higher resolution^[26].

For early diagnosis of disease, PAT light absorption for tissue imaging, and the optical absorption properties to examine biological tissues, tissue function and pathological features of a structure are closely related to differences in the parameters of optical imaging. In recent years, research on the application of PAT imaging has increased. Oraevsky *et al.*^[27] used PAT technology to examine hamster buccal squamous cell carcinoma at different stages of capsule imaging, using a wavelength of 532 nm and 12 ns of YAG (Yttrium aluminum garnet) pulsed laser excitation to clearly show photoacoustic images of pre-cancerous tissue. Wang *et al.*^[28] used three-dimensional PAT to show clear images of rat brain structures such as blood vessels, cerebellum and hippocampal processes. Further photoacoustic images of optical information reflected in the quantitative analysis and calibration were obtained with a photoacoustic signal corresponding to physiological parameters to achieve functional imaging of the rat brain. Esenaliev *et al.*^[29] performed a photoacoustic imaging study of brain structure and blood vessel dynamics in the brain by monitoring dynamic changes in cerebral blood oxygenation. Ku *et al.*^[30] used PAT to image blood vessels, to more clearly distinguish the location of a tumor. Several groups took advantage of the nature of differences in absorption and PAT to image tumor tissue and surrounding normal tissue for early diagnosis of breast cancer, showing that this technology can be combined with traditional techniques such as X-radiography and breast ultrasound imaging to produce high contrast, high resolution, non-ionizing images^[31-33]. Li *et al.*^[34] reported a molecular probe that was used as a PAT contrast agent, to show that the absorption spectra of hemoglobin are different in specific molecules in biological tissue. After calibra-

tion, an imaging experiment with multi-wavelength PAT and mathematical modeling of the contribution of the molecular probe to the optical image was used to subtract the background to achieve specific photoacoustic imaging.

Currently, despite high-resolution three-dimensional optical imaging modes, including confocal microscopy and two-photon microscopy, OCT has become fundamentally embedded in bio-medical research. However, these imaging methods cannot image deeper tissues. Photoacoustic imaging in the same signal mode, combined with a powerful optical joint ultrasound contrast and resolution, results in exceeding previous depth limits, resulting in deep tissue high-resolution optical images. At the same time, use of this technology can provide functional imaging, including analysis of oxygen use, blood flow, tumor blood vessels, and many other functions. In the future, photoacoustic imaging is expected to become the mainstream optical imaging mode and should result in development of this technology for endoscopic examination^[35].

CERENKOV LUMINESCENCE TOMOGRAPHY

Cerenkov luminescence Tomography (CLT) technology has progressed from the emergence of modern physics and detectors resulting in technological progress. In 1901, Kelvin proposed that the speed of particle radiation may exceed the speed of light^[36]. In 1933, Soviet scientist Vavilov Cerenkov used photometric technology in guided research and accidentally discovered faint blue fluorescence^[37]. In 1934, Cerenkov and colleagues confirmed that this faint blue Cerenkov radiation was fluorescence and was a new physical phenomenon. A charged particle moves in medium faster than the speed of light and emits electromagnetic radiation, known as Cerenkov radiation. In 1958 Cerenkov, Frank and Tamm won the Nobel Prize in Physics for this discovery. Since then, research involving Cerenkov radiation has been widely performed.

CLT technology is based on the principles of Cerenkov radiation physics. Small amounts of high-speed charged particles are emitted following *in vivo* injection of radioactive molecular probes. The use of low-light imaging devices in the body provides non-invasive detection of fluorescent molecular probes due to the release of Cerenkov signals. These signals are detected with a computer, which is used for data processing to produce Cerenkov luminescence imaging (CLI). Recently, *in vivo* molecular imaging probes and systems technology have been developed for CLT. ¹⁸F-FDG is a molecular probe in nuclear medicine and has played an increasingly important role in the development of low-light CCD imaging in both basic and clinical research.

In 2009, Cho *et al.*^[38] used the blue spectrum, its highly sensitive quantum effects and a dominant photomultiplier tube to detect a microchip with weak ¹⁸F-FDG-

induced fluorescence. Robertson *et al.*^[39] used a highly sensitive CCD and semiconductor cooling to detect *in vivo*-induced weak fluorescence in animals. Spinelli *et al.*^[40] used a multi-spectrum fluorescent light source in the body to successfully obtain the deep information. The essence of these experiments is the Cerenkov effect. CLT technologies employ commonly available optical detectors to observe the release of high-speed charged particles with the high sensitivity of radionuclide isotope imaging. These probes that are used in such studies in molecular nuclear medicine and functional imaging are new tools. In 2010, at the Sloan-Kettering Cancer Center in the United States Ruggiero *et al.*^[41] suggested that neither the CLI value nor positron emission is adopted to achieve gamma-ray radionuclide imaging, which can be achieved with radioactive tracers. CLI optical imaging technology is a potential new imaging mode because it can be used for quantitative assessment of exposure. In 2011, Boschi *et al.*^[42] used Cerenkov radiation in small animals *in vivo* to measure ¹⁸F-FDG uptake in tumors. This experiment showed the feasibility of using a traditional optical imaging device to study the metabolism of tumor tissue *in vivo* and that ¹⁸F-FDG PET and conventional optical imaging could be used as a dual-mode device.

Although CLT technology is still in the exploratory stage and has not been used for functional imaging in humans, functional imaging experiments in animals have shown excellent potential. In the future, the use of CLT technology combined with traditional endoscopic techniques for functional imaging may be meaningful.

ANALYSIS AND PERSPECTIVES

Endoscopy was invented 100 years ago, and has gone from hard to soft endoscopy, from endoscopy to the electronic endoscope, from ordinary white light endoscopy to new types of endoscopy, such as magnifying endoscopy, FICE endoscopy, NBI endoscopy, i-scan endoscopy, fluorescence endoscopy, confocal endoscopy, *etc.* and today's doctors are almost overwhelmed by the different types of endoscope. However, looking at the history of endoscopy over the last hundred years highlights both changing and unchanging eternal themes. One change is that high magnification endoscopy is gradually moving towards the micro-microscopic world, and endoscopy continues to develop with the unchanging goal of observing anatomic morphology. Diagnosis of gastrointestinal disease has greatly improved with endoscopy over the past 45 years. During this time, we have discovered approximately 200 types of gastrointestinal diseases. Abandoning endoscopy will be almost impossible. Therefore, the past 45 years have been a brilliant era in endoscopy^[43].

However, endoscopic diagnosis at this stage is also facing many challenges including the following: (1) early diagnosis of digestive tract cancer, because endoscopic intervention has not significantly increased; (2) the deep

mucosa and submucosa and lesions of the mucous membrane are difficult to imaging and assess; (3) microvascular imaging of the mucosa and submucosa is difficult to assess; and (4) anatomic lesions with similar endoscopic images are difficult to distinguish, etc. For these problems, the existing endoscopic imaging of morphology as the only mode has deficiencies and shortcomings. Using only gastrointestinal endoscopy for early cancer diagnosis, for example, may require introduction of new endoscopic techniques. Many experts have attempted to develop advanced endoscopic procedures to find cancer earlier despite the economic concerns and the fact that awareness of the public concerning their health has significantly improved. Today, more and more people undergo endoscopy, even though all the external conditions are favorable for developing endoscopic techniques for early diagnosis of cancer. The rate of early diagnosis of gastric cancer in China still hovers around 10%. Little has changed over the last 10 years, even with a focus on early endoscopic diagnosis of carcinoma in the top domestic endoscopy center. Similar results are seen in most of the rest of the world. Indeed, early diagnosis of cancer is a very complex issue, and in addition to endoscopy, there are many other factors. However, other factors aside, what role does endoscopy play in the early diagnosis of digestive tract cancer? Can endoscopic morphology be used solely to identify early cancer? In addition to anatomical observation, can functional endoscopic imaging also be used? Although endoscopic diagnosis of only early cancer has been discussed, other diseases, including Crohn's disease, intestinal tuberculosis, and other gastrointestinal diseases, can be diagnosed relying on existing morphology-based imaging. Endoscopic identification and diagnosis will be very difficult and challenging. Changes to the existing single anatomic endoscopic imaging modality are necessary and may include the integration of a functional imaging mode. The new Multimode endoscopy with functional imaging and anatomical imaging integration may be an effective way to solve these problems. These changes will allow earlier examination of morphological changes. In addition, functional imaging of the organism, metabolism, blood flow, and many other biological parameters offer a more comprehensive interpretation of lesions. Combined with anatomical imaging, functional imaging may permit a view of shape and function of living tissue. Changing the present single form of endoscopic morphology to include functional imaging will be a revolutionary change in modern endoscopy.

Currently, multi-modal fusion of modern medical imaging technology has become a major technology trend^[44]. Positron Emission Computed Tomography (PET-CT) is an example of this idea. Recently, multi-modal integration of new imaging technologies has emerged, such as development of Optical PET (O-PET) detectors at the University of California, Los Angeles by Prout *et al*^[45]. O-PET can detect spontaneous and gamma-ray fluorescence signals, enabling optical signals and

the integration of PET imaging. Undoubtedly, future research and development of endoscopic techniques provides a new way of thinking. A critical moment for the future of endoscopy has occurred. Should anatomical imaging continue or should it conform to multi-modal fusion imaging trends with the integration of a bolder change? Careful consideration is required. In our opinion, the future should involve functional imaging, anatomical imaging, two-dimensional imaging, and three-dimensional imaging combined with a variety of newly integrated imaging and endoscopic technologies.

CONCLUSION

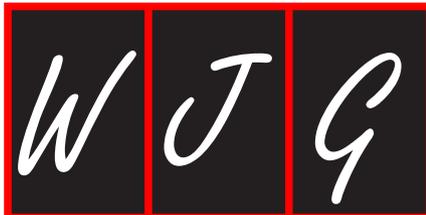
The basic anatomical observation available with existing endoscopy is a brilliant achievement, but it has also exposed many shortcomings. Development of more powerful endoscopic techniques in the future is an important issue. New optical imaging technology may soon be available for us to learn from. The future involves actively developing a set of functional imaging and anatomical imaging techniques which result in a multi-modal fusion of endoscopic techniques.

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S- Editor Tian L L- Editor O'Neill M E- Editor Zhang DN



Heme oxygenase-1 system and gastrointestinal inflammation: A short review

Xiao Zhu, Wen-Guo Fan, Dong-Pei Li, Hsiangfu Kung, Marie CM Lin

Xiao Zhu, Cancer Institute, Affiliated Tumor Hospital, Guangzhou Medical University, Guangzhou 510095, Guangdong Province, China

Xiao Zhu, Institute of Biochemistry and Molecular Biology, Guangdong Medical College, Dongguan 523808, Guangdong Province, China

Wen-Guo Fan, Guanghua School of Stomatology, Sun Yat-sen University, Guangzhou 510080, Guangdong Province, China

Dong-Pei Li, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, Guangdong Province, China

Hsiangfu Kung, Li Ka Shing Institute of Medical Sciences, the Chinese University of Hong Kong, Shatin, Hong Kong, China

Marie CM Lin, Department of Surgery, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, China

Author contributions: Zhu X and Fan WG contributed equally to this work; Zhu X, Fan WG and Li DP reviewed the literature and prepared the manuscript; Fan WG drafted the figure; Lin MCM and Kung H reviewed the final version of the paper.

Supported by National Natural Science Foundation of China, No. 81071697; Research Project of Health Bureau of Guangzhou City, No. 201102A213005 and 2010A30; Research Project of Education Bureau of Guangzhou City, No. 10A192

Correspondence to: Xiao Zhu, PhD, Cancer Institute, Affiliated Tumor Hospital, Guangzhou Medical University, Guangzhou 510095, Guangdong Province, China. biozhu@yahoo.com
Telephone: +86-20-83595032 Fax: +86-20-83591360

Received: February 9, 2011 Revised: March 21, 2011

Accepted: March 28, 2011

Published online: October 14, 2011

Abstract

Heme oxygenase-1 (HO-1) system catalyzes heme to biologically active products: carbon monoxide, biliverdin/bilirubin and free iron. It is involved in maintaining cellular homeostasis and many physiological and pathophysiological processes. A growing body of evidence indicates that HO-1 activation may play an important protective role in acute and chronic inflammation of gastrointestinal tract. This review focuses on the current understanding of the physiological significance of HO-1 induction and its possible roles in

the gastrointestinal inflammation studied to date. The ability to upregulate HO-1 by pharmacological means or using gene therapy may offer therapeutic strategies for gastrointestinal inflammation in the future.

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Key words: Heme oxygenase-1; Gastrointestinal inflammation

Peer reviewer: Chi Hin Cho, PhD, Chair Professor, School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China

Zhu X, Fan WG, Li DP, Kung H, Lin MCM. Heme oxygenase-1 system and gastrointestinal inflammation: A short review. *World J Gastroenterol* 2011; 17(38): 4283-4288 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4283.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4283>

INTRODUCTION

Heme oxygenase (HO) is the rate-limiting enzyme in heme catabolism, a process which leads to the generation of equimolar quantities of carbon monoxide (CO), Fe²⁺ and biliverdin. Three distinct HO isoforms (HO-1, HO-2 and HO-3) have been identified to date, which are the products of different genes. HO-2 is constitutively and most highly expressed in neuronal tissues contributing to cell homeostasis, whereas HO-1, also referred to as heat shock protein-32 (Hsp32), is an inducible enzyme and expressed at a relatively low level in most tissues^[1]. HO-3 has been found only in the rat brain, but no activity in humans^[2].

Unlike the constitutively expressed HO-2, HO-1 is exquisitely sensitive, not only to heavy metals^[3], but also to all kinds of stimuli and agents that cause oxidative stress and pathological conditions. Induction of the HO-1 protein has been reported to protect against a variety of stress conditions such as ischemia^[4], hemorrhagic shock^[5], heat

shock^[6], hypoxia^[7], and reactive oxygen species (ROS)^[8].

In fact, there has been no other enzyme described to date that is affected by so many stimuli of diverse nature as HO-1^[1]. The strong adaptive response of HO-1 to various stimuli suggests that pharmacologic modulation of HO-1 system may represent an effective and cooperative strategy to intervene in protection against inflammatory processes and oxidative tissue injury. HO-1 is expressed constitutively in normal gastric, intestinal and colonic mucosa^[9,10] and up-regulated in their inflamed tissues^[10]. What implications of HO-1 are in gastrointestinal inflammation and injury? In this review, we focus on this subject, and elucidated the mechanisms and some potential clinical applications to gastrointestinal inflammation.

UPREGULATION OF HO-1 IN GASTROINTESTINAL TRACT

Interestingly, expression of HO-1 is usually increased in gastrointestinal inflammation and injury. This was shown in gastric ulcers^[11], colitis^[12,13], radiation enteritis^[14], inflammatory bowel disease (IBD)^[15] of animal models or patients. Moreover, HO-1 is expressed constitutively in normal gastrointestinal tract (GIT)^[9,10].

The GIT is lined by a simple epithelium that separates the hostile processes of digestion and absorption that occur in the intestinal lumen from the aseptic environment of the internal milieu by defensive mechanisms.^[16] GIT undergoes constant oxidative stress, inflammation and cell cycle/apoptosis. The normal expression and up-regulation of HO-1 indicate that activation of HO-1 could act as a natural defensive mechanism to alleviate inflammation and tissue injury in the GIT^[13,17,18].

ROLE OF HO-1 IN GASTROINTESTINAL INFLAMMATION AND INJURY

HO-1 is commonly regarded as a potent anti-inflammatory enzyme and has anti-inflammatory properties. For example, HO-1 upregulated by hemin^[19], heme^[20] and cobalt-protoporphyrin^[21] can ameliorate experimental colitis. Conversely, administration with HO inhibitor (tin mesoporphyrin, SnMP) results in exacerbation of experimental colitis along with a reduction in HO-1 activity^[12].

In addition, the mechanism of action of 5-aminosalicylic acid (5-ASA, an anti-colitis agent used clinically) is attributed in part to the up-regulation of HO-1 enzyme expression and activity^[22]. Moreover, some agents including glutamine^[9,23], tranilast^[24], RDP58^[25], Octreotide^[26,27], lansoprazole^[28-30], Ketamine^[31] Polaprezinc (PZ, an anti-ulcer drug)^[32] and gliotoxin^[33] may contribute to the preservation of gastrointestinal mucosa in some experimental models, such as colitis, radiation enteritis, and acute gastric mucosal lesions. This protective effect is partly mediated by the induction of HO-1 expression.

Nuclear factor-erythroid 2-related factor 2 (Nrf2) has

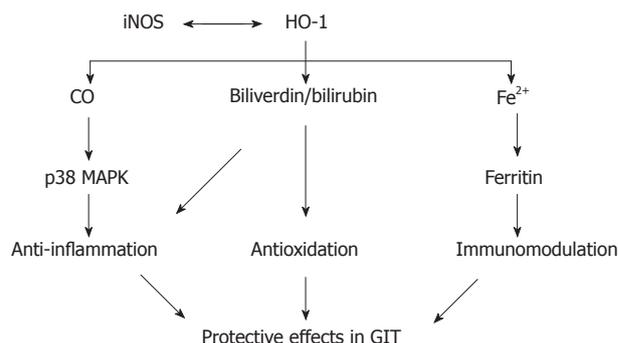


Figure 1 Cytoprotective effects of heme oxygenase-1 pathway in gastrointestinal inflammation. CO: Carbon monoxide; GIT: Gastrointestinal tract; HO-1: Heme oxygenase-1; iNOS: Inducible nitric oxide synthase; MAPK: Mitogen-activated protein kinase.

been known to be a transcriptional factor which plays a crucial role in cytoprotection against inflammation. The severity of colitis induced by dextran sulphate sodium (DSS) in Nrf2-deficient mice is found to be associated with decreased expression of HO-1^[34].

These results demonstrate that HO-1 may be implicated in cytoprotection and may be an effective agent for the treatment of diseases characterized by mucosal inflammation in GIT.

MECHANISMS OF ACTION

HO-1 seems to have an important protective role in acute and chronic inflammation of GIT. HO-1 is the key enzyme in heme degradation and plays a key role in regulating the intracellular heme level. HO-1 activity means rapid removal of free heme, which is shown to be cytotoxic. Thus, HO-1 is associated with a protective response and contributes to the preservation of GIT mucosa (Figure 1).

CO AND GASTROINTESTINAL INFLAMMATION

Almost all CO produced *in vivo* comes from the degradation of heme by HO. Evidences indicate that CO mediates many of the biological actions of HO-1^[35]. Otterbein *et al*^[36] demonstrate that CO can inhibit the production of proinflammatory cytokines [tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and macrophage inflammatory protein-1 β] and stimulate the synthesis of the anti-inflammatory cytokine interleukin-10. Other studies also suggest that CO implicates in mediating the anti-inflammatory actions^[37,38].

Hegazi *et al*^[39] have shown that CO at a low concentration mitigates chronic intestinal inflammation in a T helper-type-1 cell-mediated mouse model of murine colitis in IL 10-deficient mice and protect against the development of postoperative ileus (POI) and necrotising enterocolitis in rodents and swine^[40-42]. Moreover, Scott *et al*^[43] demonstrate that low-dose inhaled CO selectively attenuates the remote intestinal inflammatory response

elicited by hindlimb ischemia-reperfusion. And pre-treatment with CO-releasing molecules (CO-RMs) markedly reduced intestinal muscularis inflammation induced by surgical manipulation of the small intestine^[44]. The anti-inflammatory actions of CO can be in large measure mediated through p38 mitogen-activated protein kinase (MAPK) pathway^[36,37].

The knowledge of the role of CO in gastrointestinal inflammation is limited, but such a mechanism could be operative in GIT. Recently, **Chin *et al*^[45] pointed out that CO has been ascribed an additional novel role as a host defense molecule agent against microbes (bactericidal agent).**

BILIVERDIN/BILIRUBIN AND GASTROINTESTINAL INFLAMMATION

HO-1 catalyzes the rate-limiting step in heme degradation to biliverdin. Biliverdin is, in turn, converted into bilirubin by biliverdin reductase at the expense of nicotinamide adenine dinucleotide phosphate (NADPH). Biliverdin and bilirubin are reducing species and hence potential **antioxidants**^[46,47]. Several studies have demonstrated that the administration of biliverdin and/or bilirubin is potently cytoprotective in a variety of pathophysiological events, including ischemia-reperfusion injury, and **transplant rejection**^[48,49]. In addition, bilirubin is also known to modulate immune effector functions and suppress inflammatory response^[50].

Treatment with biliverdin can significantly decrease mRNA expression of inducible nitric oxide synthase (iNOS), cyclooxygenase 2, and intercellular adhesion molecule-1 as well as the inflammatory cytokines IL-6 and IL-1 β , and decreased neutrophil infiltration into the jejunal muscularis in rat syngeneic small intestinal transplants^[51]. Hayashi *et al*^[52] demonstrate that the effects of HO-1 induction on leukocyte adhesion could be mimicked by bilirubin. In addition, the study of Lee *et al*^[53] show that bilirubin exerts anti-inflammatory effects *in vitro*.

The data indicate that this product of HO reaction play an important role in the anti-inflammatory effects of HO-1. However, there has been **no report about the measurement of tissue levels of biliverdin/bilirubin in human GIT, and even the role of the biliverdin/bilirubin pathway has not been clarified in experimental model of gastrointestinal inflammation.**

Fe²⁺ AND GASTROINTESTINAL INFLAMMATION

Fe²⁺, the third product of heme decomposition, can be potentially toxic, but it **can upregulate an iron-transporter pump that removes intracellular Fe²⁺ from the cell**^[54] and induces the expression of ferritin, an iron storing protein^[55]. Expression of ferritin is originally reported to protect endothelial cells **against oxidant damage *in vitro***^[55]. In addition, over-expression of H-ferritin (heavy

chain ferritin) has also been shown to protect cultured endothelial cells from undergoing apoptosis and protect livers from transplant-associated ischemia-reperfusion injury^[56]. Increased ferritin protein levels induced by lansoprazole in endothelial cells and macrophages can reduce NADPH-dependent ROS formation, indicating that **ferritin may account for the gastric protection of lansoprazole**^[30].

Although the roles of the iron and ferritin in the overall cytoprotective effect of HO-1 are not clear, presumably both contribute in a crucial manner to the overall antioxidant effect following increased HO-1 expression in a variety of situations^[57]. Further work is clearly needed in this area.

The exact mechanisms underlying the anti-inflammatory functions of the HO-1 in gastrointestinal inflammation have not been fully elucidated. However, the signaling action of CO combined/or complemented by the antioxidant properties of biliverdin/bilirubin and the sequestration of iron by ferritin could all contribute to suppression of inflammation^[58]. It becomes **clear that upregulation of HO-1 and/or exogenous administration of one or more of its products would be therapeutic strategies for gastrointestinal inflammation.**

HO-1 AND iNOS

The inducible isoform of nitric oxide synthase (iNOS) can produce sustained high quantities of nitric oxide (NO), which may be involved in the mucosal injury associated with IBD. Indeed, upregulation of iNOS or NO release has been demonstrated in **both ulcerative colitis and Crohn's disease**^[59,60]. HO-1 inducers, cadmium and bismuth salts, heme, and nitric oxide (NO) donors, act at the transcriptional level inhibiting iNOS mRNA expression *in vitro*^[61]. Wang *et al*^[12] investigated the **possible role of HO-1 in experimental colitis in rats.** Their data show that HO-1 plays a protective role in the colonic damage, and this **effect probably result in part from inhibition of iNOS expression in colonic tissues.** Moreover, Dijkstra *et al*^[62] demonstrate opposite regulation of iNOS and HO-1 in intestinal epithelial cells in response to cytokine exposure and oxidative stress. These findings suggest that HO-1/CO and iNOS/NO system may act together in a complex, dynamic, and adaptable association in gastrointestinal inflammation, which remain to be elucidated further.

HO-1 PROMOTER POLYMORPHISM

HO-1 is known as an oxidative stress responsive protein that is upregulated by multiple stimuli, **which has been proposed to provide an important cellular response that protects cells against oxidative damage.** However, humans differ quantitatively in their ability to mount an HO-1 response.

An *HO-1* gene promoter microsatellite (GT)(*n*) dinucleotide repeat polymorphism is associated with

regulation of HO-1 in response to inflammatory stimuli. Short GT repeats (< 25) are associated with highly significant up-regulation of HO-1 in response to inflammatory stimuli^[63,64]. The investigators have studied the association between the HO-1 genotype and gastrointestinal inflammation. They investigated the variants of the HO-1 promoter region in 179 patients with Crohn's disease, 110 with ulcerative colitis and 56 control patients without inflammation. The data show that (GT)_(n) dinucleotide repeats of the HO-1 promoter region have no significance for the pathophysiology and disease course of IBD^[65]. In gastrointestinal tumors, a potential impact of the (GT)_(n) repeat polymorphism has been demonstrated^[66]. But in gastrointestinal inflammation diseases which usually associate tumors, it remains to be verified.

CONCLUSION

Chronic inflammatory disorders in GIT have been linked with an increased risk of the development of gastrointestinal tumors^[66]. It is well known that HO-1 is involved in inflammation and have protective effects in GIT against inflammation and oxidative injury; thus, the modulation of HO-1 through pharmacological means or the use of gene therapy may offer therapeutic strategies for gastrointestinal inflammation and more importantly, to prevent gastrointestinal cancer. A comprehensive understanding of the underlying mechanisms for the observed effects of HO-1 in gastrointestinal inflammation will be necessary in the future.

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S- Editor Tian L L- Editor Ma JY E- Editor Zhang DN

rAd-p53 enhances the sensitivity of human gastric cancer cells to chemotherapy

Guang-Xia Chen, Li-Hong Zheng, Shi-Yu Liu, Xiao-Hua He

Guang-Xia Chen, Shi-Yu Liu, Xiao-Hua He, Department of Gastroenterology, First People's Hospital of Xuzhou, Xuzhou 221002, Jiangsu Province, China

Li-Hong Zheng, Yantai Economic and Technological Development Zone Hospital, Yantai 264006, Shandong Province, China

Author contributions: Chen GX, Zheng LH, Liu SY and He XH conducted the experiments; Chen GX wrote the manuscript. Supported by Xuzhou Science and Technology Development Fund, No. XM07C039

Correspondence to: Dr. Guang-Xia Chen, Department of Gastroenterology, First People's Hospital of Xuzhou, Xuzhou 221002, Jiangsu Province, China. gx_chen2008@yahoo.cn

Telephone: +86-516-85803186 Fax: +86-516-85803011

Received: December 19, 2010 Revised: April 19, 2011

Accepted: April 26, 2011

Published online: October 14, 2011

Abstract

AIM: To investigate potential antitumor effects of rAd-p53 by determining if it enhanced sensitivity of gastric cancer cells to chemotherapy.

METHODS: Three gastric cancer cell lines with distinct levels of differentiation were treated with various doses of rAd-p53 alone, oxaliplatin (OXA) alone, or a combination of both. Cell growth was assessed with an 3-(4,5)-dimethylthiazoliazol-2-yl-4-methylcarbazole (MTT) assay and the expression levels of p53, Bax and Bcl-2 were determined by immunohistochemistry. The presence of apoptosis and the expression of caspase-3 were determined using flow cytometry.

RESULTS: Treatment with rAd-p53 or OXA alone inhibited gastric cancer cell growth in a time- and dose-dependent manner; moreover, significant synergistic effects were observed when these treatments were combined. Immunohistochemical analysis demonstrated that treatment with rAd-p53 alone, OXA alone or combined treatment led to decreased Bcl-2 expression and increased Bax expression in gastric cancer cells.

Furthermore, flow cytometry showed that rAd-p53 alone, OXA alone or combination treatment induced apoptosis of gastric cancer cells, which was accompanied by increased expression of caspase-3.

CONCLUSION: rAd-p53 enhances the sensitivity of gastric cancer cells to chemotherapy by promoting apoptosis. Thus, our results suggest that p53 gene therapy combined with chemotherapy represents a novel avenue for gastric cancer treatment.

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Key words: Gastric cancer; rAd-p53; Oxaliplatin; Chemoresistance; Apoptosis

Peer reviewer: Dr. Paul M. Schneider, MD, Professor of Surgery, Department of Surgery, University Hospital Zurich, Raemistrasse 100, Zurich 8091, Switzerland

Chen GX, Zheng LH, Liu SY, He XH. rAd-p53 enhances the sensitivity of human gastric cancer cells to chemotherapy. *World J Gastroenterol* 2011; 17(38): 4289-4297 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4289.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4289>

INTRODUCTION

Gastric cancer is the most common malignant tumor of the digestive system. Currently, the major therapeutic methods for the treatment of gastric cancer are surgery, radiotherapy and chemotherapy. Despite recent improvements in these treatments, the 5-year survival rate for gastric cancer patients is only 45%. Thus, the development of new therapeutic approaches for gastric cancer, such as gene therapy, is urgently needed.

p53 is known as the "genome guard" and plays important roles in various cellular processes, including cell cycle regulation, DNA damage repair and apop-

tosis. Genetic mutations in p53 are present in > 50% of human tumor tissues, and it is the most commonly detected genetic mutation in cancer^[1]. Therefore, a gene therapy strategy has been developed that employs rAd-p53, a weakened adenovirus carrying the wild-type p53 gene. rAd-p53 has been shown to inhibit tumor growth, promote apoptosis by inducing the expression of Puma, Bax, Bak and Fas, and to sensitize tumor cells to radiotherapy and chemotherapy^[2]. Clinical application of rAd-p53 has been used to treat lung cancer, breast cancer, oophoroma, liver cancer, and bladder carcinoma. However, few studies have investigated the therapeutic effects of rAd-p53 in gastric cancer.

Genetic mutation of p53 is found in > 60% of gastric cancer cases and has been shown to correlate not only with the onset and prognosis of gastric cancer, but also with the chemosensitivity of gastric cancer^[3]. Thus, we speculated that rAd-p53 could be a potential treatment for gastric cancer. In this study, we investigated the effects of rAd-p53 treatment alone or in combination with oxaliplatin (OXA) on the growth and chemosensitivity of gastric cancer cells. Our results demonstrate that rAd-p53 has antitumor properties in gastric cancer.

MATERIALS AND METHODS

Reagents

rAd-p53 was purchased from Shenzhen Saibainuo Gene Technology Co. Ltd. (Shenzhen, China); OXA was purchased from Jiangsu Hengrui Medicine Co. Ltd. (Lianyungang, China). rAd-p53 was diluted to 5×10^8 virus particles vp/mL or 5×10^{10} vp/mL in saline, and OXA was diluted to 2.5 mg/mL in 5% glucose and stored at -80 °C.

Cell culture

The human gastric cancer lines SGC-7901 (moderately differentiated), BGC-823 (poorly differentiated), and HGC-27 (undifferentiated) were purchased from the Chinese Academy of Sciences (Beijing, China). The cells were cultured in XX media containing 10% fetal bovine serum, 10^5 U/L penicillin, and 100 ng/L streptomycin at 37 °C in 5% CO₂.

MTT assay

Cells were seeded in 96-well plates at 10^4 cells/well and treated with rAd-p53 or OXA for 24, 48 or 72 h at 37 °C. Next, 150 µL MTT was added to each well and incubated for 4 h at 37 °C, followed by addition of 200 µL dimethyl sulfoxide to each well, and 10 min incubation to dissolve the formazan crystals. The absorbance was measured using an ELISA reader (EXL800; Bio-Tek, United States) at 450 nm. The data are presented as mean ± SD of triplicate samples from at least three independent experiments.

The cell growth inhibition ratio was calculated using the following formula: cell growth inhibition ratio (%) = $1 - [(A_s - A_b)/(A_c - A_b)] \times 100\%$, where A_s represents the A value of the experimental well, A_c represents the A value in the control well, and A_b represents the A

value of the blank well.

To determine whether rAd-p53 and OXA had synergistic effects, the following formula was used: $q = (E_a + b)/[(E_a + E_b) - E_a \times E_b]$, where E_a represent the inhibition ratio of rAd-p53, E_b represents the inhibition ratio of OXA, and $E_a + b$ represents the inhibition ratio of the associated group. A q value > 1.15 was considered to indicate a synergistic effect, whereas a q value < 0.85 was considered to indicate a lack of a synergistic effect, and a q value between 0.85 and 1.15 was considered to indicate an additive effect.

Immunohistochemistry

Cells were seeded in six-well plates at 10^6 cells/well and then treated with rAd-p53 or OXA for 24 h. The cells were fixed with acetone for 20 min and then stained using an SP immunohistochemistry kit (Zhongshanqiao, Beijing, China) according to the manufacturer's protocol. In the gastric cancer cells examined, p53 expression was nuclear, whereas Bcl-2 and Bax expression were located in the cytoplasm.

Flow cytometry analysis

Cells were seeded in six-well plates at 5×10^5 cells/well and then treated with rAd-p53 or OXA for 24 h. Apoptotic cells were detected with an apoptosis detection kit (Invitrogen, Eugene, OR, United States).

Statistical analysis

All data were presented as mean ± SD. Statistical analysis was performed using SPSS 13.0. Single factor analysis of variance, least significant difference methods, and Q tests were used for inside group comparisons, group comparisons, and multiple comparisons, respectively. For all analyses, the test size was set to $\alpha = 0.05$. $P < 0.05$ was considered statistically significant.

RESULTS

Treatment with rAd-p53 or OXA inhibits the growth of gastric cancer cells in a time- and dose-dependent manner

The MTT assay results showed that rAd-p53 could inhibit the growth of the gastric cancer cell lines SGC-7901 (moderately differentiated), BGC-823 (poorly differentiated) and HGC-27 (undifferentiated) in a time- and dose-dependent manner (Figure 1A-C). A similar result was observed for OXA treatment (Figure 1D-F). Among the three cell lines, we found that the inhibitory effects of rAd-p53 and OXA were both strongest in SGC-7901 and weakest in HGC-27 when treatment dose and time were kept constant, suggesting that more differentiated gastric cancer cells are more sensitive to rAd-p53 and OXA treatments.

Combined treatment with rAd-p53 and OXA shows a synergistic effect on the inhibition of gastric cancer cell growth

We next used treated the three gastric cancer cell lines

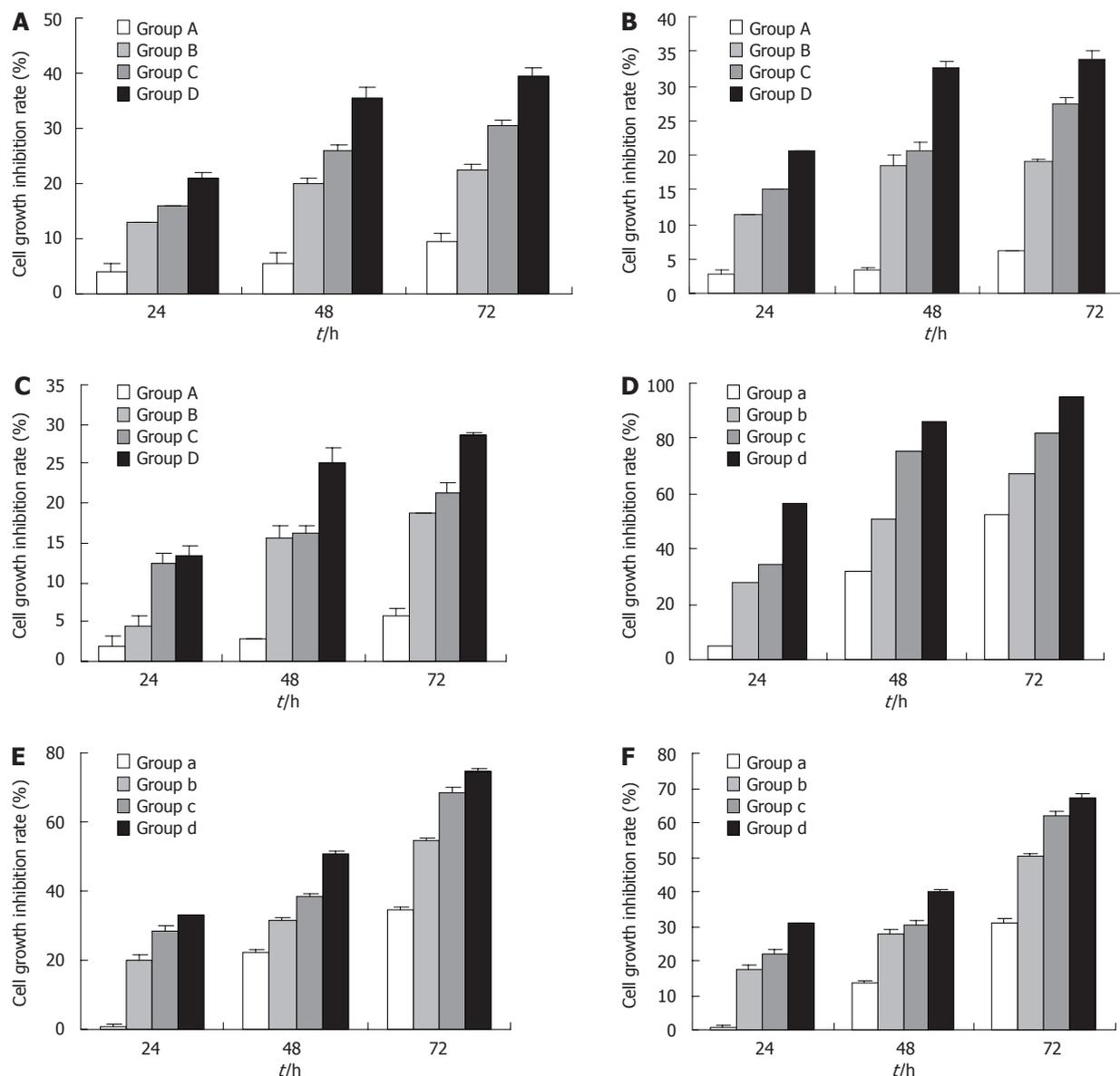


Figure 1 Treatment with rAd-p53 or oxaliplatin alone inhibits the growth of gastric cancer cells in a time- and dose-dependent manner. SGC-7910 (A), BGC-823 (B) and HGC-27 (C) cells were treated with rAd-p53 followed by the determination of cell growth inhibition rates. Groups A, B, C and D were treated with the indicated rAd-p53 dose (vp/mL) of 5×10^6 , 5×10^7 , 5×10^8 and 5×10^9 , respectively. SGC-7910 (D), BGC-823 (E) and HGC-27 (F) cells were treated with oxaliplatin (OXA), and cell growth inhibition rates were determined. Groups a, b, c and d were treated with the indicated OXA dose ($\mu\text{g/mL}$) of 3.2, 6.4, 12.8 and 25.6, respectively.

with a combination of rAd-53 and OXA and found that the inhibition of cell growth was markedly stronger at a relatively low combined dose and with a short treatment time (Figure 2), compared to treatment with rAd-p53 or OXA alone (Figure 1). A q value > 1.15 indicated that rAd-p53 and OXA had synergistic effects on the inhibition of gastric cancer cell growth.

Expression of p53 in gastric cancer cells treated with rAd-p53 or OXA alone or with rAd-p53 in combination with OXA

As expected, when the gastric cancer cell lines were treated with rAd-p53 for 48 h, immunohistochemical staining showed that p53 expression increased gradually

with respect to dose (Figure 3, Table 1). Moreover, when the same treatment doses were used, p53 expression was stronger in more differentiated gastric cancer cells. However, the combined use of OXA at 3.2 $\mu\text{g/mL}$ and rAd-p53 had no obvious, additional effects on p53 expression, indicating that the antitumor effects of OXA were not related to the upregulation of p53 expression in tumor cells.

Expression of Bax and Bcl-2 in gastric cancer cells treated with rAd-p53 or OXA alone, or rAd-p53 in combination with OXA

Immunohistochemical staining also showed that the expression of the pro-apoptotic protein Bax increased

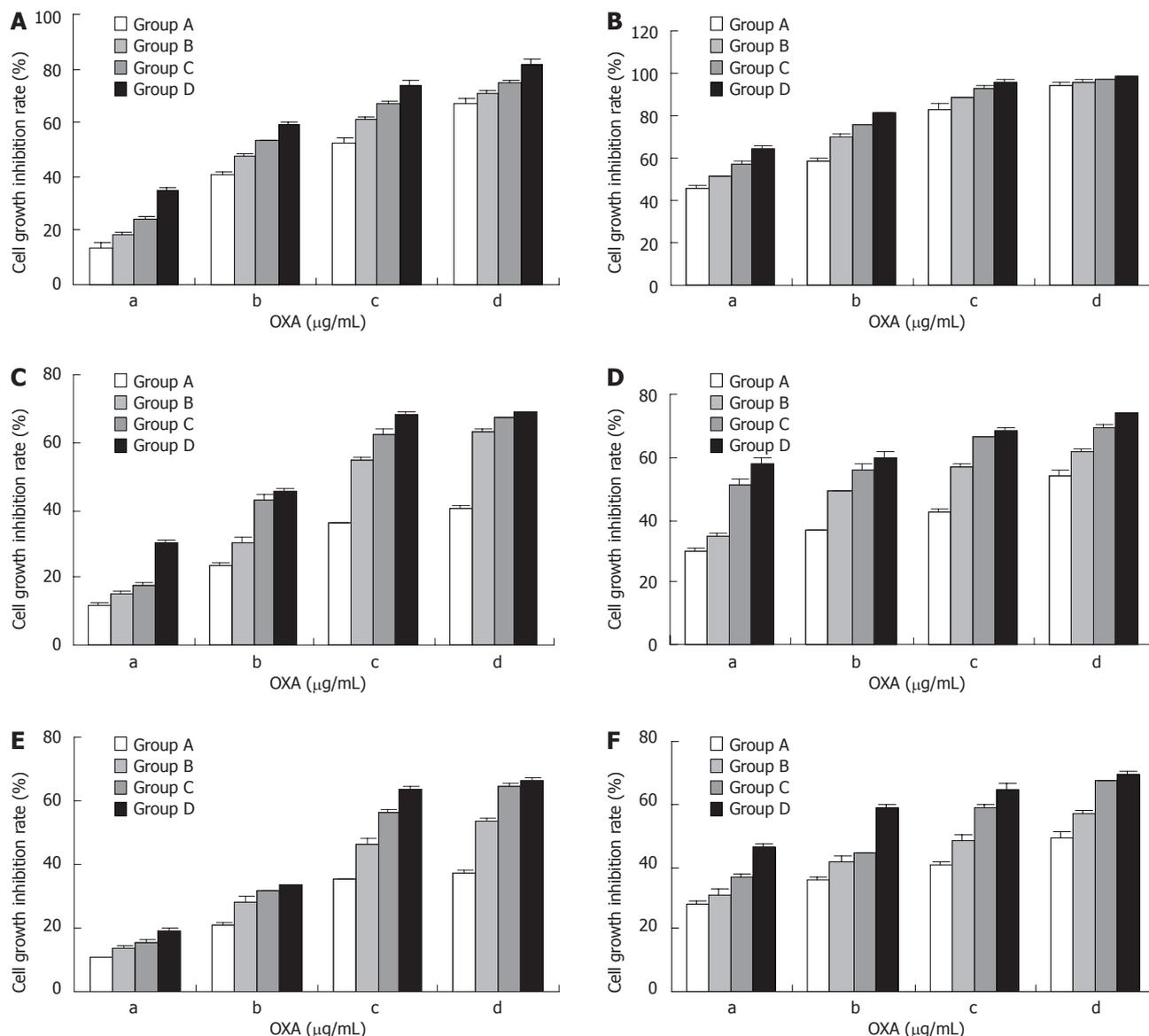


Figure 2 Combination treatment with rAd-p53 and oxaliplatin has synergistic effects on the inhibition of gastric cancer cell growth. SGC-7910 (A, B), BGC-823 (C, D) and HGC-27 (E, F) cells were treated with rAd-p53 plus oxaliplatin (OXA), and cell growth inhibition rates were determined at 24 h (A, C, E) or 48 h (B, D, F). Groups A, B, C and D were treated with the indicated rAd-p53 dose (vp/mL) of 5×10^6 , 5×10^7 , 5×10^8 and 5×10^9 , respectively. Groups a, b, c and d were treated with the indicated OXA dose (μg/mL) of 3.2, 6.4, 12.8 and 25.6, respectively.

gradually in gastric cancer cells treated with increasing doses of rAd-p53 for 48 h (Figure 4, Table 2), whereas the expression of the anti-apoptotic protein Bcl-2 decreased gradually (Figure 5, Table 3). Combination treatment with OXA at 3.2 μg/mL and rAd-p53 had modest effects on the levels of Bax and Bcl-2 expression, indicating that the antitumor effects of rAd-p53 and OXA were mediated by a mechanism that promoted gastric cancer cell apoptosis.

Apoptotic ratio and expression of caspase-3 in gastric cancer cells treated with rAd-p53 or OXA alone or with rAd-p53 in combination with OXA

To confirm that the antitumor effects of rAd-p53 and OXA were associated with induction of apoptosis in gastric cancer cells, we examined the expression of caspase-3

and the apoptotic rate in the three different gastric cancer cell lines by flow cytometric analysis. We found that caspase-3 expression was higher in treated gastric cancer cells compared to untreated cells ($P < 0.05$). Moreover, the combined treatment with rAd-p53 and OXA presented synergistic effects in the upregulation of caspase-3 expression and induction of apoptosis ($P < 0.05$) (Tables 4 and 5).

DISCUSSION

As the most important tumor suppressor gene, p53 plays an important role in the induction of apoptosis. However, the mutation rate of p53 gene is approximately 50% in human cancers^[4], leading to the loss of p53 function, including its induction of apoptosis. Available data sug-

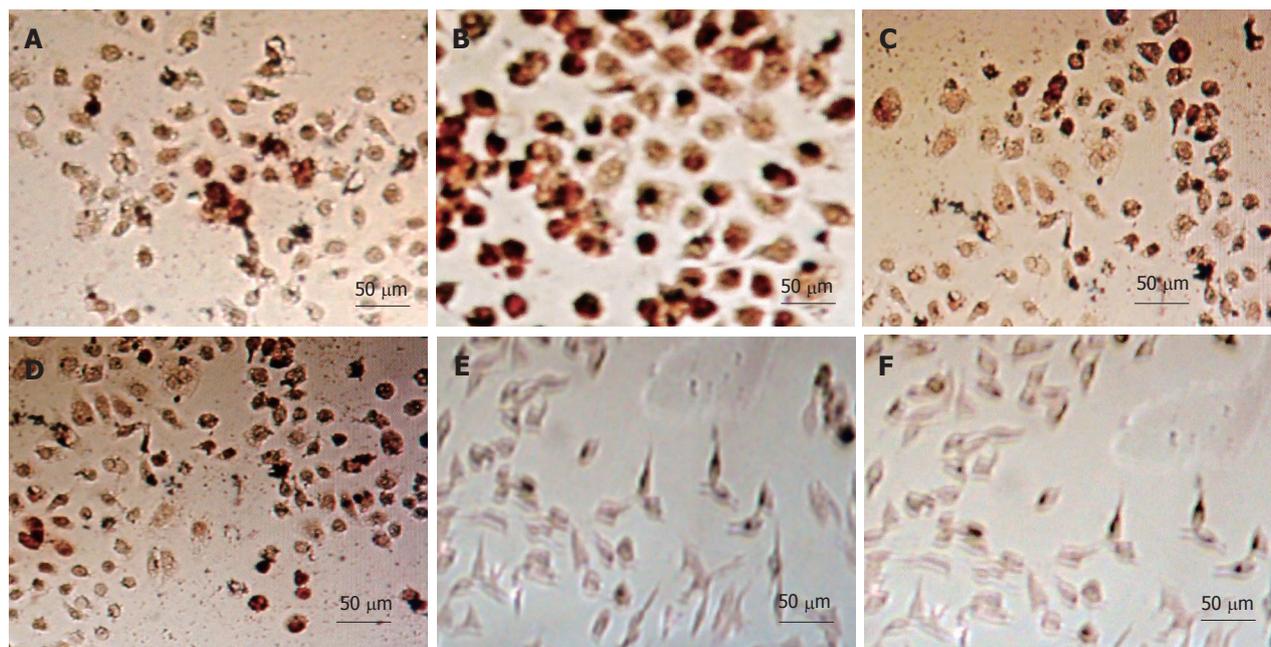


Figure 3 Detection of p53 expression in gastric cancer cells with immunohistochemistry. A: Untreated SGC-7901 cells; B: SGC-7901 cells treated with 5×10^9 vp/mL rAd-p53 plus 3.2 μ g/mL oxaliplatin (OXA); C: Untreated BGC-823 cells; D: BGC-823 cells treated with 5×10^9 vp/mL rAd-p53 plus 3.2 μ g/mL OXA; E: Untreated HGC-27 cells untreated; F: HGC-27 cells treated with 5×10^9 vp/mL rAd-p53 plus 3.2 μ g/mL OXA.

Table 1 p53 expression in gastric cancer cells 48 h after treatment with rAd-p53, oxaliplatin or rAd-p53 plus oxaliplatin

Treatment	rAd-p53 (vp/mL)	OXA (μ g/mL)	Gastric cancer cell line		
			SGC-7901	BGC-823	HGC-27
OXA	0	3.2	$11.83 \pm 1.02^{e,b}$	$8.67 \pm 1.35^{c,b}$	$6.36 \pm 1.62^{e,b}$
rAd-p53	5×10^6	0	$36.65 \pm 1.04^{c,a}$	$25.13 \pm 2.73^{c,a}$	$21.26 \pm 1.07^{c,a}$
	5×10^7	0	$40.32 \pm 1.03^{c,a}$	$32.45 \pm 2.35^{c,a}$	$25.35 \pm 1.28^{c,a}$
	5×10^8	0	$48.86 \pm 1.26^{c,a}$	$38.25 \pm 2.16^{c,a}$	$29.67 \pm 1.31^{c,a}$
	5×10^9	0	$60.38 \pm 1.14^{c,a}$	$49.37 \pm 1.07^{c,a}$	$33.25 \pm 2.05^{c,a}$
rAd-p53 + OXA	5×10^6	3.2	$37.23 \pm 1.07^{e,c,a}$	$26.54 \pm 1.53^{e,c,a}$	$22.17 \pm 1.13^{e,c,a}$
	5×10^7	3.2	$39.83 \pm 1.32^{e,c,a}$	$34.17 \pm 1.26^{e,c,a}$	$24.83 \pm 1.07^{e,c,a}$
	5×10^8	3.2	$49.03 \pm 1.26^{e,c,a}$	$40.28 \pm 1.43^{e,c,a}$	$30.45 \pm 1.32^{e,c,a}$
	5×10^9	3.2	$61.54 \pm 1.18^{e,c,a}$	$50.37 \pm 1.27^{e,c,a}$	$35.21 \pm 2.1^{e,c,a}$
Control	0	0	12.55 ± 1.15	8.23 ± 1.13	6.15 ± 1.36

^a $P < 0.05$ vs control, ^b $P > 0.05$ vs control; ^c $P > 0.05$, rAd-p53 vs rAd-p53 + oxaliplatin (OXA) with the same dose of rAd-p53; ^e $P < 0.05$, OXA vs rAd-p53 + OXA with the same dose of OXA.

Table 2 Bax expression in gastric cancer cells 48 h after treatment with rAd-p53, oxaliplatin or rAd-p53 plus oxaliplatin

Treatment	rAd-p53 (vp/mL)	OXA (μ g/mL)	Gastric cancer cell line		
			SGC-7901	BGC-823	HGC-27
OXA	0	3.2	$73.52 \pm 0.83^{e,a}$	$56.43 \pm 0.74^{e,a}$	$36.47 \pm 1.21^{e,a}$
rAd-p53	5×10^6	0	$63.25 \pm 1.32^{c,a}$	$53.86 \pm 1.54^{c,a}$	$33.71 \pm 1.41^{c,a}$
	5×10^7	0	$76.14 \pm 0.73^{c,a}$	$59.32 \pm 1.45^{c,a}$	$39.47 \pm 1.03^{c,a}$
	5×10^8	0	$79.62 \pm 1.46^{c,a}$	$64.74 \pm 1.08^{c,a}$	$41.35 \pm 1.15^{c,a}$
	5×10^9	0	$82.54 \pm 1.28^{c,a}$	$69.53 \pm 1.02^{c,a}$	$43.75 \pm 1.1^{c,a}$
rAd-p53 + OXA	5×10^6	3.2	$78.82 \pm 0.88^{e,c,a}$	$58.64 \pm 1.07^{e,c,a}$	$49.15 \pm 1.04^{e,c,a}$
	5×10^7	3.2	$84.32 \pm 1.02^{e,c,a}$	$62.74 \pm 1.19^{e,c,a}$	$52.9 \pm 1.31^{e,c,a}$
	5×10^8	3.2	$87.41 \pm 1.03^{e,c,a}$	$67.38 \pm 1.14^{e,c,a}$	$55.23 \pm 1.06^{e,c,a}$
	5×10^9	3.2	$89.71 \pm 0.36^{e,c,a}$	$75.14 \pm 1.65^{e,c,a}$	$58.67 \pm 1.12^{e,c,a}$
Control	0	0	26.32 ± 1.04	19.91 ± 0.87	16.74 ± 1.23

^a $P < 0.05$ vs control; ^c $P < 0.05$, rAd-p53 vs rAd-p53 + oxaliplatin (OXA) with the same dose of rAd-p53; ^e $P < 0.05$, OXA vs rAd-p53 + OXA with the same dose of OXA.

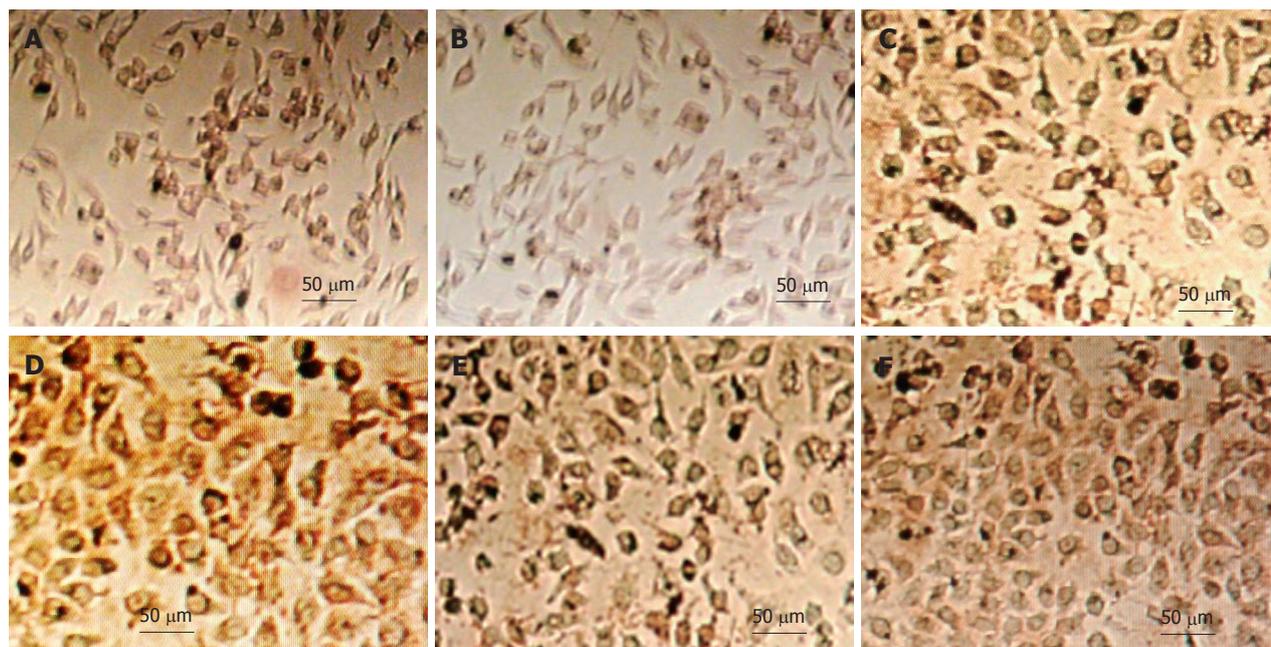


Figure 4 Detection of bax expression in gastric cancer cells with immunohistochemistry. A: Untreated SGC-7901 cells; B: SGC-7901 cells treated with 5×10^9 vp/mL rAd-p53 plus 3.2 μ g/mL oxaliplatin (OXA); C: Untreated BGC-823 cells; D: BGC-823 cells treated with 5×10^9 vp/mL rAd-p53 plus 3.2 μ g/mL OXA; E: Untreated HGC-27 cells; F: HGC-27 cells treated with 5×10^9 vp/mL rAd-p53 plus 3.2 μ g/mL OXA.

Table 3 Bcl-2 expression in gastric cancer cells 48 h after treatment with rAd-p53, oxaliplatin or rAd-p53 plus oxaliplatin

Treatment			Gastric cancer cell line		
	rAd-p53 (vp/mL)	OXA (μ g/mL)	SGC-7901	BGC-823	HGC-27
OXA	0	3.2	26.32 \pm 1.21 ^{e,a}	47.53 \pm 1.13 ^{e,a}	56.64 \pm 1.33 ^{e,a}
rAd-p53	5×10^6	0	28.62 \pm 1.07 ^{c,a}	58.23 \pm 1.04 ^{c,a}	61.23 \pm 1.07 ^{c,a}
	5×10^7	0	24.34 \pm 1.05 ^{c,a}	46.26 \pm 1.31 ^{c,a}	49.54 \pm 1.14 ^{c,a}
	5×10^8	0	18.62 \pm 1.32 ^{c,a}	40.81 \pm 1.15 ^{c,a}	47.34 \pm 1.06 ^{c,a}
	5×10^9	0	15.37 \pm 1.51 ^{c,a}	38.37 \pm 1.08 ^{c,a}	44.31 \pm 1.03 ^{c,a}
rAd-p53+ OXA	5×10^6	3.2	21.76 \pm 1.16 ^{e,c,a}	35.63 \pm 1.41 ^{e,c,a}	38.18 \pm 1.08 ^{e,c,a}
	5×10^7	3.2	18.34 \pm 1.24 ^{e,c,a}	32.37 \pm 1.07 ^{e,c,a}	35.71 \pm 2.02 ^{e,c,a}
	5×10^8	3.2	16.22 \pm 1.02 ^{e,c,a}	29.27 \pm 1.13 ^{e,c,a}	32.91 \pm 1.24 ^{e,c,a}
	5×10^9	3.2	13.14 \pm 1.07 ^{e,c,a}	26.74 \pm 1.02 ^{e,c,a}	29.84 \pm 1.57 ^{e,c,a}
Control	0	0	38.97 \pm 1.06	73.71 \pm 2.02	84.03 \pm 1.02

^a*P* < 0.05 vs control; ^c*P* < 0.05, rAd-p53 vs rAd-p53 + oxaliplatin (OXA) with the same dose of rAd-p53; ^e*P* < 0.05, OXA vs rAd-p53 + OXA with the same dose of OXA.

Table 4 Caspase-3 expression in gastric cancer cells 48 h after treatment with rAd-p53, oxaliplatin or rAd-p53 plus oxaliplatin

Treatment			Gastric cancer cell line		
	rAd-p53 (vp/mL)	OXA (μ g/mL)	SGC-7901	BGC-823	HGC-27
OXA	0	3.2	12.32 \pm 0.8 ^{e,a}	11.21 \pm 1.05 ^{e,a}	8.86 \pm 1.01 ^{e,a}
rAd-p53	5×10^6	0	7.89 \pm 1.13 ^{c,a}	6.07 \pm 0.97 ^{c,a}	4.32 \pm 1.03 ^{c,a}
	5×10^7	0	10.03 \pm 1.03 ^{c,a}	8.38 \pm 1.04 ^{c,a}	6.03 \pm 0.99 ^{c,a}
	5×10^8	0	12.34 \pm 1.05 ^{c,a}	10.52 \pm 0.89 ^{c,a}	8.31 \pm 1.02 ^{c,a}
	5×10^9	0	15.04 \pm 1.03 ^{c,a}	11.34 \pm 0.55 ^{c,a}	10.12 \pm 1.01 ^{c,a}
rAd-p53 + OXA	5×10^6	3.2	22.05 \pm 1.01 ^{e,c,a}	15.67 \pm 1.03 ^{e,c,a}	13.48 \pm 1.01 ^{e,c,a}
	5×10^7	3.2	25.13 \pm 1.06 ^{e,c,a}	18.83 \pm 1.02 ^{e,c,a}	15.32 \pm 1.07 ^{e,c,a}
	5×10^8	3.2	27.24 \pm 1.73 ^{e,c,a}	21.07 \pm 1.01 ^{e,c,a}	18.93 \pm 1.06 ^{e,c,a}
	5×10^9	3.2	35.67 \pm 1.03 ^{e,c,a}	26.16 \pm 1.05 ^{e,c,a}	22.34 \pm 1.13 ^{e,c,a}
Control	0	0	1.32 \pm 1.02	1.29 \pm 0.97	1.27 \pm 0.68

^a*P* < 0.05 vs control; ^c*P* < 0.05, rAd-p53 vs rAd-p53 + oxaliplatin (OXA) with the same dose of rAd-p53; ^e*P* < 0.05, OXA vs rAd-p53 + OXA with the same dose of OXA.

Table 5 Apoptotic rate in gastric cancer cells 48 h after treatment with rAd-p53, oxaliplatin or rAd-p53 plus oxaliplatin

Treatment	rAd-p53 (vp/mL)	OXA ($\mu\text{g/mL}$)	Gastric cancer cell line		
			SGC-7901	BGC-823	HGC-27
OXA	0	3.2	$33.52 \pm 1.6^{e,a}$	$23.28 \pm 1.35^{e,a}$	$18.72 \pm 1.61^{e,a}$
rAd-p53	5×10^6	0	$7.89 \pm 1.13^{c,a}$	$6.51 \pm 0.97^{c,a}$	$4.07 \pm 0.83^{c,a}$
	5×10^7	0	$12.47 \pm 1.43^{c,a}$	$8.78 \pm 1.34^{c,a}$	$6.43 \pm 0.79^{c,a}$
	5×10^8	0	$21.84 \pm 1.05^{c,a}$	$14.24 \pm 0.89^{c,a}$	$11.72 \pm 1.12^{c,a}$
rAd-p53 + OXA	5×10^6	3.2	$36.73 \pm 1.03^{c,a}$	$28.64 \pm 1.75^{c,a}$	$21.82 \pm 1.81^{c,a}$
	5×10^6	3.2	$42.38 \pm 1.51^{e,c,a}$	$35.72 \pm 1.13^{e,c,a}$	$28.84 \pm 1.21^{e,c,a}$
	5×10^7	3.2	$54.84 \pm 1.26^{e,c,a}$	$48.63 \pm 1.62^{e,c,a}$	$34.51 \pm 1.47^{e,c,a}$
	5×10^8	3.2	$58.41 \pm 1.13^{e,c,a}$	$51.71 \pm 1.41^{e,c,a}$	$38.5 \pm 1.16^{e,c,a}$
Control	0	0	$63.91 \pm 1.23^{e,c,a}$	$55.73 \pm 1.35^{e,c,a}$	$42.92 \pm 1.33^{e,c,a}$
	0	0	4.67 ± 1.32	1.74 ± 0.67	1.15 ± 0.58

^a $P < 0.05$ vs control; ^c $P < 0.05$, rAd-p53 vs rAd-p53 + oxaliplatin (OXA) with the same dose of rAd-p53; ^e $P < 0.05$, OXA vs rAd-p53 + OXA at the same dose of OXA.

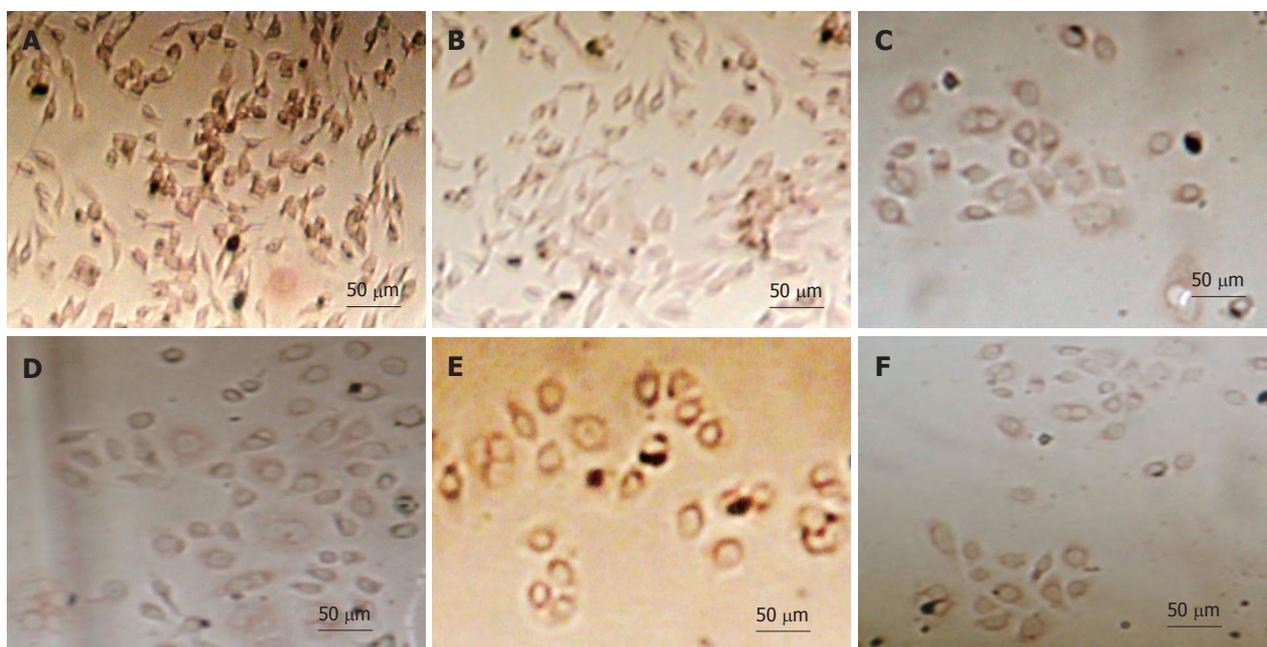


Figure 5 Detection of Bcl-2 expression in gastric cancer cells with immunohistochemistry. A: Untreated SGC-7901 cells; B: SGC-7901 cells treated with 5×10^9 vp/mL rAd-p53 plus 3.2 $\mu\text{g/mL}$ oxaliplatin (OXA); C: Untreated BGC-823 cells; D: BGC-823 cells treated with 5×10^9 vp/mL rAd-p53 plus 3.2 $\mu\text{g/mL}$ OXA; E: Untreated HGC-27 cells; F: HGC-27 cells treated with 5×10^9 vp/mL rAd-p53 plus 3.2 $\mu\text{g/mL}$ OXA.

gest that p53 mutations are linked to the development of multiple malignant tumors, such as liver cancer, breast cancer, bladder carcinoma, gastric cancer, colon carcinoma, prostatic carcinoma, ovarian cancer, brain cancer, esophageal cancer, lung cancer, lymphocyte tumor, soft tissue sarcoma, and osteogenic sarcoma^[5-20].

rAd-53, which is an adenovirus carrier containing the p53 tumor suppressor gene, is the first gene therapy drug. In this therapy, the adenovirus is used to deliver the p53 gene to target cells; restoration of p53 expression in the targeted cells results in antitumor effects. The mechanisms of p53 action include: (1) inhibition of cell cycle progression and induction of apoptosis in tumor cells through the modulation of the expression of apoptosis- and cell-cycle-related genes; (2) sensitization of tumor cells to radiotherapy and chemotherapy;

and (3) stimulation of antitumor immunity through the bystander effect. Clinical application studies have demonstrated that rAd-p53 not only strengthens tumor cell sensitivity to radiotherapy and chemotherapy, but also reduces side effects of chemotherapy. For these reasons, a combination of p53 gene therapy and chemotherapy has been successfully applied to cure a variety of cancers, including lung adenocarcinoma, liver cancer and oophoroma^[21,22].

In the present study, we treated three different gastric cancer cell lines with a combination of rAd-p53 and OXA and found that these agents had significant inhibitory effects on cancer cell growth that were dependent on treatment time and dose. In addition, we observed that more differentiated cells were more sensitive to rAd-p53 and OXA treatment. To investigate whether

the antitumor effects of rAd-p53 and OXA are related to the induction of apoptosis in gastric cancer cells, we examined the expression of apoptosis-related proteins. Bcl-2 is the most important anti-apoptotic protein^[23,24], whereas Bax is a pro-apoptotic protein^[25]. Furthermore, it is well known that caspase-3 is critical in chemotherapy-induced apoptosis of cancer cells^[26-30]. Therefore, we examined the expression of Bcl-2, Bax and caspase-3 in gastric cancer cells treated with rAd-p53. As expected, our results demonstrated that the expression Bax and caspase-3 was increased, whereas the expression of Bcl-2 was decreased in a dose-dependent manner. Consistent with these data, we found that the apoptosis of gastric cancer cells was increased.

In conclusion, in the present study, we demonstrated that rAd-p53 inhibited gastric cancer cell growth and sensitized these cells to the chemotherapeutic agent OXA. The underlying mechanisms of these effects involved the induction of apoptosis, which was achieved via downregulation of Bcl-2 and upregulation of Bax and caspase-3. Our results suggest that the combination of p53 gene therapy and chemotherapy represents a novel avenue for gastric cancer treatment.

COMMENTS

Background

Gastric cancer is the most common malignant tumor of the digestive system. Current major therapeutic methods for gastric cancer are surgery, radiotherapy and chemotherapy. Despite recent improvements in these treatments, the > 5-year survival rate for gastric cancer patients is only up to 45%. Thus, it is urgent to develop new therapeutic approaches such as gene therapy for gastric cancer.

Research frontiers

p53 is known as the "genome guard" that plays important roles in various cellular processes, including cell cycle regulation, DNA damage repair and apoptosis. p53 genetic mutation exists in > 50% human tumor tissues and it is the most common detected genetic mutation in cancer. Therefore, a gene therapy strategy has been developed to employ rAd-p53, a weakened adenovirus that carries the wild-type p53 gene, to make tumor cells sensitive to radiotherapy and chemotherapy. Clinical application of rAd-p53 has been carried out on lung cancer, breast cancer, oophoroma, liver cancer, and bladder carcinoma. However, few studies have investigated the therapeutic effects of rAd-p53 on gastric cancer.

Innovations and breakthroughs

In the present study, the authors demonstrated that rAd-p53 inhibited gastric cancer cell growth and sensitized them to chemotherapy by oxaliplatin (OXA). The underlying mechanisms were concerned with induction of apoptosis achieved via downregulation of bcl-2 and upregulation of Bax and caspase-3.

Applications

Given that p53 genetic mutation exists in > 60% of gastric cancers and is correlated with onset and prognosis of gastric cancer, and with chemosensitivity of gastric cancer, the authors' findings that rAd-p53 had antitumor effects in gastric cancer is important for the application of rAd-p53 to clinical treatment of gastric cancer.

Terminology

Apoptosis is a process of programmed cell death that occurs in multicellular organisms. In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis confers advantages during an organism's life cycle by maintaining the balance of cell survival and death. However, an insufficient amount of apoptosis results in uncontrolled cell proliferation, such as cancer. Apoptosis is regulated by a balance between pro-apoptotic and anti-apoptotic molecules.

Peer review

In this paper, the authors reported that rAd-p53 enhanced the sensitivity of

gastric cancer cells to chemotherapy by promoting apoptosis. These results suggest that p53 gene therapy combined with chemotherapy is more effective for gastric cancer treatment than regular chemotherapy.

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S- Editor Sun H L- Editor Kerr C E- Editor Zhang DN

Casticin-induced apoptosis involves death receptor 5 upregulation in hepatocellular carcinoma cells

Jun Yang, Yun Yang, Li Tian, Xi-Feng Sheng, Fei Liu, Jian-Guo Cao

Jun Yang, Department of Pathology, The Third Xiangya Hospital of Central South University, Changsha 410013, Hunan Province, China

Yun Yang, Li Tian, Xi-Feng Sheng, Fei Liu, Jian-Guo Cao, Laboratory of Medical Engineering, Medical College, Hunan Normal University, Changsha 410013, Hunan Province, China

Author contributions: Yang J, Yang Y, Tian L and Liu F performed the majority of experiments; Sheng XF provided the vital reagents and analytical tools and was involved in editing the manuscript; Cao JG designed the study and wrote the manuscript.

Supported by The Scientific Research Project of Hunan Provincial Administration Bureau of Traditional Chinese Medicine, No. 2010081; Scientific Research Project of Hunan Provincial Health Department, No. B2010-030; Major Projects of Scientific Research of Hunan Provincial Department of Education, No. 09A054

Correspondence to: Jian-Guo Cao, Professor, Laboratory of Medical Engineering, Medical College, Hunan Normal University, Changsha 410013, Hunan Province, China. caojianguo2005@yahoo.com.cn

Telephone: +86-731-8912434 Fax: +86-731-8912417

Received: January 26, 2011 Revised: June 9, 2011

Accepted: June 16, 2011

Published online: October 14, 2011

Abstract

AIM: To investigate the apoptotic activities of casticin in hepatocellular carcinoma (HCC) cells and its molecular mechanisms.

METHODS: PLC/PRF/5 and Hep G2 cell lines were cultured *in vitro* and the inhibitory effect of casticin on the growth of cells was detected by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. The apoptotic cell death was examined using the cell apoptosis enzyme linked immunosorbent assay (ELISA) detection kit, flow cytometry (FCM) after propidium iodide (PI) staining and DNA agarose gel electrophoresis. The caspase activities were measured using ELISA. Reactive oxygen species (ROS) production was evalu-

ated by FCM after dichlorodihydrofluorescein diacetate (DCFH-DA) probe labeling. Intracellular glutathione (GSH) content was measured using a glutathione assay kit. The expression of death receptor (DR)4 and DR5 proteins was analyzed by Western blotting and FCM.

RESULTS: Casticin significantly inhibited the growth of human HCC (PLC/PRF/5 and Hep G2) cells in a dose-dependent manner ($P < 0.05$). Casticin increased the percentage of the sub-G1 population in HCC cells in a concentration-dependent manner. The potency of casticin to PLC/PRF/5 cells was higher than that of 5-fluorouracil ($26.8\% \pm 4.8\%$ vs $17.4\% \pm 5.1\%$) at $10 \mu\text{mol/L}$ for 24 h. Casticin increased the levels of Histone/DNA fragmentation and the levels of active caspase-3, -8 and -9 in a concentration-dependent manner ($P < 0.05$). Treatment with $30 \mu\text{mol/L}$ casticin for 24 h resulted in the formation of a DNA ladder. Casticin reduced the GSH content ($P < 0.05$), but did not affect the level of intracellular ROS in PLC/PRF/5 and Hep G2 cells. The thiol antioxidants, acetylcysteine (NAC) and GSH restored GSH content and attenuated casticin-induced apoptosis. In contrast, the nonthiol antioxidants, butylated hydroxyanisole and mannitol failed to do so. In the HCC cells treated with casticin for 24 h, DR5 protein level was increased. The expression of DR5 protein induced by casticin was inhibited by NAC. Pretreatment with DR5/Fc chimera protein, a blocking antibody, effectively attenuated the induction of apoptosis by casticin.

CONCLUSION: Casticin-induced apoptosis of HCC cells is involved in GSH depletion and DR5 upregulation.

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Key words: Hepatocellular carcinoma; Casticin; Glutathione; Death receptor 5

Peer reviewer: Kotaro Miyake, MD, PhD, Department of Surgery, Institute of Health Biosciences, The University of

Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8503, Japan

Yang J, Yang Y, Tian L, Sheng XF, Liu F, Cao JG. Casticin-induced apoptosis involves death receptor 5 upregulation in hepatocellular carcinoma cells. *World J Gastroenterol* 2011; 17(38): 4298-4307 Available from: URL: <http://www.wjnet.com/1007-9327/full/v17/i38/4298.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4298>

INTRODUCTION

Hepatocellular carcinoma (HCC) is currently the fifth most common malignant neoplasm in the world^[1], causing over 600 000 deaths each year^[2]. HCC is prevalent in Asia and Africa and its incidence has steadily increased in European and American populations^[3,4]. The majority of patients with HCC die within one year after the diagnosis was established. Unfortunately, HCC is often diagnosed at its late stage when potentially curative therapies are least effective. The 5-year relative survival rate is only 7%^[5]. Patients with surgically resectable localized HCC have a better prognosis, but their 5-year survival rate is only 15%-39%^[6], thus, new therapeutic agents for this malignant disease are urgently needed.

Casticin is one of the main components from *Fructus Viticis* (Manjingzi in Chinese name), a traditional Chinese medicine prepared from the fruit of *Vitex trifolia* L. (family *Verbenaceae*) that is also used as an anti-inflammatory agent and for the treatment of certain cancers in China^[7]. Its chemical structure is shown in Figure 1. Casticin has been shown to inhibit lymphocyte proliferation *in vitro*^[8] and has an anti-inflammatory effect *in vivo*^[9]. In recent years, many studies have demonstrated its anti-carcinogenic activity in breast cancer^[10], lung cancer and colon cancer^[11]. Casticin was also reported to inhibit the growth of human myelogenous leukemia cells^[12] and induce cell death of leukemia cells through induction of apoptosis or mitotic catastrophe^[13]. However, the precise mechanisms underlying casticin inducing apoptosis of HCC cells are still unclear. In the present study, we investigated the effects and molecular mechanism of casticin on the apoptotic cell death of HCC cells *in vitro*. We found that casticin significantly induced apoptosis of HCC cells by glutathione (GSH) depletion and upregulation of DR5.

MATERIALS AND METHODS

Cell culture and reagents

PLC/PRF/5 (*p53* mutant) and Hep G2 (*p53* wild type) human HCC cells were obtained from American Type Culture Collection (Rockville, MD, United States) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin 100 U/mL and streptomycin 100 µg/mL (Life Technologies, Inc., Shanghai, China) in an incubator containing 50 mL/L CO₂ at 37 °C. Casticin was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China), and has a molecular weight

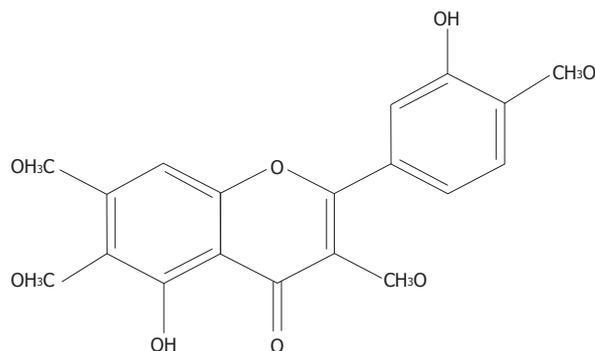


Figure 1 Chemical constitution of casticin.

of 374.3 kDa, appears as yellow crystals and has a purity of 98.0%. Casticin was prepared in dimethyl-sulfoxide (DMSO) as a 10 mmol/L stock solution and diluted in a medium to the indicated concentration before use. The followings were purchased from Hunan Clonetimes Biotech Co., Ltd. (Changsha, China): RPMI-1640 medium (Invitrogen, CA, United States), fetal bovine serum (Invitrogen), Cell Apoptosis enzyme linked immunosorbent assay (ELISA) Detection Kit (Roche), N-acetylcysteine (NAC; Sigma, MO, United States), glutathione (GSH; Sigma), propidium iodide [propidium iodide (PI); Sigma], ethidium bromide (EB; Sigma), N-(4-hydroxyphenyl) retinamide (4HPR; Sigma), butylated hydroxyanisole [butylated hydroxyanisole (BHA); Sigma], mannitol (Sigma), Glutathione Assay kit (Calbiochem, Darmstadt, Germany), Apoptotic DNA Ladder Detection Kit (Bodataike Company, Beijing, China), Caspase 3 Activity Detection Kit (Millipore, MA, United States), Caspase 8 Colorimetric Activity Assay Kit 25 (Millipore), Caspase 9 Colorimetric Activity Assay Kit (Millipore), zVAD-fmk (R and D Systems, MN, United States), zIETD-fmk (R and D Systems), zLEHD-fmk (R and D Systems), 5-fluorouracil (5-FU; Sigma), death receptor (DR)5/Fc chimera protein (R and D Systems), 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes Inc., OR, United States), mouse anti-human DR5 and DR4 (Santa Cruz Biotechnology, CA, United States), Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Zymed Laboratories, CA, United States), mouse IgG1 immunoglobulin (Dako Cytomation, CA, United States).

MTT assay

Cells were seeded in a 96-well plate at a density of 0.5×10^4 cells/well and incubated for 24 h, followed by treatment with various concentrations of casticin or 5-fluorouracil for 24 h. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) colorimetric analysis was performed as described previously^[14]. The IC₅₀ value, i.e., 50% of the cell growth inhibition compared with (DMSO) control, was calculated by nonlinear regression analysis using GraphPad Prism software (San Diego, CA).

Flow cytometry using PI staining

Cells were seeded at a density of 4×10^6 cells/mL in

100 mL culture flasks for 24 h and then treated with the medium containing various concentrations of casticin or 5-fluorouracil for the indicated time. Propidium iodide staining for DNA content analysis was performed as described previously^[15].

Histone/DNA fragment ELISA

The cell apoptosis ELISA detection kit was used to detect apoptosis in cells treated with casticin according to the manufacturer's protocol. Briefly, cells were seeded in a 96-well plate at a density of 1×10^4 cells/well for 24 h, added with the medium containing various concentrations of casticin. After 24 h, we transferred the cytoplasm of the control and treatment group to the 96-well plate peridumed by the streptavidin, incubated with the biotinylated histone antibody and peroxidase-tagged mouse anti-human DNA for 2h at room temperature. The absorbance at 405 nm was measured with EXL-800 type Enzyme-Linked Immunosorbent apparatus.

DNA fragmentation assay

Cells were seeded at a density of 4×10^6 cells/mL in 100 mL culture flasks for 24 h and treated with medium containing various concentrations of casticin for 24 h. This assay was performed as described previously^[15].

Analysis of caspase-3, -8 and -9 activities

To evaluate caspase activity, cell lysates were prepared after their respective treatment with the testing agents. Assays were performed in 96-well plates by incubating 20 μ g cell lysates in 100 μ L reaction buffer (1% NP-40, 20 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, 10% glycerol) containing a 5 μ mol/L caspase-3 substrate Ac-DEVD-*p*NA or caspase-8 substrate Ac-IETD-*p*NA or caspase-9 substrate Ac-LEHD-*p*NA. Lysates were incubated at 37 °C for 2 h. Thereafter, the absorbance at 405 nm was measured with an enzyme-labeling instrument (ELX-800 type). In the caspase inhibitor assay, cells were pretreated with a caspase inhibitor (20 μ mol/L zVAD-fmk or zIETD-fmk or zLEHD-fmk) for 1 h prior to the addition of casticin.

Determination of reactive oxygen species

Intracellular reactive oxygen species (ROS) accumulation was measured by flow cytometry using the fluorescent probe DCFH-DA^[15]. Cells were incubated with 10 μ mol/L DCFH-DA for 30 min at 37 °C in dark. After incubation, the cells were washed with phosphate buffered saline (PBS) and analyzed within 30 min using FACScan (Becton Dickinson, San Jose, CA, United States) equipped with an air-cooled argon laser tuned to 488 nm. The specific fluorescence signals corresponding to DCFH-DA were collected with a 525-nm band pass filter. As a rule, 10 000 cells were counted in each determination.

Measurement of intracellular glutathione

Intracellular GSH contents were measured using a Glutathione Assay kit. In brief, 5×10^6 cells were homogenized in 5% metaphosphoric acid using a Teflon

pestle (Racine, WI). Particulate matter was separated by centrifugation at $4000 \times g$. The supernatant solution was used for GSH measurement according to the manufacturer's instructions. The GSH content was expressed as nmol/106 cells.

Analysis of cell surface receptor expression

Cells were cultured at an indicated concentration for 24 h, and then collected. Five hundred thousand cells for each receptor analysis were transferred to polystyrene tubes, washed twice with PBS and resuspended in PBS containing 0.5% bovine serum albumin (BSA) (Sigma). A specific monoclonal antibody to either DR5, DR4 or unspecific mouse IgG1 as isotype control was applied at 5 μ g/mL. Cells were incubated for 20 min with gentle rocking at room temperature. Cells were washed twice in PBS, and secondary fluorescein isothiocyanate-conjugated polyclonal goat antibody to mouse IgG1 (1:200 in PBS containing 0.5% BSA) was added, followed by incubation protected from light for 30 min with gentle rocking at room temperature. Cells were then washed and resuspended in PBS containing 0.5% BSA. All analyses were carried out on FACScan using CellQuest software (Pharmingen BD Biosciences, CA, United States).

Western blotting analysis

Total cell extracts were obtained as described previously^[15]. Cell lysate containing 50 μ g of protein was separated on a 10% SDS-polyacrylamide gel for electrophoresis and then blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, United States). Anti-DR5, -DR4 and - β -actin (1:1000 dilutions for each) were used as primary antibodies. Signals were detected using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, United States). Images were scanned followed by densitometric analysis with Alphazmager 2200 software (Silk Scientific Inc., Utah, United States). The ratios of DR5 or DR4/ β -actin were determined for the expression level of DR5 or DR4.

Statistical analysis

The database was set up with the SPSS 15.0 software package (SPSS Inc, Chicago, IL, United States) for analysis. Data were presented as mean \pm SD. The means of multiple groups were compared with one-way analysis of variance (ANOVA), after the equal check of variance, and the two-two comparisons among the means were performed using the least-significant difference (LSD) method. Statistical comparison was also performed with two-tailed *t* test when appropriate. *P* < 0.05 was considered statistically significant.

RESULTS

Effects of casticin on growth of hepatocellular carcinoma lines

To characterize the effect of casticin on cell growth, two kinds of cell lines, including PLC/PRF/5 (*p53* mutant) and Hep G2 (*p53* wild type) cells, were treated with various

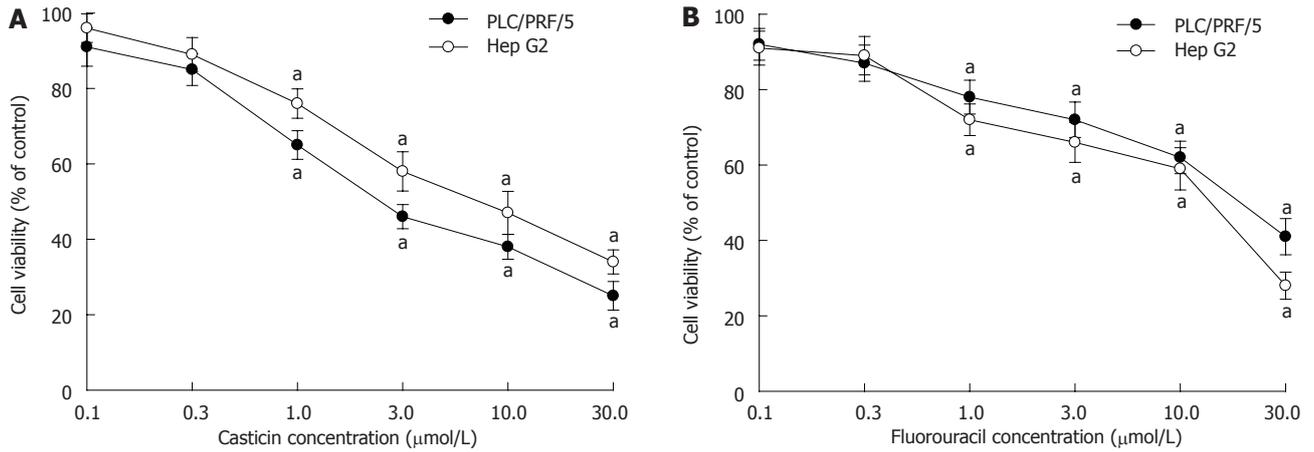


Figure 2 Effects of casticin (A) and fluorouracil (B) on the growth of hepatocellular carcinoma cells using 3-(4,5)-dimethylthiaziazolo (2,4)-bis(4-methyl-5-pyridyl) tetrazolium bromide assay (mean \pm SD, $n = 9$). ^a $P < 0.05$ vs treatment with dimethyl-sulfoxide.

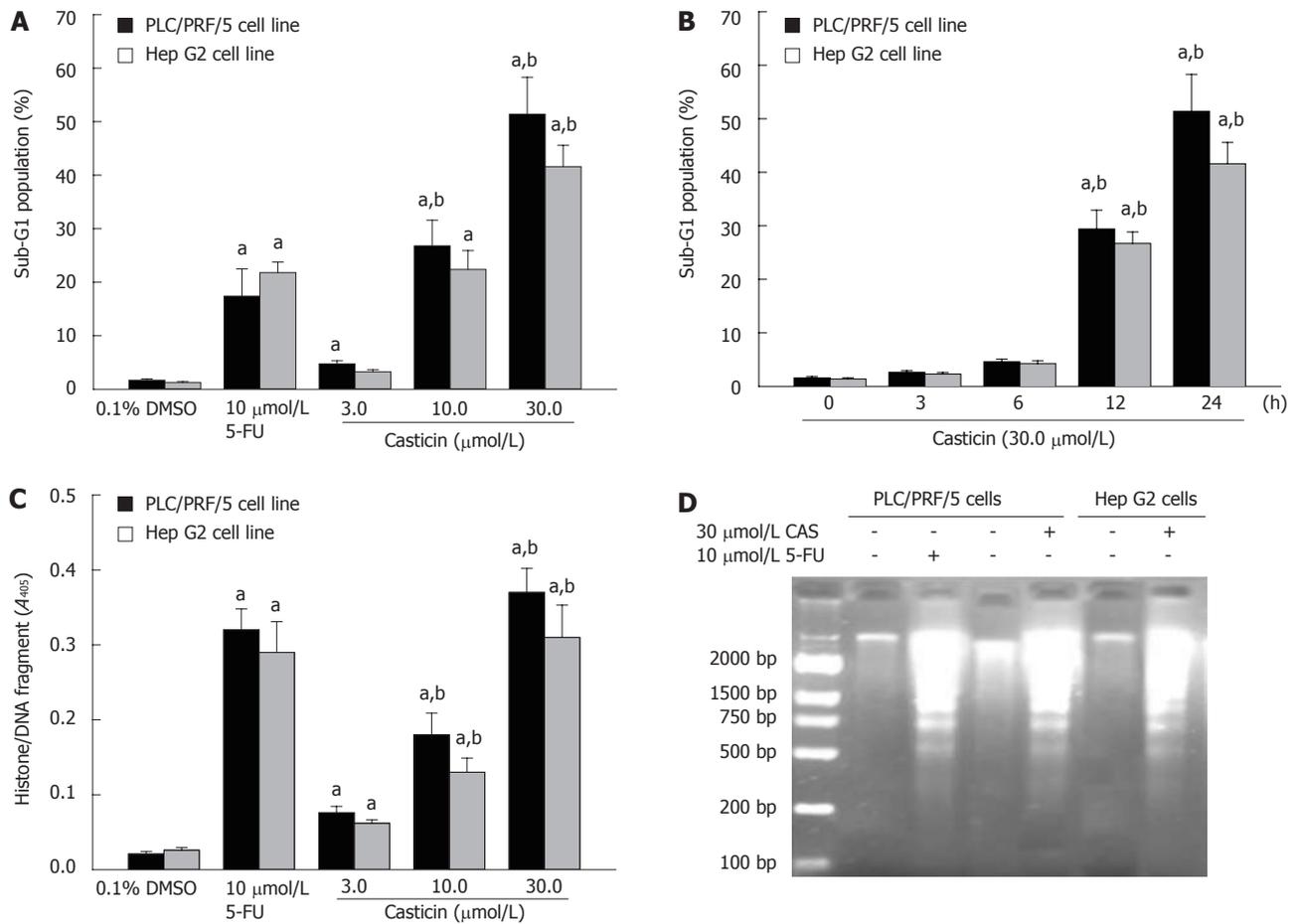


Figure 3 Effects of casticin on the percentage sub-G1 cell population (A and B), histone/DNA fragment (C) and DNA fragmentation (D) in PLC/PRF/5 and Hep G2 cells (mean \pm SD, $n = 3$). ^a $P < 0.05$ vs treatment with dimethyl-sulfoxide (DMSO) or 0 h; ^b $P < 0.05$ vs treatment with 3 μ mol/L casticin or 6 h. 5-FU: 5-fluorouracil.

concentrations of casticin for 24 h, and cell viability was assessed by MTT assay. Figure 2A shows that casticin significantly inhibited the growth of human HCC (PLC/PRF/5 and Hep G2) cells in a dose-dependent manner. When the IC₅₀ for 24 h was 9.4 and 13.6 μ mol/L, respectively, the potency of casticin to PLC/PRF/5 cells was stronger than that of 5-FU with an IC₅₀ of 16.8 μ mol/L (Figure 2B).

Effects of casticin on apoptosis of hepatocellular carcinoma cells

To investigate whether apoptosis was involved in cell growth inhibition by casticin, we detected apoptosis increase using flow cytometric analysis in hypodiploid cell populations. Figure 3A shows that casticin increased the percentage of the sub-G1 cell population in PLC/PRF/5

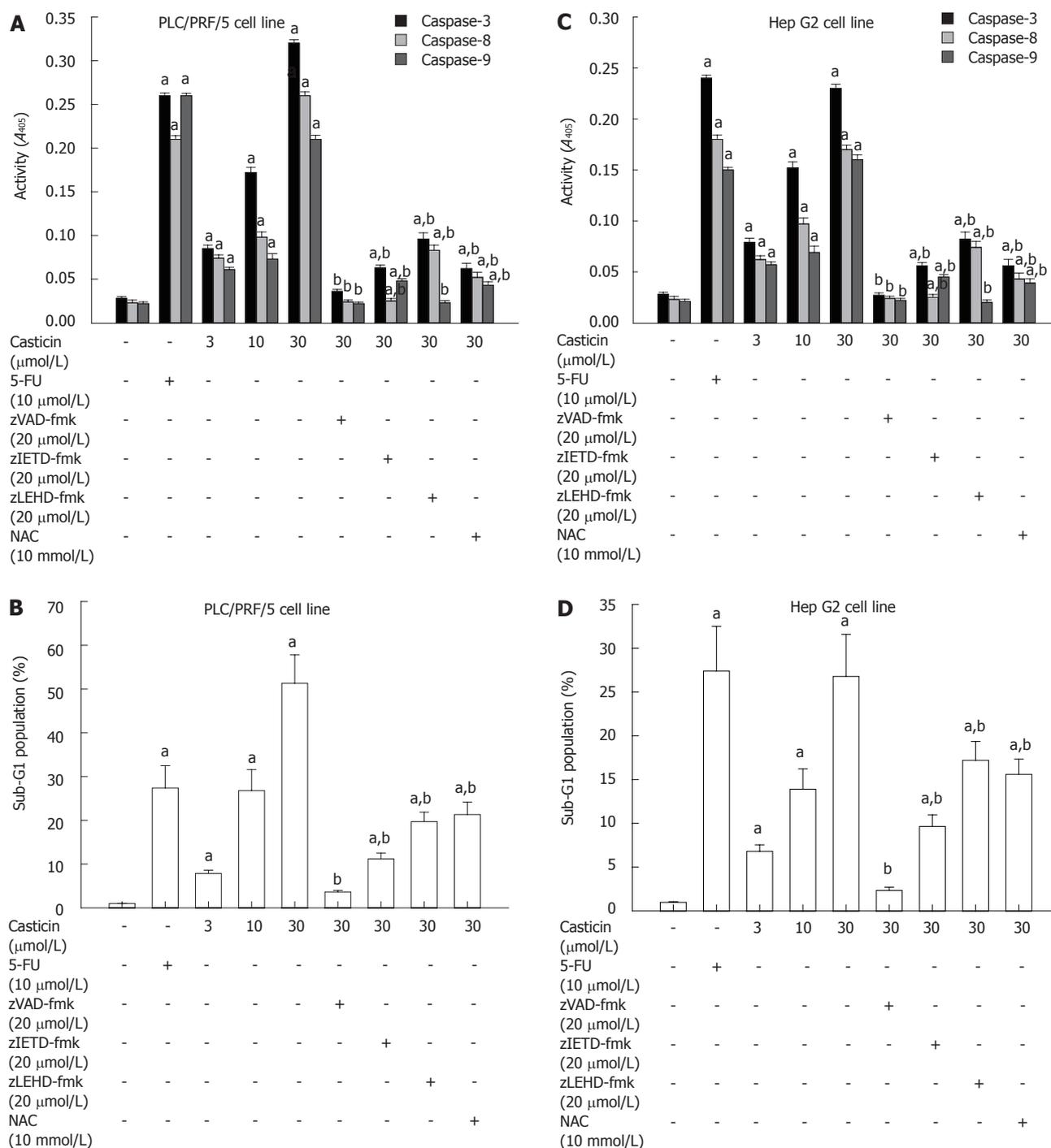


Figure 4 Effects of casticin on the activities of caspases (A and C) and the percentage sub-G1 cell population (B and D) in PLC/PRF/5 and Hep G2 cells (mean \pm SD, $n = 3$). ^a $P < 0.05$ vs treatment with dimethyl-sulfoxide (DMSO); ^b $P < 0.05$ vs treatment with 30 μ mol/L casticin alone. 5-FU: 5-fluorouracil; NAC: N-acetyl-cysteine.

and Hep G2 cells in a concentration-dependent manner ($P < 0.05$). The potency of casticin to PLC/PRF/5 cells was higher than that of 5-fluorouracil (26.8% \pm 4.8% *vs* 17.4% \pm 5.1%) at 10 μ mol/L for 24 h. The sub-G1 population in PLC/PRF/5 and Hep G2 cells by casticin was increased at 12 h and peaked at 24 h (Figure 3B). Histone/DNA fragment of PLC/PRF/5 and Hep G2 cells, as measured by the cell apoptosis ELISA detection kit, was increased in a dose-dependent manner ($P < 0.05$) after treatment with casticin (Figure 3C). Furthermore, DNA fragmentation analysis by agarose gel electrophoresis showed a typical ladder pattern of internucleosomal DNA

fragments in PLC/PRF/5 and Hep G2 cells treated with 30 μ mol/L casticin for 24 h (Figure 3D). These results suggested that casticin inhibited HCC cell growth through a mechanism involving the induction of apoptosis.

Effects of casticin on caspases activities of hepatocellular carcinoma cells

To determine the effectors active in casticin-induced apoptotic pathways, we examined whether caspases were actually activated during casticin-induced cell death of HCC cells. Figure 4A shows that treatment of PLC/PRF/5 cells with casticin for 24 h increased the levels of

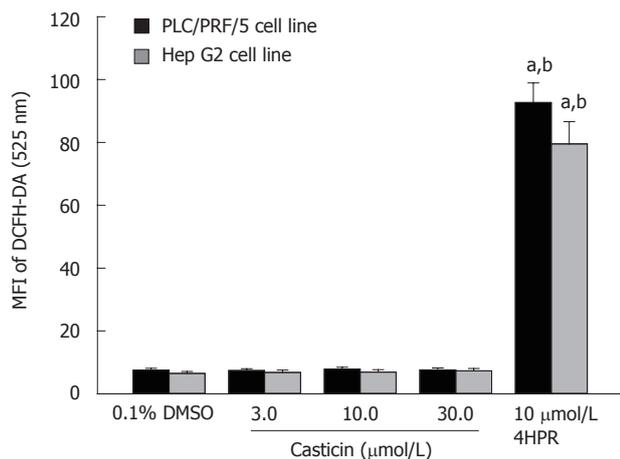


Figure 5 Effects of casticin on the production of reactive oxygen species in PLC/PRF/5 and Hep G2 cells (mean \pm SD, $n = 3$). ^a $P < 0.05$ vs treatment with dimethyl-sulfoxide (DMSO); ^b $P < 0.05$ vs treatment with 30 $\mu\text{mol/L}$ casticin. 4HPR: N-(4-hydroxyphenyl) retinamide.

active caspase-3, -8 and -9 ($P < 0.05$) in a concentration-dependent manner ($P < 0.05$).

We further examined the role of caspases activated during apoptosis induced by casticin treatment using the pan-caspase inhibitor zVAD-fmk, the caspase-8 inhibitor zIETD-fmk and the caspase-9 inhibitor zLEHD-fmk. Figure 4B shows that zVAD-fmk abrogated apoptosis induced by casticin and zIETD-fmk and zLEHD-fmk attenuated casticin-induced apoptosis. The similar findings were observed in the Hep G2 cell line (Figure 4C and D). These data indicate that casticin-induced apoptosis was essentially dependent on the activation of caspase-3, -8 and -9.

Effects of casticin on reactive oxygen species generation of hepatocellular carcinoma cells

Because NAC, an antioxidant, could attenuate induction of apoptosis by casticin (Figure 4B and D), it was plausible to speculate that casticin-induced apoptosis by promoting intracellular ROS generation. Thus, we examined whether casticin promoted ROS generation in HCC cells. Unexpectedly, we failed to detect any increase of intracellular ROS generation in PLC/PRF/5 and Hep G2 cells treated with casticin, at concentrations ranging from 3.0 to 30.0 $\mu\text{mol/L}$. 4HPR, an agent known to promote ROS generation in cancer cells^[16,17], did increase the ROS generation in HCC cell lines (Figure 5).

Effects of casticin on intracellular glutathione content of hepatocellular carcinoma cells

Earlier studies demonstrated that flavonoid toxicity was strictly dependent on the intracellular GSH content^[18,19]. We measured the intracellular GSH in cells treated with casticin. In PLC/PRF/5 cells treated with casticin (3.0, 10.0 and 30.0 $\mu\text{mol/L}$) for 24 h, the intracellular GSH content significantly was decreased in a concentration-dependent manner (Figure 6A). In addition, time-course studies indicated that casticin caused a progressive decrease in GSH content from 1 h onwards (Figure 6B).

Importantly, the pan-caspase inhibitor z-VAD-fmk, which successfully prevented apoptosis execution, failed to prevent GSH decrease (Figures 4A and B, 6A and C). This excludes the possibility that GSH depletion in casticin-treated cells could be a trivial, secondary consequence of cell death.

To shed light on the mechanisms accounting for GSH depletion as well as on the relationship between GSH depletion and apoptosis induction, we determined the effects of thiol antioxidants including NAC and GSH, and nonthiol antioxidants including butylated hydroxyanisole (BHA) and mannitol on GSH content and apoptosis by casticin treatment. Figure 6A and C show that thiol antioxidants, NAC and GSH, restored GSH content and attenuated casticin-induced apoptosis. In contrast, nonthiol antioxidants, BHA and mannitol, failed to do so (Figure 6A and C). The similar findings were observed in Hep G2 cell line (Figure 6D-F). The results suggest that casticin-induced apoptosis of HCC cells is at least partially dependent on GSH depletion.

Effects of casticin on DR5 expression of hepatocellular carcinoma cells

Because casticin induced caspase-8 activation (Figure 4A), we wondered whether casticin induced DR5 upregulation. Therefore, we compared the effects of casticin on the expression of DR4 and DR5 using Western blotting. In PLC/PRF/5 cell line, casticin increased the expression of DR5 at protein levels, but not affected the expression of DR4 protein; however, it failed to do so in the presence of NAC (Figure 7A). In addition, flow cytometry (FCM) analysis showed that casticin increased DR5 protein levels on the surface of Hep G2 cells by casticin treatment, but DR4 expression levels were not obviously altered (Figure 7 C).

To further define the role of DR5 upregulation in casticin-induced apoptotic cell death, we examined the effects of DR5/Fc chimera protein, a blocking antibody on induction of apoptosis by casticin. Figure 7B shows that the pretreatment with 1 $\mu\text{g/mL}$ DR5/Fc chimera protein effectively attenuated induction of apoptosis by casticin in PLC/PRF/5 and Hep G2 cells. Our findings suggest that casticin-induced apoptosis of HCC cells is involved in upregulation of DR5.

DISCUSSION

The polymethoxyflavone from *Fructus Vitis*, casticin is a potent novel molecule with a wide range of actions, many of which are potentially useful for cancer prevention or treatment^[20-23]. In the present study, we investigated the effects of casticin on the cell growth using two kinds of human HCC (PLC/PRF/5 with *p53* mutant and HepG2 with *p53* wild type) cell lines. We have demonstrated that casticin was a potent agent in inhibiting the growth of human HCC cells. The cancer suppressor *p53* is an important factor that affects the cell response to drug effects on growth inhibition and

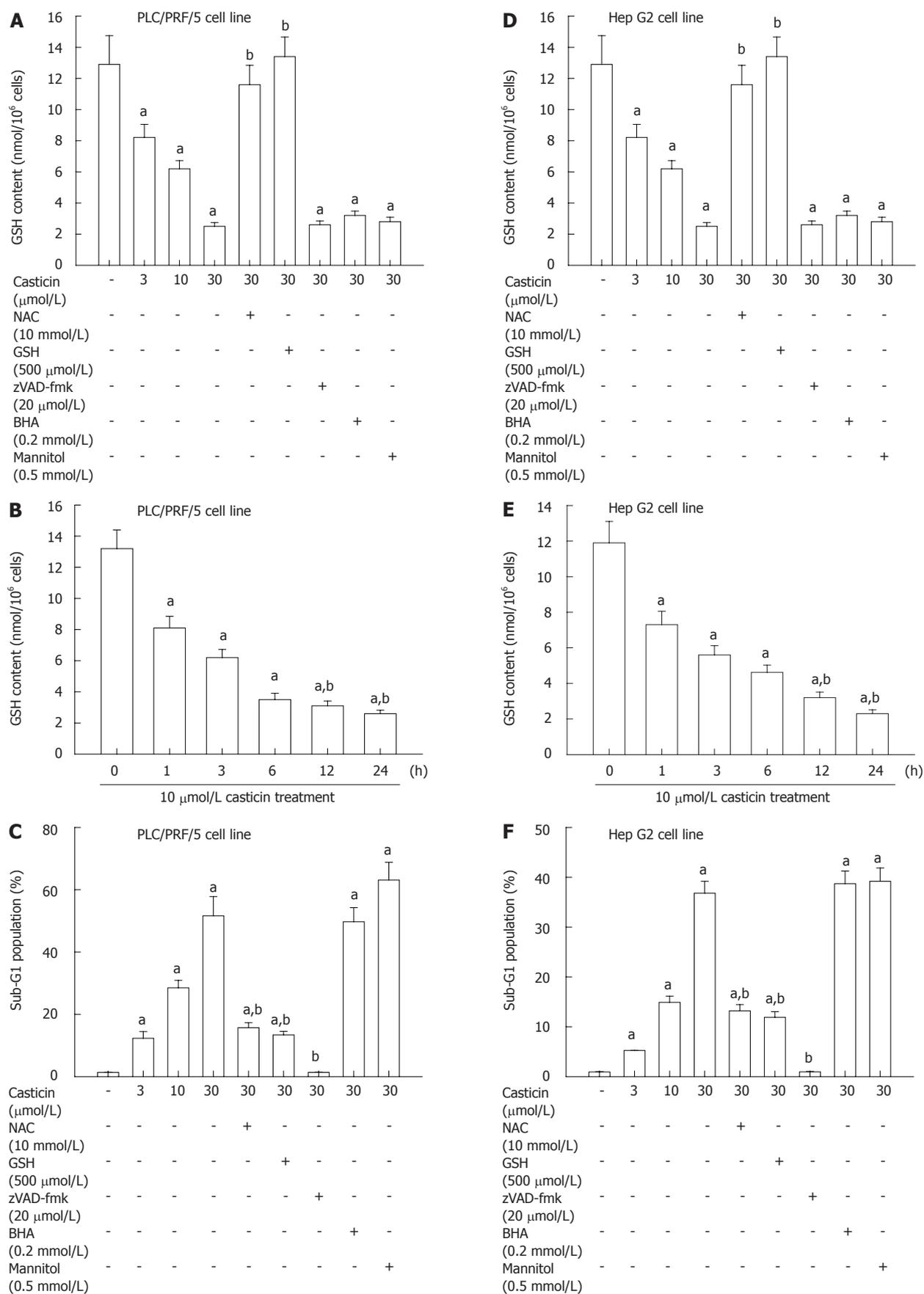


Figure 6 Effects of casticin on intracellular glutathione content (A, B and D, E) and the percentage sub-G1 cell population (C and F) in PLC/PRF/5 and Hep G2 cells (mean \pm SD, $n = 3$). ^a $P < 0.05$ vs treatment with dimethyl-sulfoxide (DMSO) or 0 h; ^b $P < 0.05$ vs treatment with 30 $\mu\text{mol/L}$ casticin alone or treatment with 10 $\mu\text{mol/L}$ casticin for 1 h. GSH: Glutathione; BHA: Butylated hydroxyanisole; NAC: N-acetyl-cysteine.

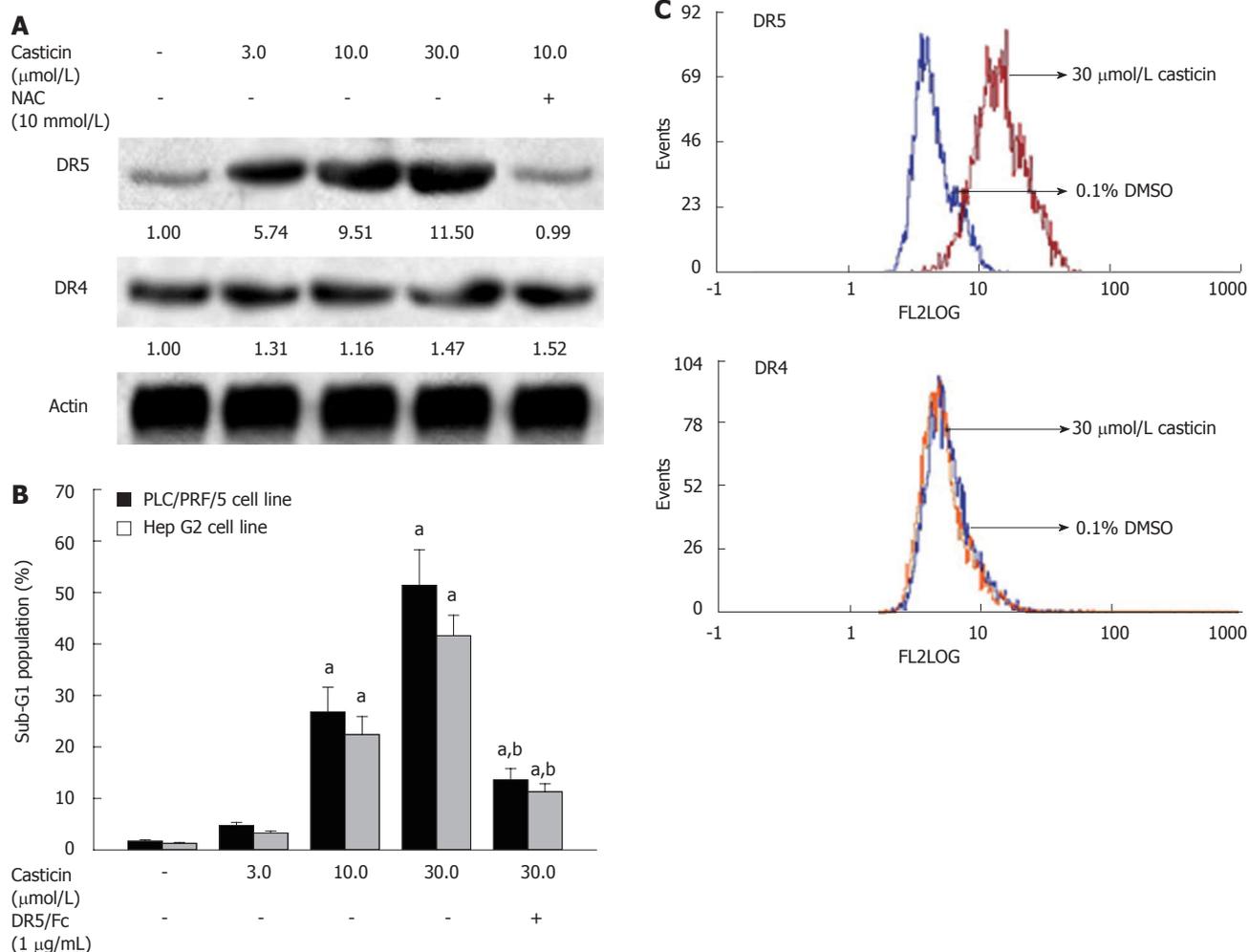


Figure 7 Effects of casticin on the levels of death receptors (DR4 and DR5) expression(A) using Western blotting and the percentage sub-G1 cell population (B) in PLC/PRF/5 cells (mean ± SD, n = 3). ^aP < 0.05 vs treatment with dimethyl-sulfoxide (DMSO); ^bP < 0.05 vs treatment with 30 μmol/L casticin alone. C: Effects of casticin on the levels of death receptor (DR)4 and DR5 protein levels on the surface of Hep G2 cells using flow cytometry. NAC: N-acetyl-cysteine.

apoptosis induction^[24,25]. The majority of evidence supports the notion that cells with wild-type *p53* exhibit increased sensitivity to radiation or chemotherapeutic agents, whereas cells lacking wild-type *p53* expression still undergo apoptosis but need a relatively high dose of radiation or chemotherapeutic drugs^[24,25]. The study by Häidara *et al*^[10] demonstrated the apoptotic effect of casticin in *p53* mutant breast cancer cell lines. Our results indicated that casticin inhibited cell growth and induced apoptosis regardless of *p53* status in human HCC cells. Therefore, we conclude that casticin-induced apoptosis in human cancer cells is *p53*-independent.

Apoptotic cells presented some common characteristics. In the stage of apoptosis, activation of a cascade of various caspases, was followed by DNA fragmentation, nuclear fragmentation, the appearance of apoptotic bodies and cellular shrinkage. Hence, caspase-3 activation, sub-G1 cell population, Histon/DNA fragment and DNA ladder were regarded as the characters specific for apoptosis. Our present study showed activation of caspase-3, increase of the percentage of sub-G1 population and Histone/DNA fragment and presentation of DNA

ladder in casticin-treated HCC cells, demonstrating that casticin inhibited HCC cell growth through induction of apoptosis.

We also demonstrated that casticin induced apoptosis by depleting intracellular GSH content, rather than by promoting ROS generation, in HCC cells. Our findings are supported by the following lines of evidence: (1) casticin did not promote intracellular ROS generation although it decreased intracellular GSH content; and (2) Thiol-containing antioxidants rather than nonthiol antioxidants reduced casticin-induced apoptosis; this suppression correlated with their ability to prevent a casticin-induced decrease of GSH content. NAC, an aminothiols and synthetic precursor of intracellular cysteine and GSH, functions through either its antioxidative/radical scavenging properties as an antioxidant or its thiol-disulfide exchange activity as a reductant^[26,27]. We found that only thiol containing antioxidants with reducing activity, including NAC and GSH, rather than nonthiol antioxidants, including BHA and mannitol, suppressed casticin-induced apoptosis. Therefore, it is likely that NAC and GSH inhibit casticin-induced apoptosis *via* their reducing activ-

ity. It appears that our findings have clinical implications regarding the rational use of casticin in combination with other agents for cancer prevention and/or treatment. The polymethoxyflavone should not be used in combination with the agents with reducing activity, such as NAC, in order to avoid the potential contradictory interaction.

The activation of caspases plays an important role in apoptosis triggered by various proapoptotic signals^[28,29]. It is generally recognized that there are two major apoptotic pathways: one involves death signals transduced through death receptors, and the other relies on a signal from the mitochondria^[22,28]. Both pathways are involved in an ordered activation of a set of caspases, which in turn cleave cellular substrates leading to the morphological and biochemical changes of apoptosis. The activation of caspase-8 and caspase-9 has been documented to play central roles in mediating apoptosis signaled by death receptors and by mitochondria, respectively^[15,28]. However, caspase-8 can activate the caspase-9-mediated apoptotic pathway *via* activating or cleaving the Bid protein^[28,29]. We found that casticin activated caspase-8 and -9. The presence of the caspase-8 inhibitor z-IETD-fmk and the caspase-9 inhibitor zLEHD-fmk attenuated the apoptosis induced by casticin, indicating that caspase-8 and -9 activation were required for the apoptosis induced by casticin in PLC/PRF/5 cells. Moreover, casticin up-regulated DR5 expression, and DR5/Fc chimera protein reduced casticin-induced apoptosis, which shows that DR5 upregulation is required for casticin-induced apoptosis. Therefore, our results, for the first time, highlight a novel simultaneous both death receptor and mitochondria-mediated mechanism by casticin-induced apoptosis in HCC cells. The study by Zou *et al.*^[30] demonstrated that the synthetic triterpenoid methyl-2-cyano-3,12-dioxoolean-1,9-dien-28-oate induced upregulation of DR5 expression by activation of the CCAAT/enhancer binding protein homologous protein (CHOP) *via* intracellular GSH depletion-induced the endoplasmic reticulum stress. Quercetin, a flavonoid compound decreases intracellular GSH content and potentiates the apoptotic action of the antileukemic drug, arsenic trioxide, in human leukemia cell lines^[31]. However, why and how the intracellular GSH depletion induces DR5 expression up-regulation need to be further investigated.

In summary, our studies have demonstrated that the polymethoxyflavone from *Fructus viticis*, casticin is a potent apoptosis-inducing agent in human HCC cells, which acts through depleting intracellular GSH content and upregulating DR5, and subsequent activation of caspase-3, -8 and -9. Moreover, casticin inhibited the growth of HCC cells independent of *p53* status. For this reason, we suggest that casticin may be a good candidate for additional evaluation as a cancer therapeutic agent for human HCC as well as other types of cancer.

neoplasm in the world, leading to over 600 000 deaths each year. HCC is prevalent in Asia and Africa and its incidence has steadily increased in European and American populations. New therapeutic agents for this malignant disease are urgently needed.

Research frontiers

Casticin is one of the main components from *Fructus Viticis* (Manjingzi in Chinese name), a traditional Chinese medicine prepared from the fruit of *Vitex trifolia* L. (family *Verbenaceae*) that is also used as an anti-inflammatory agent and for the treatment of certain cancers in China. In recent years, many studies have demonstrated its anti-carcinogenic activity in breast cancer, lung cancer and colon cancer. However, the precise mechanisms underlying casticin-induced apoptosis of HCC cells is still not clear.

Innovations and breakthroughs

In this study, the effects and molecular mechanism of casticin on apoptotic cell death of HCC cells *in vitro* were studied. Casticin significantly induced apoptosis of HCC cells by glutathione (GSH) depletion and upregulation of reactive oxygen species.

Applications

Casticin is a potent apoptosis-inducing agent in human HCC cells, which acts through depleting intracellular GSH content and upregulating DR5, and subsequent activation of caspase-3, -8 and -9. Moreover, casticin inhibited the growth of HCC cells independent of *p53* status. The authors suggested that casticin may be a good candidate for additional evaluation as a cancer therapeutic agent for human HCC as well as other types of cancer.

Terminology

Casticin is one of the main components from *Fructus Viticis* (Manjingzi in Chinese name), a traditional Chinese medicine prepared from the fruit of *Vitex trifolia* L. (family *Verbenaceae*).

Peer review

In this manuscript, the authors demonstrate that the treatment with casticin, main components from *Fructus Viticis*, induced apoptosis through the depletion of glutathione DR5 up-regulation in HCC cells. A series of experiments were well planned and well performed, and the manuscript is well written. The findings are important to those with closely related research interest.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is currently the fifth most common malignant

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High resolution colonoscopy in a bowel cancer screening program improves polyp detection

Matthew R Banks, Rehan Haidry, M Adil Butt, Lisa Whitley, Judith Stein, Louise Langmead, Stuart L Bloom, Austin O'Bichere, Sara McCartney, Kalpesh Basherdas, Manuel Rodriguez-Justo, Laurence B Lovat

Matthew R Banks, Rehan Haidry, M Adil Butt, Lisa Whitley, Judith Stein, Louise Langmead, Stuart L Bloom, Austin O' Bichere, Sara McCartney, Kalpesh Basherdas, Laurence B Lovat, Department of Gastrointestinal Services, University College London Hospitals NHS Foundation Trust, London NW1 2BU, United Kingdom

Manuel Rodriguez-Justo, Department of Histopathology, University College London Hospitals NHS Foundation Trust, London NW1 2BU, United Kingdom

Author contributions: Banks MR and Lovat LB designed the study; Banks MR, Whitley L, Stein J, Langmead L, Bloom SL, O'Bichere A, McCartney S, Rodriguez-Justo M, and Basherdas K performed the research; Banks MR, Haidry R, Butt MA and Lovat LB wrote and edited the paper and related articles.

Supported by Proportion of UCLH/UCL funding from the Department of Health's NIHR Biomedical Research Centres funding scheme; A grant from the UCL experimental cancer medicine centre; Unrestricted educational grant support from Pentax United Kingdom (Lovat LB)

Correspondence to: Dr. Laurence B Lovat, Department of Gastrointestinal Services, University College London Hospitals NHS Foundation Trust, London NW1 2BU, United Kingdom. l.lovat@uclh.nhs.uk

Telephone: +44-203-4567890 Fax: +44-207-8132828

Received: February 14, 2011 Revised: May 19, 2011

Accepted: May 26, 2011

Published online: October 14, 2011

Abstract

AIM: To compare high resolution colonoscopy (Olympus Lucera) with a megapixel high resolution system (Pentax HiLine) as an in-service evaluation.

METHODS: Polyp detection rates and measures of performance were collected for 269 colonoscopy procedures. Five colonoscopists conducted the study over a three month period, as part of the United Kingdom bowel cancer screening program.

RESULTS: There were no differences in procedure du-

ration ($\chi^2 P = 0.98$), caecal intubation rates ($\chi^2 P = 0.67$), or depth of sedation ($\chi^2 P = 0.64$). Mild discomfort was more common in the Pentax group ($\chi^2 P = 0.036$). Adenoma detection rate was significantly higher in the Pentax group (χ^2 test for trend $P = 0.01$). Most of the extra polyps detected were flat or sessile adenomas.

CONCLUSION: Megapixel definition colonoscopes improve adenoma detection without compromising other measures of endoscope performance. Increased polyp detection rates may improve future outcomes in bowel cancer screening programs.

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Key words: High resolution colonoscopy; Bowel cancer screening; Polyp detection

Peer reviewer: Marc D Basson, Dr., Department of Surgery, Wayne State University and John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48201, United States

Banks MR, Haidry R, Butt MA, Whitley L, Stein J, Langmead L, Bloom SL, O'Bichere A, McCartney S, Basherdas K, Rodriguez-Justo M, Lovat LB. High resolution colonoscopy in a bowel cancer screening program improves polyp detection. *World J Gastroenterol* 2011; 17(38): 4308-4313 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4308.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4308>

INTRODUCTION

Colorectal cancer is one of the most common cancers worldwide, particularly in Europe and the United States^[1]. Detection of cancer at an early stage, as well as detection and removal of polyps, is likely to decrease mortality from the disease. Colonoscopy is now established as the gold standard for the identification of both colorectal cancer and

polyps^[2]; therefore, the accuracy of the procedure is very important. The UK bowel cancer screening program has been established to reduce deaths from colorectal cancer utilising colonoscopy for patients screened positive by faecal occult blood tests. However, the colonoscopic miss rate of adenomas is as high as 24%^[3] and the false negative colonoscopy rate for colorectal cancers appears to be up to 6%^[4]. It is therefore desirable to investigate methods that could improve the accuracy of colonoscopy, particularly as a higher adenoma detection rate is associated with lower rates of subsequent development of colon cancer^[5]. It has been suggested that screening efficacy requires a high quality examination and removal of all visible neoplastic lesions. It is plausible that higher image resolution will help achieve these aims^[6-8]. For bowel cancer screening, we currently use Olympus Lucera colonoscopes and Scope Guide system for colorectal cancer screening. The new Pentax HiLine colonoscopes have a higher image resolution and might, therefore, provide better detection of visible polyps and early cancers. Pentax scope handling is different to Olympus scopes, and patient comfort and procedure performance may therefore be altered. This in-service evaluation of the new Pentax HiLine colonoscopes aimed to compare procedure duration, caecal intubation rates, patient comfort, and polyp detection with those obtained by the Olympus Lucera system.

MATERIALS AND METHODS

All patients undergoing colonoscopy in the bowel cancer screening program at University College London Hospitals NHS Foundation Trust between August and November 2009 were included in this prospective study. Routine bowel cancer screening colonoscopies are usually performed in our unit with the Olympus Lucera series of colonoscopes (CF-Q260DL colonoscopes and CLV 260-SL processor). There are five bowel cancer screening colonoscopy lists per week. During the study period, one of the screening lists was allocated to be performed with a Pentax HiLine colonoscopy (EC-3890i).

A specialist bowel cancer screening nurse collected data on completeness of insertion to caecum or terminal ileum, duration of insertion, withdrawal of colonoscopy, and total length of procedure in real time. In addition, the nurse noted the amount of sedation used, the level of conscious sedation (awake, drowsy, asleep), and degree of discomfort suffered by the patient during the procedure. This was classified as minimal, mild, moderate, or severe using a nurse-evaluated score in line with the National Bowel Cancer Screening standards. The location and size of polyps, as well as removal and retrieval rates, were collected. Polyps were classified by the histopathologist in charge of running the bowel cancer screening program at UCLH.

Statistical analysis was performed using non parametric Mann Whitney tests, where Gaussian approximation did not occur, and unpaired *t* tests where it did, for ex-

ample in comparing polyp sizes. Linear regression analysis was used to assess learning curves, and χ^2 contingency tests were used to compare patient parameters between scope types.

The University College London hospitals research ethics committee considered this study to be an in-service evaluation. Ethical approval was therefore not required.

RESULTS

A total of 269 procedures were recorded. Forty-four were performed with the new Pentax HiLine colonoscopes and the rest were performed with Olympus Lucera series colonoscopes. Five colonoscopists performed the procedures. An important limitation to our in-service evaluation was that most of the procedures performed with the Pentax Scopes were completed by a single colonoscopist. This colonoscopist also performed a significant number of colonoscopies with the Olympus Lucera scopes. We therefore analysed all parameters for this single endoscopist between the two scopes, as well as for all procedures performed by all endoscopists. We found no difference between these analyses and therefore present the overall findings only. All the study colonoscopists are accredited for the UK bowel cancer screening programme and during the study, all detected adenomas in at least 40% of procedures, demonstrating their competence^[5,9].

The Pentax HiLine colonoscopes were new in our unit, and these have different handling characteristics to the Olympus Lucera Scopes; therefore, we analysed the learning curve, as measured by duration of procedure and time to reach the caecum, as none of the endoscopists in this study had used this colonoscopy before. There was no significant change in either of these parameters throughout the study, suggesting that there was no significant learning curve. There was no difference in caecal intubation time, duration of withdrawal, or in total procedure duration with either type of scope or between endoscopists.

No statistically significant difference was found in the caecal intubation rate, which was 100% with Olympus Scopes and 95% with the Pentax Scopes [$\chi^2 P = 0.67$, not significant (NS)]. Terminal ileal intubation was 54% with Olympus and 55% with Pentax scopes ($\chi^2 P = 0.38$, NS).

Equivalent doses of midazolam or fentanyl were used with both types of scope, with a median dose of 2 mg midazolam and 50 μ g fentanyl. The depth of sedation was equivalent ($\chi^2 P = 0.64$) and the majority of patients were drowsy in both groups. More patients suffered mild discomfort with Pentax scopes (44%) compared to Olympus colonoscopies (16%), ($\chi^2 P = 0.036$). There was no increase in moderate discomfort, and no patients in either group suffered severe discomfort during the procedures (Figure 1).

Although all colonoscopists demonstrated a high pick up rate of adenomas with both colonoscopes; a higher proportion of patients had polyps picked up when examined with the Pentax scopes (66%) compared to

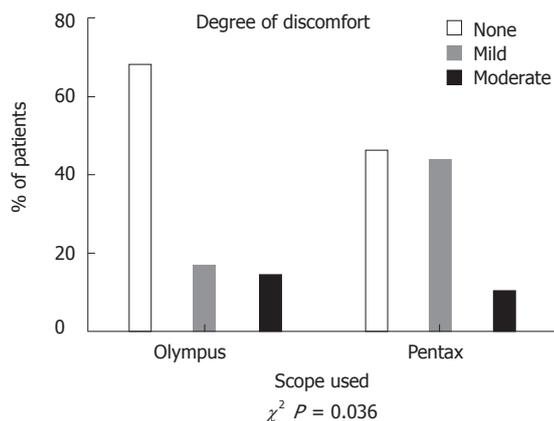


Figure 1 Discomfort scores during colonoscopy. The degree of mild discomfort was worse for patients undergoing colonoscopy with Pentax scopes; however, there was no increase in moderate discomfort and no patients suffered severe discomfort with either scope.

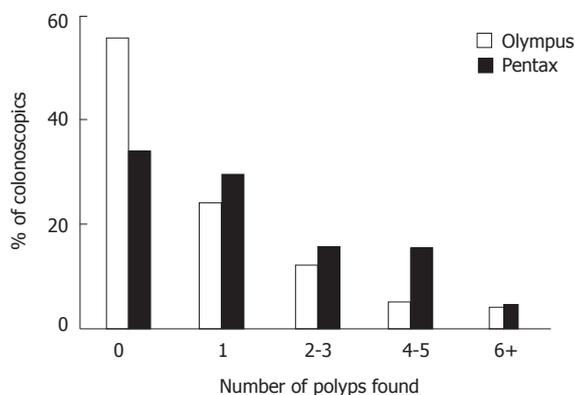


Figure 2 Polyp detection rates. More polyps were found with Pentax HiLine colonoscopes than with Olympus Lucera colonoscopes. χ^2 test for trend $P = 0.01$.

Olympus scopes (44%). The median number of polyps detected per procedure was also higher at one (IQR 0-3) for Pentax compared to zero (IQR 0-1) for Olympus (χ^2 for trend $P = 0.01$) (Figure 2). The median size of polyps was identical at 4 mm in the Olympus group (IQR 2-8) and 3 mm in the Pentax group (IQR 2-8) ($\chi^2 P = 0.98$) (Figure 3). In both groups, approximately one quarter of the polyps were pedunculated and the other three quarters were sessile in nature (Fisher exact test $P = 0.74$. NS). More importantly, the majority of polyps found with both colonoscopes were adenomas. Although smaller polyps were more likely to be hyperplastic, there was no statistically significant difference in polyp histology whichever scope was used.

DISCUSSION

The principal aim of the bowel cancer screening programme in the United Kingdom is to reduce the mortality from colorectal cancer by the early detection of cancerous or pre-cancerous lesions. The accuracy of colonoscopy in identifying these lesions is vital to the success of the program. Factors important in the optimisation of the test

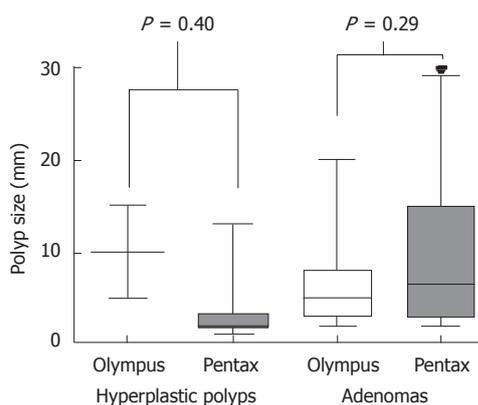


Figure 3 Sizes of polyps. Box plot demonstrating that the median size of polyps was identical for both hyperplastic and adenomatous polyps for both type of colonoscope. Median, interquartile range, and minimum and maximum polyp sizes are shown for each polyp type and colonoscope.

include the bowel preparation, operator skill, withdrawal time, and the image quality^[5,7-11]. On the basis of prevalence of adenomas and caecal intubation rates in studies of screening colonoscopy, threshold values for rates of adenoma detection of 15% amongst women and 25% amongst men over the age of 50 have been proposed in the United States^[7,12]. These have received support from a recent European Study^[5]. All endoscopists taking part in the United Kingdom bowel cancer screening services must demonstrate a minimum level of competence that exceeds these thresholds.

Several studies have demonstrated the potential advantage of utilising additional optical technology, such as narrow band imaging in Olympus scopes, to improve polyp detection, although results are conflicting^[13-16]. Pentax HiLine colonoscopes also have enhanced optical imaging capability, using the iScan surface and contrast enhancements^[17]; however, our aims were to investigate whether white light colonoscopy with increased image resolution alone may be sufficient to improve detection, as neither narrow band imaging nor iScan enhancements are used routinely.

The quality of the final endoscopy image viewed on the screen is dependent upon all the components in the system, including the charge coupled device (CCD) chip within the scope, the processor, the cables, and the screen. CCD chips in the newer “high resolution” scopes contain more pixels, and have increased by an order of magnitude from 100 000 pixels in the older standard definition scopes to 1.3 million pixels in the latest scopes^[18]. The current displays are “high definition” displaying 1080 lines, thereby further improving image quality. The Olympus Lucera colonoscope series has been available in the United Kingdom since 2003 and were the first high resolution endoscopes. The Pentax HiLine series has been marketed since 2009. The images from both video systems are viewed on a high-definition TV screen (1080 lines), but the Olympus colonoscopes have a resolution range from 400 000 to 700 000 pixels whereas the Pentax Scopes have a much higher resolution at 1.3 megapixels. It is therefore expected that the new Scopes would have

a better detection rate for colonic polyps. This study has confirmed this finding, showing that there is a significantly increased chance of detecting polyps with the HiLine system compared to the Lucera system. More importantly, these polyps are significant in that they are adenomas and of a similar size to those detected with the Olympus Lucera Scopes.

The American Gastroenterological Association “Guidelines on screening and surveillance for colorectal cancer”^[19] consider any adenomatous polyp, irrespective of size, to be a significant risk factor for the development of further high risk polyps or colorectal cancer. The prevalence in one large study reporting on 4967 patients identified that the majority (69%) of advanced adenomas detected were < 10 mm size. Even among polyps \leq 5 mm, there was an appreciable prevalence of advanced adenomas (10%)^[20]. Combining this with the ability to now accurately predict polyp type *in vivo* with the modified Kudo pit pattern and vascular colour intensity (VCI) analysis^[21], enables colonoscopists to decide on which polyps to remove *in vivo*, irrespective of size. Consistent with this, most polyps removed in this paper were adenomas on histology.

The estimated 10-year CRC risk for unresected diminutive (< 5 mm), small (5-9 mm), and large (\geq 10 mm) polyps in a decision analysis for CT colonography (CTC) in the United States was 0.08%, 0.7%, and 15.7%, respectively; however, this analysis considered all polyps detected at CTC for these estimations. With modified Kudo pit pattern classification and VCI, accurate *in vivo* characterisation of polyps < 10mm can be predicted with 94% sensitivity and 89% specificity^[22]. This allows non-adenomatous polyps to be resected and discarded without the need for histological assessment. Full economic modelling would be needed to assess the overall cost savings; however, the potential cost savings of not sending diminutive polyps for formal histopathology is thought to exceed 95 million dollars per year in the United States alone^[23].

Rembacken and colleagues^[24] have demonstrated that flat and depressed polyps are more likely to contain high grade dysplasia or invasive cancer than polypoid lesions; however, they are less easily identified and, therefore, are more likely to be missed on colonoscopy. Moreover, it is suggested that the advanced cancers appearing within three years of a negative colonoscopy may have developed from these subtle lesions^[24,25]. A recent population-based study showed that 8% of colorectal cancers were missed by colonoscopy performed within the previous three years^[26]. The improved overall polyp detection rate of megapixel high resolution colonoscopies demonstrated by our study, particularly for small flat adenomas could significantly improve outcomes of the bowel cancer screening program, although this hypothesis clearly needs to be formally tested in a prospective randomised controlled trial.

The Lucera colonoscopes have variable stiffness and are the standard scopes used by the majority of endoscopists in United Kingdom. The Pentax colonoscopes do not have a variable stiffness feature and feedback from in-

dividual endoscopists prior to the start of the study suggested that patients may find these scopes more uncomfortable than the Lucera Scopes. This study confirmed this finding, although the degree of added discomfort was only mild and only occurs in one quarter of patients. For the majority therefore, there was no difference in discomfort score between the two types of colonoscope. Importantly, patients did not require any more sedation. We routinely use ScopeGuide with our Lucera colonoscopes, which helps us to manage scope looping. We do not have this feature with the Pentax scopes and this might also explain the increase in mild discomfort in this group of patients.

Operators also suggested that endoscopists’ performance with the new Pentax scopes may be reduced due to changes in handling from the Olympus scopes. The performance, as measured by caecal intubation rates and procedural times, were no different between the two scopes. Moreover, no performance learning curve was detected.

Missing polyps when performing a colonoscopy is a serious problem. Several advanced imaging techniques have therefore been developed, including dye sprays, narrow band imaging, and endomicroscopy, amongst others. However, these techniques can be time consuming and require training and experience. Only techniques that are easy to perform and can be done without “high end” expertise by all appropriately trained endoscopists are suitable for screening programs. For this reason, the introduction of better image quality may be a simple solution to the problem of missed polyps at colonoscopy.

It is worth remembering that all colonoscopists who are accredited for bowel cancer screening have to demonstrate very high standards of caecal intubation and polyp detection. The fact that scope handling was no different between the scopes may not be generally applicable to all endoscopists, but it is likely to be applicable to all colonoscopists who are accredited to do bowel cancer screening, even if they are not trained to use enhanced optical detection techniques.

There are obvious limitations to this study. Although the data were collected prospectively, this is a single site study and most of the Pentax HiLine procedures were performed by a single endoscopist. All analyses, however, were carried out between the two types of endoscope for all five colonoscopists taking part in the bowel cancer screening programme, and also for the individual colonoscopists who performed procedures with both Pentax and Olympus colonoscopes. No difference was found between the two types of analysis, suggesting that the findings are robust. Nonetheless, it would be wise to confirm these findings with a multicentre, prospective, randomised controlled study involving multiple endoscopists. In addition, it would be optimal to have assessed either endoscopy system in the same patient performed by the same operator to test for a significant difference in the measured outcomes, but this would not be ethically viable. Finally, it is not certain that detecting more small

polyps would actually translate to better outcomes from a national bowel cancer screening programme. It would take a very large study to prove this. Nonetheless, if the procedure takes no more time, carries minimal extra discomfort, and detects significantly more polyps, it seems reasonable to consider using this in routine practice. A prospective trial to compare the performance of these two colonoscopes for bowel cancer screening is therefore required.

COMMENTS

Background

Colorectal cancer is one of the most common cancers worldwide, particularly in Europe and the United States, and is the 7th most common cause of death worldwide in high earning countries. To reduce the number of deaths from this disease, strategies have been designed to detect cancer at an earlier stage, and detect and remove polyps, thought to be the precursors for a large proportion of future colorectal cancers.

Research frontiers

In the United Kingdom, the national bowel cancer screening program was established to reduce deaths from colorectal cancer, utilising colonoscopy for patients screened positive with tests designed to detect minute quantities of blood in their stool. Colonoscopy was chosen as the screening modality, as it is now established as the gold standard both for the identification of colorectal cancer and polyps. The miss rate during colonoscopy, however, is unsuitably high for both the detection of significant polyps, known as adenomas (24%) and colorectal cancers (up to 6%). It is therefore desirable to investigate methods that may improve the accuracy of colonoscopy, particularly as a higher adenoma detection rate is associated with lower rates of colon cancer developing later.

Innovations and breakthroughs

Although colonoscopy is now accepted as the gold standard for the detection of colorectal cancer, a number of colonoscopes with varying technologies are now available in the market. Previous studies have compared high definition (HD) with standard video colonoscopy to show how high definition colonoscopy could detect significantly more patients with colorectal neoplasia (38% vs 13%), and significantly more adenomatous and cancerous polyps. Furthermore high definition endoscopy with surface enhancement could also predict the final histology with high accuracy (98.6%) within the HD+ group. There are few studies comparing different high definition systems that have varying resolutions.

Applications

This study demonstrates that higher definition colonoscopes improve adenoma detection without compromising other measures of endoscope performance. Increased polyp detection rates may improve the outcomes of bowel cancer screening programs in the future. Future research is still needed to identify the cost effectiveness of megapixel high-resolution endoscopy systems in larger clinical studies.

Terminology

Polyps: a polyp is an abnormal growth of tissue arising from the lining of the bowel; Adenoma: an adenoma is benign tumour arising from glands inside the lining of the bowel; Histology: the microscopic study of cells and tissues.

Peer review

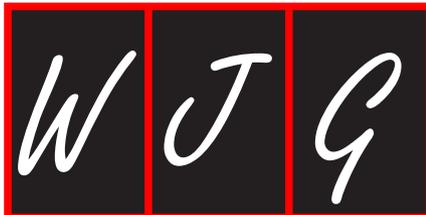
This article addresses the very important issue of investigating the best equipment available to endoscopist in order to adequately equip them to perform their duties to the best of their abilities. Historically, studies have concentrated on comparing groups or individuals practicing procedures. This study is unique and important as it compared two independently available endoscopy systems that are used in the United Kingdom. It demonstrates that the Pentax system is superior to the Olympus system when specifically looking at the question of polyp detection. Although the Olympus scopes are widely used and have other excellent qualities that have made them very popular in the United Kingdom, it is vital that, as endoscopists and practitioners, the authors question the quality of their equipment. This study allows bowel cancer colonoscopists to make a better and more informed decision about which endoscopy system is best for allowing them to accurately detect these pre-cancerous polyps, in their practice.

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S- Editor Sun H L- Editor Stewart GJ E- Editor Xiong L



Role of surgical intervention in managing gastrointestinal metastases from lung cancer

Po-Chu Lee, Chiao Lo, Ming-Tsan Lin, Jin-Tung Liang, Been-Ren Lin

Po-Chu Lee, Department of Trauma, National Taiwan University Hospital and College of Medicine, Taipei 10002, Taiwan, China

Chiao Lo, Ming-Tsan Lin, Division of General Surgery, Department of Surgery, National Taiwan University Hospital and College of Medicine, Taipei 10002, Taiwan, China

Jin-Tung Liang, Been-Ren Lin, Division of Colorectal Surgery, Department of Surgery, National Taiwan University Hospital and College of Medicine, Taipei 10002, Taiwan, China

Author contributions: Lee PC and Lo C performed the majority of the data collection and the initial analysis; Lin MT and Liang JT performed the literature review and wrote the draft of the manuscript; Lin BR coordinated the study, analyzed the data, and wrote the manuscript.

Correspondence to: Dr. Been-Ren Lin, Division of Colorectal Surgery, Department of Surgery, National Taiwan University Hospital and College of Medicine, No. 7, Chung-Shan South Road, Taipei 10002, Taiwan, China. beenrenlin@ntu.edu.tw

Telephone: +886-2-23123456 Fax: +886-2-23934358

Received: January 15, 2011 Revised: May 5, 2011

Accepted: May 12, 2011

Published online: October 14, 2011

Abstract

AIM: To investigate the clinicopathological characteristics of late-stage lung cancer patients with gastrointestinal (GI)-tract metastases, focusing on therapeutic options and outcomes.

METHODS: Our institution (the National Taiwan University Hospital) diagnosed 8159 patients with lung cancer between 1987 and 2008, of which 21 developed symptomatic GI metastases. This study reviewed all of the patients' information, including survival data, pathological reports, and surgical notes.

RESULTS: The most common histological type of lung cancer was adenocarcinoma, and 0.26% of patients with lung cancer developed GI metastases. The median duration from lung cancer diagnosis to GI metastases was three months (range, 0-108 mo), and the average time

from diagnosis of GI metastasis to death was 2.8 mo. Most patients with symptomatic gastric and/or duodenal metastases exhibited GI bleeding and were diagnosed by panendoscopy. In contrast, small bowel metastases typically presented as an acute abdomen and were not diagnosed until laparotomy. All patients with small bowel or colonic metastases underwent surgical intervention, and their perioperative mortality was 22%. Our data revealed a therapeutic effect in patients with solitary GI metastasis and a favorable palliative effect on survival when metastases were diagnosed preoperatively. In patients with multiple GI metastases, the presentation varied according to the locations of the metastases.

CONCLUSION: Surgical treatment is worthwhile in a select group of patients with bowel perforation or obstruction. Physicians should be more alert to symptoms or signs indicating GI metastases.

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Key words: Gastrointestinal metastasis; Lung cancer; Palliative effect; Prognosis; Surgical intervention

Peer reviewer: Michael Leitman, MD, FACS, Chief of General Surgery, Beth Israel Medical Center, 10 Union Square East, Suite 2M, New York, NY 10003, United States

Lee PC, Lo C, Lin MT, Liang JT, Lin BR. Role of surgical intervention in managing gastrointestinal metastases from lung cancer. *World J Gastroenterol* 2011; 17(38): 4314-4320 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4314.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4314>

INTRODUCTION

Lung cancer is a major cause of cancer-related death worldwide. In Taiwan, it is the second leading cause of cancer death, with nearly 8700 new cases and 7500 deaths per year^[1]. Approximately one-half of lung cancer pa-

tients develop metastases^[2,3], the most common sites of which are the lymph nodes, liver, adrenal glands, bone, and brain^[2]. Symptomatic gastrointestinal (GI) tract metastases are not uncommon^[4,5], although GI metastases from lung cancer are rare. The reported incidence of GI metastasis from lung cancer varies from 0.5%-10%, and mainly depends on the evaluation method used (endoscopy, surgical specimens, or autopsy)^[2,5-7]. The typical presentations of GI metastases are abdominal pain, bleeding, obstruction, and perforation, of which perforation is the most serious complication, necessitating surgical intervention to prevent a life-threatening event. GI tract involvement with lung cancer is generally considered to be associated with a late or advanced stage of the disease. Therefore, the question of how to manage patients with symptomatic GI metastases from lung cancer is important; however, management of the disease remains a controversial topic^[2,3,6].

The present study describes the clinicopathological features of a large series of primary lung cancers manifesting GI metastases and discusses therapeutic options, with a particular focus on the role of surgeons in this uncommon oncological setting.

MATERIALS AND METHODS

This research reviewed the surgical pathology reports of patients diagnosed with GI metastases from 1987 to 2008 at the National Taiwan University Hospital. Only those cases in which the primary source of the metastases was documented as lung cancer were included in our analysis. The diagnostic criteria were (1) radiological demonstration of a primary lung tumor and exclusion of tumors elsewhere, and (2) the morphology and immunohistochemical profiles were consistent with a primary pulmonary tumor. Of 8159 patients diagnosed with lung cancer, 21 were found to have GI metastases. Metastatic disease originating from a lung primary tumor was confirmed from biopsy specimens or from surgical resection of the GI tract in patients.

Patient information, including age, sex, pathology, initial lung cancer stage, interval between lung tumor diagnosis and discovery of GI involvement, clinical presentation, other metastatic site locations at the time of GI metastases, and patient survival, was recorded and reviewed retrospectively. Clinical and other follow-up data were obtained in all cases from patient records and referring physicians. Analysis of the survival data was performed by the Kaplan-Meier method. Differences between subgroups were compared for statistical significance using the log-rank test. *P* values were two-sided and the significance level was set at 0.05.

RESULTS

Patient characteristics

Table 1 summarizes the patient data, including clinical manifestations, diagnosis, treatment, and follow-up.

The patients ranged in age from 40 to 81 years (median, 69 years), and of the five women and 16 men, 14 had a smoking history. Nine patients (one woman, eight men) developed symptomatic gastric and/or duodenal metastases. Six patients (all male) had symptomatic small bowel metastases. Three patients (two women, one man) had symptomatic colonic metastases. The other three patients (two women, one man) had multiple GI tract metastases.

Interval between lung cancer diagnosis and gastrointestinal metastasis

Studies have reported GI tract metastasis to occur in the later stages of lung cancer^[1]. In our study, the median duration from lung cancer diagnosis to GI tract metastasis in the eight patients with initial stage I to III disease was 8 mo (ranging from 2 to 108 mo). In contrast, 5 of the 13 patients with stage IV lung cancer developed simultaneous GI metastases, and the median duration of the other eight patients with GI metastases was 3.5 mo (ranging from 1 to 17 mo). The average time from GI metastasis diagnosis to death was 2.8 mo, similar to the 130.3 d reported by Yang *et al*^[8].

Clinical presentations and diagnosis

Gastrointestinal hemorrhage was the most common symptom of GI metastasis (11 of 21, 52.4%). Panendoscopic biopsy easily diagnosed nine patients with gastric or duodenal metastases. Three of the six patients with small bowel metastases exhibited intestinal perforations and the others exhibited GI obstructions. Of these patients, 67% (4/6) were diagnosed by laparotomy, including three cases of intestinal perforation, which could reflect the fact that small bowel metastases are more difficult to diagnose before surgery, and the typical clinical manifestations are an acute abdomen or peritonitis. In contrast to patients with colonic metastases, the diagnosis of patients with GI metastasis could be made before surgery by computed tomography (CT) or colonoscopy. This result is possibly attributable to the slow progression of clinical manifestations and physician awareness. Clinical presentations of multiple GI involvements were variable, and depended on the major location of the metastatic GI tumors. For example, patient #19 had stage IV lung cancer and experienced mild abdominal pain for weeks. She did not pay attention to the pain, thinking it was an adverse effect of chemotherapy. Her symptoms progressed rapidly to poor appetite and little oral intake; therefore, she was admitted for further management. A CT scan revealed multiple intra-abdominal tumors involving the ovaries, pancreas, and stomach. After discussing the options with the patient and her family, they all agreed to hospice care, and the patient passed away three months later.

Histological subtypes and other metastatic sites

Half of the patients in this series (12 of 21) had primary lung adenocarcinoma and five had squamous cell carcinoma; the other four included one pleomorphic carcinoma, one small cell carcinoma, one undifferentiated carcinoma,

Table 1 Clinical and pathological features of patients with gastrointestinal metastases

Patient number	Age (yr) /sex	Histology	Initial pTNM staging	Time period (mo) from diagnosis of lung tumor to major GI metastasis	Clinical presentation	Diagnostic procedure	Other extra-thoracic metastatic sites	Surgery	Survival
1	58/F	Adenocarcinoma	IV	1/stomach	Abdominal pain	PES	Brain, bone, liver, adrenal gland	Nil	¹ 15 mo
2	79/M	Pleomorphic carcinoma	IV	Simultaneous/stomach	Hemorrhage	PES	Bone, liver, gall bladder	Nil	1 mo
3	81/M	Adenocarcinoma	IV	5/stomach	Hemorrhage	PES	Bone	Nil	1 mo
4	73/M	Adenocarcinoma	IIIb	5/stomach	Hemorrhage	PES	Brain	Nil	1 mo
5	71/M	Squamous cell carcinoma	I b	9/stomach	Hemorrhage	PES, CT	Spleen, liver	Proximal gastrectomy	16 d
6	59/M	Adenocarcinoma	IV	17/stomach	Hemorrhage	PES	Bone, liver	Nil	2 mo
7	71/M	Squamous cell carcinoma	IIIa	108/stomach and duodenum	Hemorrhage	Laparotomy	Liver	Wedge resection of stomach	15 d
8	70/M	Small cell carcinoma	IIIb	7/stomach and duodenum	Abdominal pain hemorrhage	PES	Bone, pancreas	Nil	3 mo
9	66/M	Adenocarcinoma	IV	Simultaneous/duodenum	Hemorrhage	PES	Adrenal gland	Nil	1 mo
10	72/M	Undifferentiated	IV	Simultaneous/small bowel	Bowel obstruction	CT	Brain	Segmental resection	1 mo
11	63/M	Adenocarcinoma	IIIb	3/small bowel	Perforation	Laparotomy	Peritoneal seeding	Primary repair	3 d
12	47/M	Adenocarcinoma	IV	2/small bowel	Bowel obstruction	Laparotomy	Nil	Segmental resection	2 mo
13	66/M	Adenosquamous carcinoma	IV	3/small bowel	Bowel obstruction	Echo	Brain	Segmental resection	3 mo
14	73/M	Adenocarcinoma	IV	14/small bowel	Perforation	Laparotomy	Liver	Segmental resection	1 d
15	71/M	Squamous cell carcinoma	IIIb	2/small bowel	Perforation	Laparotomy	Peritoneal seeding	Segmental resection	3 mo
16	61/F	Adenocarcinoma	IIIb	52/colon	Abdominal pain	CT	Nil	Right hemicolectomy	8 mo
17	66/M	Squamous cell carcinoma	IV	4/colon	Hemorrhage	Colonoscopy	Liver, brain	Right hemicolectomy	5 mo
18	54/F	Adenocarcinoma	IIIb	32/colon	Ileus	CT, Colonoscopy	Bone, brain	Right hemicolectomy	4 mo
19	40/F	Adenocarcinoma	IV	1/multiple GI involvement	Ileus, Hemorrhage	PES, CT	Ovary, adrenal gland, pancreas	Nil	3 mo
20	69/M	Squamous cell carcinoma	IV	Simultaneous/multiple GI involvement	Hemorrhage	PES, CT	Bone	Nil	2 mo
21	80/F	Adenocarcinoma	IV	Simultaneous/multiple GI involvement	Tenesmus	CT	Ovary, uterus	Nil	3 mo

¹Patient still alive. M: Male; F: Female; pTNM: Pathological tumor-lymph node-metastasis; GI: Gastrointestinal; PES: Panendoscopy; CT: Computed tomography.

and one adenosquamous carcinoma.

All but two of the patients (19/21, 90.5%) had metastases to other locations at the time of GI metastasis diagnosis. The most common extrathoracic metastatic sites were the liver, brain, bone, adrenal glands, spleen, and pancreas. The two cases (patients #12 and #16) with only one GI metastasis underwent surgical resection with therapeutic intent and one was still alive at the time of writing.

Surgical procedures and clinical outcomes

Upon diagnosis of stomach or duodenal involvement, conservative treatment was prescribed for symptoms, and imaging studies were performed to evaluate other possible metastatic sites. All nine patients in this subgroup had other simultaneous metastases that were considered to represent the late stage of lung cancer. If the symptoms (GI bleeding or abdominal pain) could be controlled by medication, supportive treatment was continued. Two

patients underwent surgical intervention due to massive hemorrhage; patient #5 received a proximal gastrectomy for a 6.7-cm ulcerative mass over the body of the stomach with direct invasion of the spleen, and patient #7 received a wedge resection for an ulcerative lesion over the cardia of the stomach. Unfortunately, these two patients died within one month of surgery owing to postoperative complications. The other seven patients received only supportive treatment, and the median survival was one month (range, 1 to 15 mo). We observed that patients treated with conservative therapy exhibited a significant survival benefit in comparison with those who received surgical treatment ($P = 0.005$).

Nine patients with small bowel or colonic metastases underwent laparotomy. As mentioned above, four patients were not diagnosed until laparotomy. Patients who required immediate surgical intervention exhibited an acute abdomen, such as perforation or peritonitis. Survival comparisons of these patients, based on the timing

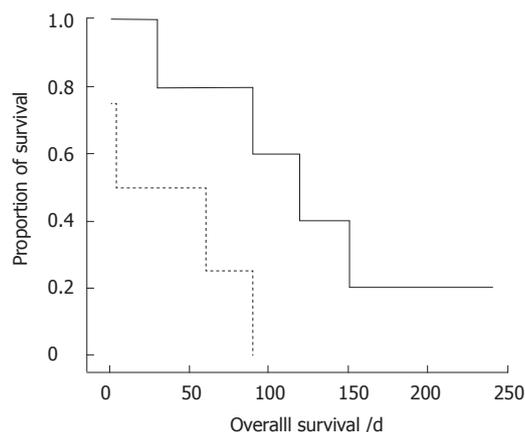


Figure 1 Kaplan-Meier plot of the survival curve for nine patients according to the diagnostic time of gastrointestinal metastasis before surgery (solid line) or after surgery (dashed line).

of diagnosis, showed that the survival benefit was significantly increased in the subgroup with GI metastases diagnosed before surgery ($P = 0.032$, Figure 1). Accordingly, early GI metastasis detection and timely surgical intervention are important for lung cancer patients. Of the three patients with multiple GI tract metastases, all received palliative treatment and died of the disease.

As an example, one patient (case #16 in Table 1) was diagnosed with lung adenocarcinoma in October of 2003. At that time, chest radiography showed massive left pleural effusion. A chest CT revealed a patchy consolidation with pleural thickening in the left upper lung lobe. Adenocarcinoma was confirmed by CT-guided biopsy and pleural effusion collected *via* aspiration for cytological analysis. After a complete staging work-up, stage IIIb lung cancer was confirmed and the patient underwent multiple courses of chemotherapy. However, the patient began experiencing abdominal pain, which was aggravated after meals, in March of 2008. A physical examination revealed diffuse abdominal tenderness without rebounding pain, and an abdominal and pelvic CT showed a single ill-defined mass with local infiltration into the ascending colon (Figure 2A). Colonoscopy revealed a circular tumor with luminal obstruction (Figure 2B), and a biopsy indicated adenoma with malignant change. Colon cancer was the tentative diagnosis; however, the actual origin of the tumor could not be confirmed. After surgical intervention, a 4-cm irregular-shaped mass invading the adjacent small bowel in the ascending colon was noted during the laparotomy, and a right hemicolectomy with a segmental resection of the small bowel was performed without complications. The pathological report revealed that the tumor involving the colon was mainly located in the pericolonic soft tissue and invaded inwards into the colonic muscular layer (Figure 3A). The morphology of the tumor was distinct, characterized by pleomorphic oval nuclei, scant cytoplasm, and an irregular glandular pattern. Immunohistochemical staining revealed the tumor cells to be positive for thyroid transcription factor-1 (TTF-1) and cytokeratin 7 (CK7), but negative for CK20

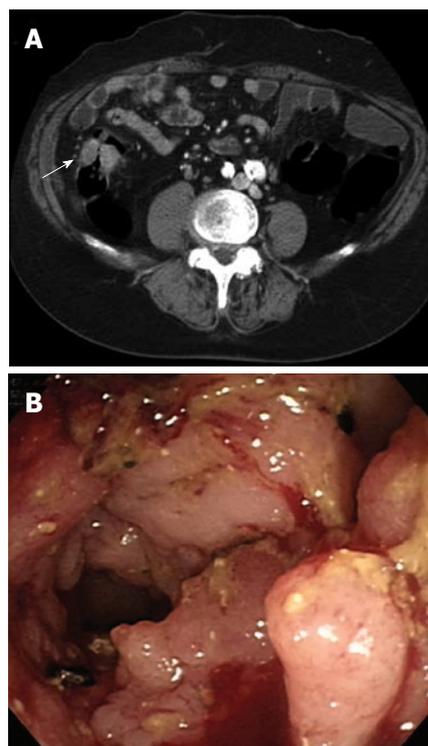


Figure 2 Imaging study of one patient with colonic metastases from lung cancer. A: An abdominal computed tomography showed a 4-cm ill-defined tumor at the ascending colon (arrow); B: Colonoscopic feature of a circular mass with luminal stenosis and easy-touch bleeding.

(Figure 3B-D). The diagnosis of a metastatic adenocarcinoma of pulmonary origin was confirmed.

DISCUSSION

Lung cancer is the leading cause of death from cancer worldwide, and about one-half of lung cancer patients have metastatic disease at the initial diagnosis^[2,3]. The most common sites of metastatic disease are the lymph nodes (48%), liver (45%), adrenal glands (41%), bone (31%), and brain (25%)^[2]. Before the 1980s, GI tract involvement was considered an unusual metastasis site from lung cancer^[9,10], and the pathogenesis of bowel metastases was considered to be due to tumor-cell spreading via the hematogenous or lymphatic routes. A review of autopsy data by Antler *et al*^[7] reported the incidence of GI metastasis to be 14%; however, 14% may be higher than the true incidence of GI metastasis, because a large number of esophageal metastases in their study were the result of direct invasion. The incidence of symptomatic small bowel metastasis has been reported to be 0.4%-0.5%^[4,5]. In our series, there were 21 cases of symptomatic gastrointestinal metastases among the 8159 lung cancer patients (0.26%). The disparity between subclinical and clinical GI metastases is still high.

Gastric and/or duodenal metastases from lung cancer are very rare, and there are only a few cases of varying malignant cell types reported in the literature^[11-13]. These cases exhibited symptoms of abdominal pain, chronic

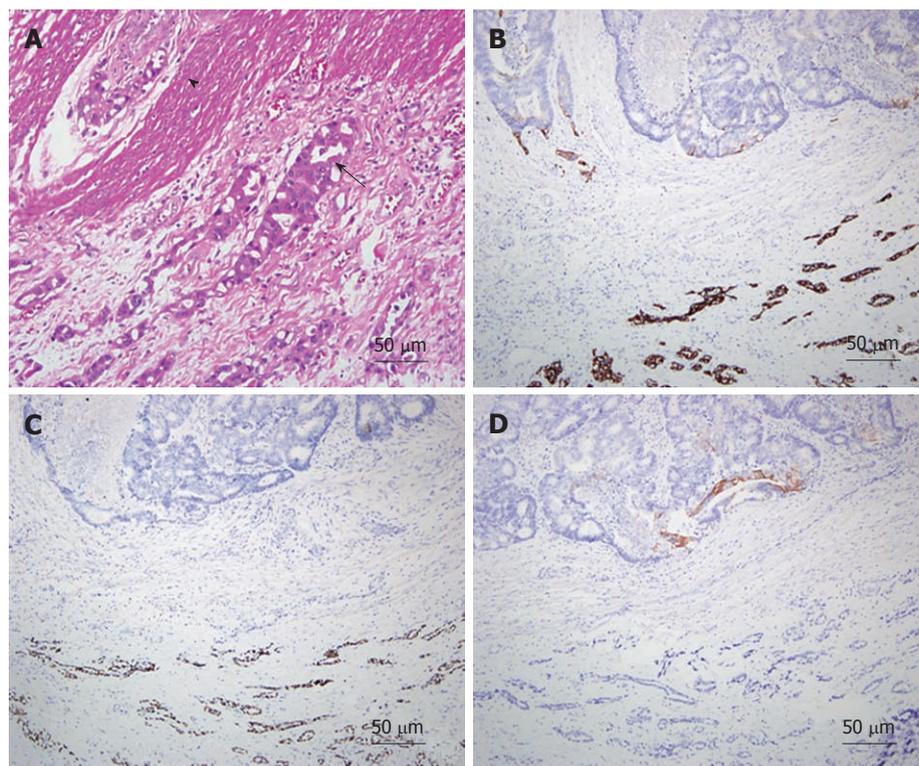


Figure 3 Microscopic findings of metastatic lung adenocarcinoma in the colon. A: Hematoxylin-eosin staining; $\times 200$. This tumor was characterized by pleomorphic oval nuclei, scant cytoplasm, an irregular glandular pattern (arrow), and invasion of the muscle layer (arrow head) of the colon; B-D: Staining for thyroid transcription factor (TTF)-1 (B), cytokeratin (CK)7 (C), CK20 (D); $\times 100$. In general, CK20 positive means that the tumor originates from the gastrointestinal tract. By immunohistochemistry, the tumor cells located in the lower part of the figure were found to be positive for TTF-1 (B) and CK7 (C), but negative for CK20 (D). The glandular structures in the upper part belong to the colon mucosa.

bleeding, anemia, or hematemesis, and the pathological results of panendoscopic biopsy provided accurate diagnoses. In contrast to the small bowel, the most common metastatic site of lung cancer in the GI tract^[4,5,14,15], there have been few reports of lung cancer metastases to the colon, appendix, or anus^[16-19]. Small intestine involvement often leads to an acute abdomen as a result of perforation or obstruction^[4,5,15], whereas colon metastases usually result in vague symptoms. In our cases of small bowel or colon metastases, all patients required surgery to relieve the obstruction or control peritonitis. In general, the use of CT, abdominal sonography, or endoscopy plays an important role in identifying lesions.

Even with endoscopy, lung cancer involving the GI tract has no specific features, appearing as a diffuse involvement of the mucosa and multiple nodules with or without mucosa ulceration^[20,21]. An experienced pathologist might be able to conclude a metastatic tumor based on morphological study of tumor tissue from surgical resection. However, in most cases histological examination with staining using cell type-specific markers is the only way to identify metastatic tumors of the GI tract. Several different CK and other protein markers are widely used to distinguish carcinomas of different origins. Rossi *et al.*^[21] concluded that lung carcinomas usually demonstrate a CK7+/CK20- immunoprofile, whereas intestinal carcinomas have a different CK7-/CK20+ pattern. Thus, CK7 is a good marker for distinguishing those cell types. In gen-

eral, primary lung tumors can be identified by CK7; however, studies have demonstrated that primary adenocarcinomas of the rectum or small intestine may also express CK7 in a significant number of cases, and may even lose CK20 expression^[22,23]. Therefore, to exclude a possibility like this, employing a more specific marker of lung tumor origin, such as TTF-1^[24], together with CK7 and CK20 could more effectively differentiate metastatic GI tumors from lung cancer.

Every type of lung cancer can result in GI metastasis. McNeill *et al.*^[2] and Berger *et al.*^[5] reported that squamous cell carcinoma causes small bowel metastases more frequently than other lung tumor cell types. In the series of Antler *et al.*^[7], small cell carcinoma and large cell carcinoma were more likely to result in GI metastases. However, in our series, adenocarcinoma was the most frequent type resulting in GI metastases. In accordance with our results, Garwood *et al.*^[4] reported that adenocarcinoma (23.7%) and squamous cell carcinoma (22.7%) were the most common histological types causing small bowel perforations.

Our results showed that the median time from lung cancer diagnosis to GI metastasis was three months and the longest period of time was 108 mo (patient #7). Observations of previous data show that, in patients with lung metastases from colon cancer, a longer interval between colon cancer diagnosis and lung metastasis was associated with a significantly longer survival^[25]. However, our findings did not show a similar survival difference

when patients with an interval greater than 1 year were compared with those with an interval less than 1 year ($P = 0.79$). Our results also revealed no significant difference in overall survival in patients with initial stage I–III lung cancer upon GI metastasis diagnosis in comparison with those with stage IV disease. Taken together, these findings indicate that patients with GI metastases from lung cancer have a poor prognosis.

The perioperative mortality rate reported in the literature ranges from 60% to 100%^[2,15]. The series of Berger *et al.*^[5] indicated no perioperative mortality in patients with small bowel metastases who underwent resection. However, in our experience, the perioperative mortality rate was 100% in the two patients with gastric and/or duodenal metastases and 22% (2/9) in patients with small bowel or colon metastases. We observed longer survival in patients with gastric and/or duodenal metastases that were managed by supportive treatment without surgery ($P = 0.005$). However, the relatively small sample size in this retrospective subgroup analysis and the nature of the different underlying conditions (these nine patients had other metastases at the same time) may limit the conclusion that surgical treatment is not suitable for patients with gastric or duodenal metastases from lung cancer. On the other hand, in two of our patients, radiological work-up did not show other metastases. These patients then underwent surgical resection and one is still alive. Although surgery for localized extrathoracic metastasis from lung cancer still has a palliative intent^[21], some authors have reported prolonged survival in cases of surgically-resected isolated bone^[26], brain^[27], or small bowel^[14] metastasis from lung cancer. Our results (Figure 1) are the first to show a longer survival or more favorable outcome in patients diagnosed with GI metastasis before surgery. Consequently, surgical treatment still plays an important role in lung cancer patients with GI metastases that cause bowel obstruction, perforation, or massive hemorrhage.

In summary, patients with GI metastases from lung cancer are in the latter stages of the disease. However, physicians and surgeons should be aware that surgical intervention is typically required for patients exhibiting bowel perforation or an acute abdomen. Therefore, early detection of GI metastasis in lung cancer patients and timely surgical management may provide symptom palliation in patients with a life-threatening GI event and long-term survival in those with only a solitary GI metastasis.

COMMENTS

Background

Lung cancer is a major cause of cancer-related death worldwide. Lung cancer patients with gastrointestinal (GI)-tract metastases are generally considered to be at a late or advanced stage of the disease. Management of the disease remains controversial. Study of the management in patients with symptomatic GI metastases from lung cancer is important.

Research frontiers

Histological examination using cell type-specific markers is the best way to identify the origin of metastatic tumors of the GI tract. Staining using thyroid transcription factor-1 (TTF-1) as a specific marker of lung tumor origin, together with cytokeratin 7 (CK7) and CK20, could more effectively differentiate meta-

static GI tumors from lung cancer.

Innovations and breakthroughs

Surgical treatment plays an important role in lung cancer patients with a GI metastasis that causes bowel obstruction, perforation, or massive hemorrhage. The results of this study were the first to show a longer survival or more favorable outcome in patients diagnosed with GI metastasis before surgery.

Applications

Surgical intervention is typically required for patients exhibiting bowel perforation or an acute abdomen. Therefore, early detection of GI metastasis in lung cancer patients and timely surgical management may provide symptom palliation in patients with a life-threatening GI event and improve long-term survival in those with only solitary GI metastasis.

Terminology

CK7 and CK20 are low molecular weight cytokeratins. Their distribution is generally restricted to epithelia and neoplasms. The specific expression patterns of CKs are correlated with different pathways of epithelial differentiation, and therefore can be used to classify epithelial cells into different subtypes or origins. TTF-1 is a 38-kDa nuclear transcription protein that influences organogenesis and the maintenance of the differentiated phenotypes of various tissues, including thyroid, lung and brain.

Peer review

The paper was well written and it is enhanced by the photomicrographs, radiological and endoscopic images.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Xiong L

A meta-analysis of lamivudine for interruption of mother-to-child transmission of hepatitis B virus

Lei Han, Hong-Wei Zhang, Jia-Xin Xie, Qi Zhang, Hong-Yang Wang, Guang-Wen Cao

Lei Han, Hong-Wei Zhang, Jia-Xin Xie, Qi Zhang, Guang-Wen Cao, Department of Epidemiology, Second Military Medical University, Shanghai 200433, China

Hong-Yang Wang, Laboratory for Signal Transduction, the 3rd Affiliated Hospital, Second Military Medical University, Shanghai 200433, China

Author contributions: Han L and Cao GW designed research; Han L and Zhang HW extracted and analyzed data; Xie JX and Zhang Q checked accuracy of data; Wang HY provided analytic tools; and Cao GW wrote the paper.

Supported by National Natural Science Foundation of China, No. 81025015 and No. 30921006

Correspondence to: Guang-Wen Cao, MD, PhD, Professor and Chairman, Department of Epidemiology, Second Military Medical University, 800 Xiangyin Rd, Shanghai 200433, China. gcao@smmu.edu.cn

Telephone: +86-21-81871060 Fax: +86-21-81871060

Received: February 18, 2011 Revised: April 7, 2011

Accepted: April 14, 2011

Published online: October 14, 2011

Abstract

AIM: To determine the therapeutic effect of lamivudine in late pregnancy for the interruption of mother-to-child transmission (MTCT) of hepatitis B virus (HBV).

METHODS: Studies were identified by searching available databases up to January 2011. Inclusive criteria were HBV-carrier mothers who had been involved in randomized controlled clinical trials (RCTs) with lamivudine treatment in late pregnancy, and newborns or infants whose serum hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) or HBV DNA had been documented. The relative risks (RRs) for interruption of MTCT as indicated by HBsAg, HBV DNA or HBeAg of newborns or infants were calculated with 95% confidence interval (CI) to estimate the efficacy of lamivudine treatment.

RESULTS: Fifteen RCTs including 1693 HBV-carrier

mothers were included in this meta-analysis. The overall RR was 0.43 (95% CI, 0.25-0.76; 8 RCTs; $P_{\text{heterogeneity}} = 0.04$) and 0.33 (95% CI, 0.23-0.47; 6 RCTs; $P_{\text{heterogeneity}} = 0.93$) indicated by newborn HBsAg or HBV DNA. The RR was 0.33 (95% CI, 0.21-0.50; 6 RCTs; $P_{\text{heterogeneity}} = 0.46$) and 0.32 (95% CI, 0.20-0.50; 4 RCTs; $P_{\text{heterogeneity}} = 0.33$) indicated by serum HBsAg or HBV DNA of infants 6-12 mo after birth. The RR (lamivudine vs hepatitis B immunoglobulin) was 0.27 (95% CI, 0.16-0.46; 5 RCTs; $P_{\text{heterogeneity}} = 0.94$) and 0.24 (95% CI, 0.07-0.79; 3 RCTs; $P_{\text{heterogeneity}} = 0.60$) indicated by newborn HBsAg or HBV DNA, respectively. In the mothers with viral load $< 10^6$ copies/mL after lamivudine treatment, the efficacy (RR, 95% CI) was 0.33, 0.21-0.53 (5 RCTs; $P_{\text{heterogeneity}} = 0.82$) for the interruption of MTCT, however, this value was not significant if maternal viral load was $> 10^6$ copies/mL after lamivudine treatment ($P = 0.45$, 2 RCTs), as indicated by newborn serum HBsAg. The RR (lamivudine initiated from 28 wk of gestation vs control) was 0.34 (95% CI, 0.22-0.52; 7 RCTs; $P_{\text{heterogeneity}} = 0.92$) and 0.33 (95% CI, 0.22-0.50; 5 RCTs; $P_{\text{heterogeneity}} = 0.86$) indicated by newborn HBsAg or HBV DNA. The incidence of adverse effects of lamivudine was not higher in the mothers than in controls ($P = 0.97$). Only one study reported side effects of lamivudine in newborns.

CONCLUSION: Lamivudine treatment in HBV carrier-mothers from 28 wk of gestation may interrupt MTCT of HBV efficiently. Lamivudine is safe and more efficient than hepatitis B immunoglobulin in interrupting MTCT. HBV MTCT might be interrupted efficiently if maternal viral load is reduced to $< 10^6$ copies/mL by lamivudine treatment.

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Key words: Hepatitis B virus; Lamivudine; Mother-to-child transmission; Efficacy; Meta-analysis

Peer reviewer: Rosemary Joyce Burnett, MPH, Department of

Epidemiology National School of Public Health, University of Limpopo, Medunsa Campus PO Box 173, MEDUNSA, Pretoria 0204, South Africa

Han L, Zhang HW, Xie JX, Zhang Q, Wang HY, Cao GW. A meta-analysis of lamivudine for interruption of mother-to-child transmission of hepatitis B virus. *World J Gastroenterol* 2011; 17(38): 4321-4333 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4321.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4321>

INTRODUCTION

Hepatitis B virus (HBV) infection is a global issue of public health. More than 350 million people suffer from chronic HBV infection, the commonest cause of hepatocellular carcinoma^[1]. Mother-to-child transmission (MTCT) of HBV, the commonest mode of transmission worldwide, may occur either in utero or perinatally. In East and Southeast Asia, in utero transmission of HBV is rare, whereas perinatal transmission is common. MTCT of HBV is associated with a very high rate of chronicity, especially in countries where HBV is endemic. This is attributed to the high rate of hepatitis B e antigen (HBeAg)-positive infection in women of child-bearing age in these areas and the efficient transmission of HBV from mothers to their newborns^[2-4]. Therefore, prevention of MTCT is the most important strategy in the eradication of HBV infection.

Joint immunoprophylaxis with hepatitis B immunoglobulin (HBIG) and three doses of hepatitis B vaccines to infants born to hepatitis B surface antigen (HBsAg)-positive mothers are known to be safe and effective. However, 5%-10% of infants of HBV-positive mothers become infected even with proper vaccination^[5]. Very high maternal viremia, in utero infection, or escape mutants are possible reasons for vaccination failure, while immunocompromised hosts also risk vaccination failure^[6]. Of those, maternal high viral load and HBeAg positivity contribute greatly to MTCT despite the use of passive-active immunoprophylaxis in newborns. A meta-analysis of individual patient data of the three randomized trials indicated that the passive-active immunoprophylaxis had 100% protective efficacy if maternal serum HBV DNA was lower than 150 pg/mL compared with 68% if maternal serum HBV DNA was higher than 150 pg/mL^[7]. The recommended dose of HBIG may be insufficient to neutralize the huge virus load that the infants are exposed to at the time of birth in cases whose mothers have very high serum HBV DNA levels^[8]. In addition, immunized children born to genotype C HBV-infected mothers may have a higher rate of breakthrough infection than those born to genotype B-infected mothers in Southeast Asia^[9]. Thus, administration of antiviral therapy to lower the maternal serum HBV DNA levels may reduce the rate of perinatal infection in newborns born to mothers who have high serum HBV DNA levels or who are infected with HBV genotype C, the major HBV

genotype endemic in mainland China^[10].

The antiviral drug with a record of safe use in pregnant women is lamivudine^[11]. In HBeAg-positive mothers who had serum HBV DNA > 10⁹ copies/mL, lamivudine treatment started between weeks 34 and 38 of pregnancy until delivery might greatly decrease the perinatal infection of their newborns who routinely received the combined immunoprophylaxis, as compared with historical controls^[12]. However, some studies have demonstrated that treatment with lamivudine or HBIG did not reduce the perinatal infection rate significantly among women with an extremely high HBV DNA load, and among those with reduced maternal HBV DNA, even with undetectable status, this treatment could not guarantee exemption of their newborns' HBV infection in late pregnancy^[13,14]. These controversial results necessitate meta-analyses by pooling data of the available studies to address the following questions^[8]. (1) At which maternal serum HBV DNA level does the antiviral therapy have a clear beneficial effect? (2) How early in pregnancy should antiviral therapy be initiated? (3) Is lamivudine safe in pregnancy or in nursing mothers? A recent meta-analysis of randomized controlled trials (RCTs) using the data up to October 2009 has demonstrated that lamivudine treatment in HBV-infected mothers with a high degree of infectiousness in late pregnancy effectively prevented MTCT^[15]. However, the first two questions have not been fully answered. In this study, we performed a meta-analysis of the randomized, placebo controlled trials up to January 2011, evaluated the efficacy of lamivudine in late pregnancy as compared with placebo or control in interruption of MTCT of HBV and determined the maternal HBV DNA level that lamivudine treatment has a clear beneficial effect. We also investigated the safety of lamivudine treatment in mothers and their newborns in an attempt to provide useful data for interrupting MTCT of HBV.

MATERIALS AND METHODS

Search strategy and selection criteria

We searched MEDLINE, EMBASE, the Cochrane controlled trials register, the Cochrane Library, and China Biological Medicine Database for publications (including abstracts) in English or Chinese, up to January 2011. The keywords used for searching were "MTCT (vertical transmission, perinatal transmission or intrauterine transmission)" and "hepatitis B virus (HBV or hepatitis B)" and "antiviral treatment (lamivudine)". We also did a full manual search from bibliographies of selected studies to identify additional studies. We contacted some of the authors to collect further information.

The following studies were included: RCTs; lamivudine treatment for HBV-carrier mothers in late pregnancy; passive-active immunoprophylaxis for newborns after birth; MTCT; MTCT diagnosed based on the serum parameters including HBsAg, HBeAg and HBV DNA; and relative risks (RRs) with the 95% confidence intervals (CIs). The following studies were excluded: unclear his-

tory of the immunoprophylaxis of newborns or infants; the patients co-infected with hepatitis C or hepatitis D virus or human immunodeficiency virus; participants who had received antiviral treatment before pregnancy or without control subjects. We only included the most recent studies or studies with a larger number of participants when more than one studies were published by the same authors.

Data extraction

Data were independently extracted by two investigators (Han L and Zhang HW) and checked by the other authors. The concordance rate between the two investigators was more than 90%. Discrepancies were resolved by consensus. The following information was extracted using a standardized form: the details of the study (study design, citation, publication date); the characteristics of the subjects (number of included mothers, maternal serum HBV HBsAg and HBeAg); the interventions on mothers (lamivudine treatment and comparative treatment regimen used for each arm, dosage, and duration) and the outcomes (serum HBsAg, HBeAg and HBV DNA of newborns within 24 h and infants within 6-12 mo after birth) and adverse events.

Quality assessment

Two investigators (Han L and Zhang HW) independently rated the quality of each retrieved study. Disagreement was resolved by discussion among the investigators. Trials of high quality (with low risk of bias) should fulfil two or three of the following elements: adequate generation of the allocation sequence, adequate allocation concealment, and adequate blindness. Trials of low quality (with high risk of bias) were those having one or none of these elements^[16,17].

Statistical analysis

Freeware program Review Manager (Version 5.0 for Windows, Cochrane Collaboration, Oxford, United Kingdom, 2010) was applied to conduct statistical analysis. The effect measures of interest were RRs and the corresponding 95% CIs. Statistical heterogeneity among studies was evaluated using χ^2 test, P values, and I^2 statistics^[18]. Statistical heterogeneity was defined as $P < 0.10$ or $I^2 > 50\%$. A random-effect model was used to obtain summary RRs. The random-effect model adjusted for variability of results among trials provided a more conservative estimate of an effect using wider CI^[19]. Publication bias was evaluated using funnel plots which displayed the studies in a plot of effect size against sample size, which mapped the log standard error against the log RR of individual studies^[20]. All statistical tests were two-sided.

RESULTS

We identified 106 citations from the literature. The relevant trials are shown in Figure 1. Eighteen irrelevant ci-

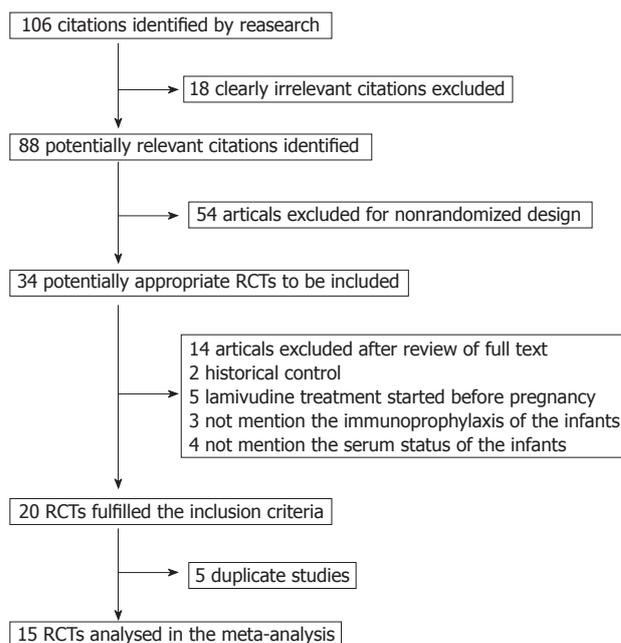


Figure 1 Flow chart of study recruitment. RCTs: Randomized controlled clinical trials.

tations were excluded after abstract preview. Among the remaining 88 articles, 54 were excluded because of non-randomized design after the full text review. Of the remaining 34 potentially appropriate RCTs, 2 were excluded because of historical controls, 5 were excluded because the lamivudine treatment started before pregnancy, 3 were excluded for not having detailed information on the immunoprophylaxis of newborns, and 4 were excluded because the serum HBV parameters of the infants were not mentioned. After removing the 5 duplicate studies, a total of 15 RCTs were included eventually^[21-35]. Of the 15 RCTs, 2 were from PubMed^[25,35], 12 from China Biological Medicine Database published in Chinese^[21-24,27-34], and 1 from reference lists^[26]. Of those, 10 investigated MTCT by measuring the newborn's blood within 24 h after birth^[21-23,25,26,28-30,34-35], 11 investigated MTCT by measuring the infant's sera 6-12 mo after birth^[21-25,27-28,30-33], 11 measured maternal HBV DNA levels when they were enrolled in the trials before lamivudine treatment^[21,23,25-27,29-31,33-35], 10 measured maternal HBV DNA levels after treatment before delivery^[21,25-27,29-31,33-35]. The regimen of lamivudine treatment varied and some RCTs had several intervention groups such as HBIG or lamivudine plus HBIG groups. Three articles reported adverse events in mothers with lamivudine treatment^[25,26,34], only one article reported adverse events in the infant whose mother received lamivudine treatment^[25]. A total of 1693 HBV-carrier mothers were included. The characteristics of the included studies are summarized in Table 1. Of the 15 trials, 6 adequately described the generation of the allocation sequence^[26-29,34,35], 2 concealed treatment allocation and double blinded methods were described^[25,26]. The remaining trials did not report the methodological quality.

Table 1 General information of included randomized controlled trials

First author, year ^[Ref.]	Group (n)	Interventions on mothers	Maternal HBV DNA level ¹		Newborns within 24 h			Infants within 6-12 mo			Adverse events	
			Before intervention	Before delivery	HBsAg (+)	HBeAg (+)	HBV DNA (+)	HBsAg (+)	HBeAg (+)	HBV DNA (+)	Mothers	Infants
Zhang, 2010 ^[21]	Arm 1:50	3TC 100 mg od from week 28	6.83 ± 0.90	3.65 ± 0.54	6/50	-	5/50	1/50	-	1/50	0	0
	Arm 2:50	No treatment	6.87 ± 1.67	6.88 ± 1.08	17/50	-	18/50	8/50	-	8/50	-	-
Han, 2010 ^[22]	Arm 1:52	3TC 100 mg od from week 20	-	-	5/52	-	1/52	0/42	-	0/42	0	0
	Arm 2:61	200 IU HBIG every 2 wk from week 28	-	-	26/61	-	7/61	9/55	-	9/55	0	0
Han, 2009 ^[23]	Arm 1:57	3TC 100 mg od from week 20	7.5 ± 0.50	-	6/57	-	1/57	0/46	-	0/46	0	0
	Arm 2:66	200 IU HBIG every 2 wk from week 28	7.5 ± 0.72	-	27/66	-	8/66	10/59	-	10/59	0	1/66
Su, 2009 ^[24]	Arm 1:128	3TC 100 mg od from week 32, 200 IU HBIG at week 28, 32, 36	-	-	-	-	-	6/128	-	-	0	0
	Arm 2:120	200 IU HBIG at week 28, 32, 36	-	-	-	-	-	17/120	-	-	-	-
Xu, 2009 ^[25]	Arm 1:63	3TC 100 mg od from week 32	9.35 ± 0.21	7.71 ± 1.49	17/56	-	7/56	10/56	-	11/56	7/89	10/56
	Arm 2:62	Placebo	9.43 ± 0.21	9.34 ± 0.22	14/59	-	24/59	23/59	-	27/59	6/61	12/59
Shi, 2009 ^[26]	Arm 1:49	3TC 100 mg od from week 28	7.24 ± 1.90	4.49 ± 3.25	3/49	-	1/49	-	-	-	2/51	-
	Arm 2:116	100 IU HBIG at week 28, 32, 36	6.31 ± 2.13	5.86 ± 2.62	8/116	-	4/116	-	-	-	3/146	-
	Arm 3:43	Placebo	6.40 ± 2.12	6.19 ± 2.57	10/43	-	5/43	-	-	-	2/84	-
Yang, 2008 ^[27]	Arm 1:45	3TC 100 mg od from week 24	6.99 ± 0.84	5.10 ± 0.80	-	-	-	1/45	-	-	-	0
	Arm 2:42	100 IU HBIG at week 28, 32, 36	6.87 ± 0.92	6.87 ± 0.92	-	-	-	6/42	-	-	-	0
Guo, 2008 ^[28]	Arm 1:70	3TC 100 mg od from week 28	-	-	6/70	-	8/70	4/70	-	6/70	-	-
	Arm 1:40	No treatment	-	-	10/40	-	13/40	12/40	-	18/40	-	-
Xiang, 2007 ^[29]	Arm 1:21	3TC 100 mg od from week 28	8.02 ± 1.15	4.58 ± 1.22	1/21	3/21	-	-	-	-	-	-
	Arm 2:25	200 IU HBIG every month from month 4	7.63 ± 1.23	5.12 ± 1.07	2/25	2/25	-	-	-	-	-	-
	Arm 3:18	No treatment	7.16 ± 0.79	6.88 ± 1.36	5/18	3/18	-	-	-	-	-	-
Feng, 2007 ^[30]	Arm 1:48	3TC 100 mg od from week 28	8.34 ± 1.23	4.85 ± 1.27	8/48	-	9/48	7/48	-	7/48	0	0
	Arm 1:42	No treatment	8.26 ± 1.87	8.56 ± 1.08	17/42	-	19/42	16/42	-	16/42	0	0
Li, 2006 ^[31]	Arm 1:36	3TC 100 mg od from week 24	6.89 ± 0.82	5.08 ± 0.76	-	-	-	1/36	-	-	0	0
	Arm 2:44	No treatment	> 5.00	> 5.00	-	-	-	7/44	-	-	0	0
Li, 2006 ^[32]	Arm 1:40	3TC 100 mg od from week 28 and 200 IU HBIG at week 28, 32, 36	-	-	-	-	-	1/35	1/35	1/35	0	0
	Arm 2:37	200 IU HBIG at week 28, 32, 36	-	-	-	-	-	7/32	6/32	6/32	0	0
Han, 2005 ^[33]	Arm 1:43	3TC 100 mg od from week 28	7.15 ± 0.91	5.43 ± 0.85	-	-	-	0/43	-	0/43	0	0
	Arm 1:35	No treatment	> 5.60	> 5.60	-	-	-	5/35	-	-	0	0
Shi, 2005 ^[34]	Arm 1:21	3TC 100 mg od from week 28	8.72 ± 0.69	6.59 ± 1.06	1/21	3/21	2/21	-	-	-	1/21	0
	Arm 1:18	No treatment	8.93 ± 1.12	9.05 ± 0.26	1/18	2/18	8/18	-	-	-	0	0
Li, 2003 ^[35]	Arm 1:56	200 IU HBIG every 4 wk from week 28	7.38 ± 1.17	5.28 ± 2.77	3/56	7/56	-	-	-	-	0	0
	Arm 2:43	3TC 100 mg od from week 28	7.49 ± 0.54	5.33 ± 1.34	1/43	7/43	-	-	-	-	0	0
	Arm 3:52	No treatment	7.05 ± 1.29	6.23 ± 3.66	8/52	11/52	-	-	-	-	0	0

3TC: Lamivudine; HBIG: Hepatitis B immunoglobulin; IU: International unit; mg: Milligram; od: Once daily; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; -: Data not available. ¹Log₁₀ HBV DNA (mean ± SD).

Table 2 Methodological quality of included randomized clinical trials

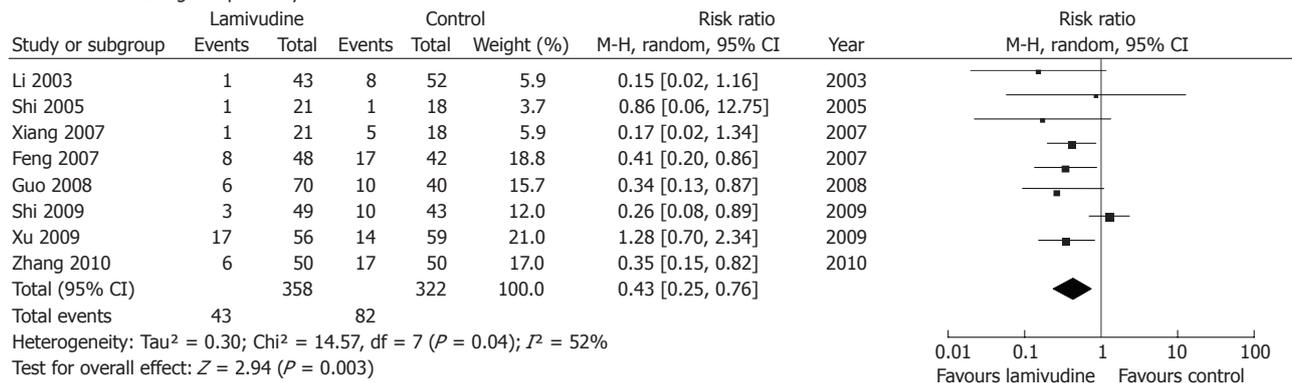
First author, year ^[Ref.]	Generation of allocation sequence	Allocation concealment	Blinding	Methodological quality
Han, 2010 ^[22]	Unclear	Unclear	Unclear	Low
Zhang, 2010 ^[21]	Unclear	Unclear	Unclear	Low
Han, 2009 ^[23]	Unclear	Unclear	Unclear	Low
Su, 2009 ^[24]	Unclear	Unclear	Unclear	Low
Xu, 2009 ^[25]	Unclear	Adequate	Adequate	High
Shi, 2009 ^[26]	Adequate	Adequate	Adequate	High
Yang, 2008 ^[27]	Adequate	Unclear	Unclear	Low
Guo, 2008 ^[28]	Adequate	Unclear	Unclear	Low
Xiang, 2007 ^[29]	Adequate	Unclear	Unclear	Low
Feng, 2007 ^[30]	Not done	Not done	Not done	Low
Li, 2006 ^[31]	Not done	Not done	Not done	Low
Li, 2006 ^[32]	Not done	Not done	Not done	Low
Han, 2005 ^[33]	Not done	Not done	Not done	Low
Shi, 2005 ^[34]	Adequate	Unclear	Unclear	Low
Li, 2003 ^[35]	Adequate	Unclear	Unclear	Low

As a result, 2 trials were classified as of high quality and the remaining 13 trials were of low quality (Table 2).

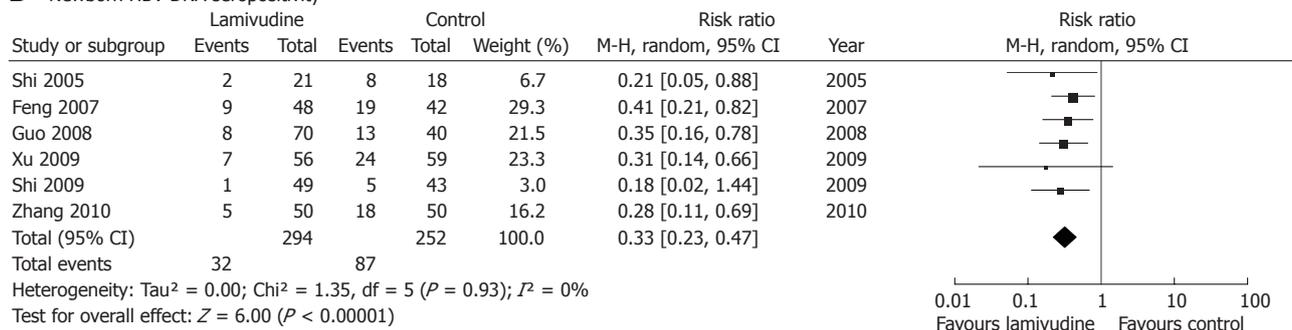
Effects of lamivudine on interruption of MTCT indicated by serum HBsAg, HBeAg or HBV DNA of newborns within 24 h after birth

Compared with controls (placebo or no treatment), lamivudine treatment for the HBV-carrier mothers significantly interrupted MTCT. The efficacy (RR, 95% CI) of lamivudine treatment *vs* control in 8 RCTs was 0.43, 0.25-0.76; *P* < 0.01, with significant heterogeneity (*P* = 0.04, *I*² = 52%) as indicated by serum HBsAg (Figure 2A). It was 0.33, 0.23-0.47 in 6 RCTs; *P* < 0.01, with minimum heterogeneity (*P* = 0.93, *I*² = 0) indicated by serum HBV DNA (Figure 2B). However, the corresponding values shown by HBeAg was not significant in 3 RCTs (0.86, 0.43-1.69; *P* = 0.65) with minimum heterogeneity (*P* = 0.87, *I*² = 0) (Figure 2C). The funnel

A Newborn HBsAg seropositivity



B Newborn HBV DNA seropositivity



C Newborn HBeAg seropositivity

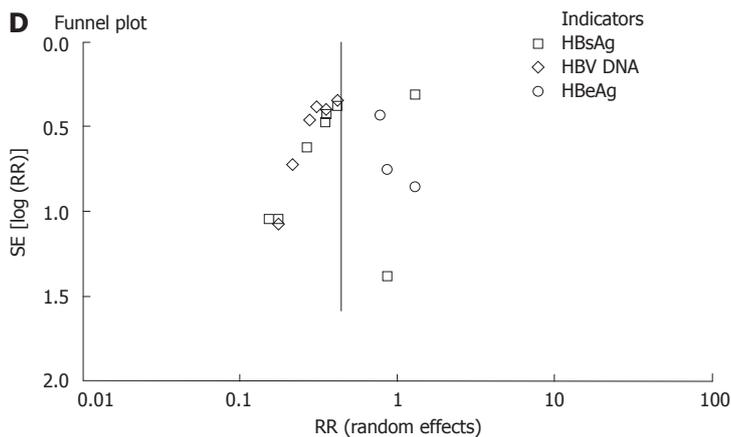
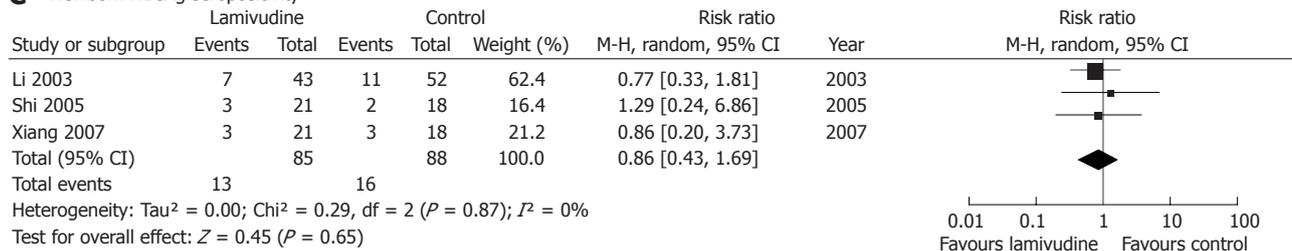


Figure 2 Effect of lamivudine treatment vs control (placebo or no intervention) on interruption of hepatitis B virus mother-to-child transmission as indicated by newborn serum hepatitis B surface antigen or hepatitis B virus DNA. Vertical line indicates no difference between compared treatment. Horizontal lines show 95% CIs. Squares indicate point estimates, and the size of the squares indicates the weight of each study in the meta-analysis. HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; M-H random: Mantel-Haenszel random-effects model; CI: Confidence interval; HBV: Hepatitis B virus; RR: Risk ratio.

plots showed possible publication bias (Figure 2D).

Lamivudine treatment for the mothers receiving

HBIG before delivery significantly interrupted MTCT.

The efficacy of lamivudine *vs* HBIG in interruption

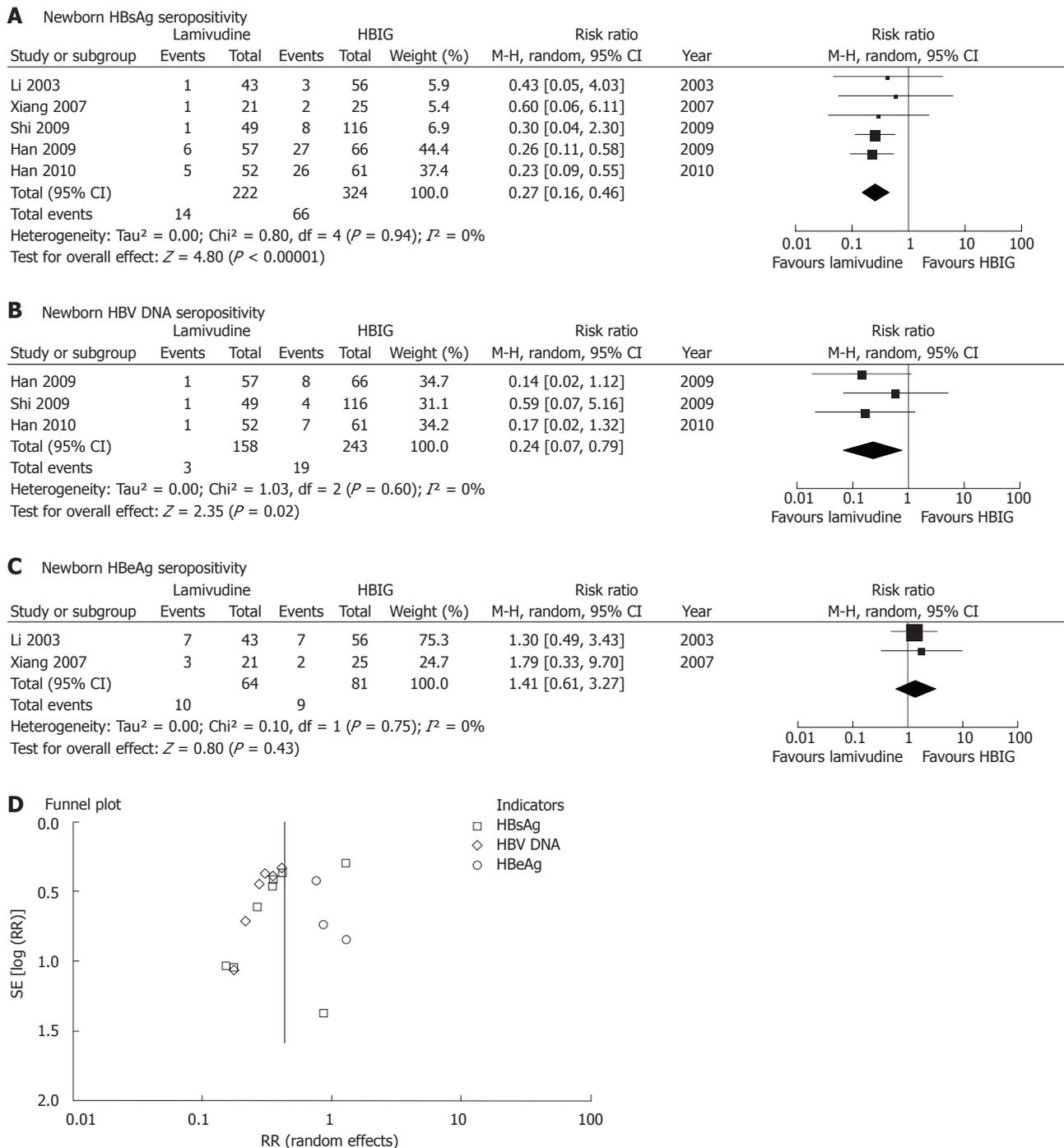


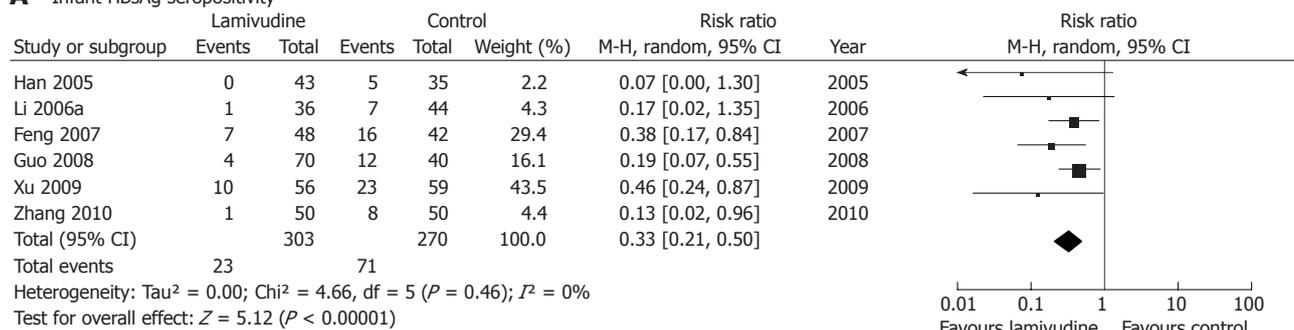
Figure 3 Lamivudine treatment vs hepatitis B immunoglobulin in interruption of hepatitis B virus mother-to-child transmission as indicated by newborn serum hepatitis B surface antigen or hepatitis B virus DNA. CI: Confidence interval; HBIG: Hepatitis B immunoglobulin; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; M-H random: Mantel-Haenszel random-effects model; RR: Risk ratio.

of MTCT indicated by serum HBsAg or HBV DNA was 0.27, 0.16-0.46 in 5 RCTs; *P* < 0.01, with minimum heterogeneity (*P* = 0.94, *I*² = 0); and 0.24, 0.07-0.79 in 3 RCTs; *P* = 0.02, with minimum heterogeneity (*P* = 0.60, *I*² = 0) (Figure 3A and B). However, the corresponding value indicated by HBeAg was not significant (1.41, 0.61-3.27 in 3 RCTs; *P* = 0.43) with minimum heterogeneity (*P* = 0.75, *I*² = 0) (Figure 3C). The funnel plots showed possible publication bias (Figure 3D).

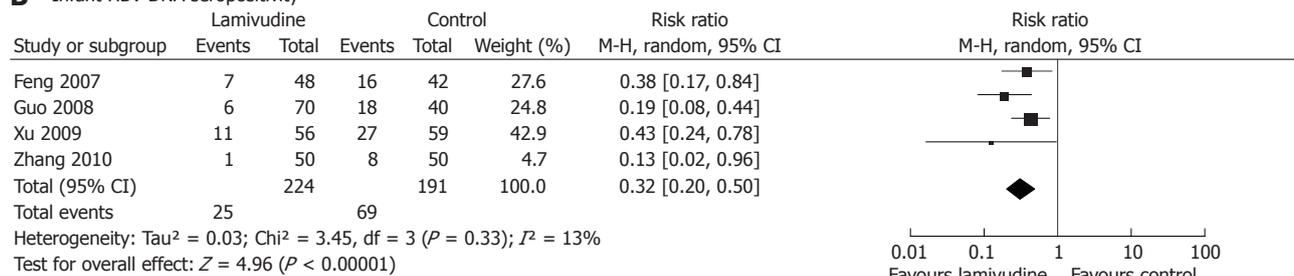
Effect of lamivudine on interruption of MTCT indicated by serum HBsAg or HBV DNA of infants 6-12 mo after birth

Compared with controls (placebo or no treatment), lamivudine treatment significantly interrupted MTCT. The efficacy (RR, 95% CI) of lamivudine treatment *vs* controls was 0.33, 0.21-0.50 in 6 RCTs; *P* < 0.01, with medium heterogeneity (*P* = 0.46, *I*² = 0) as shown by serum HBsAg (Figure 4A). It was 0.32, 0.20-0.50 in 4 RCTs; *P* <

A Infant HBsAg seropositivity



B Infant HBV DNA seropositivity



C Funnel plot

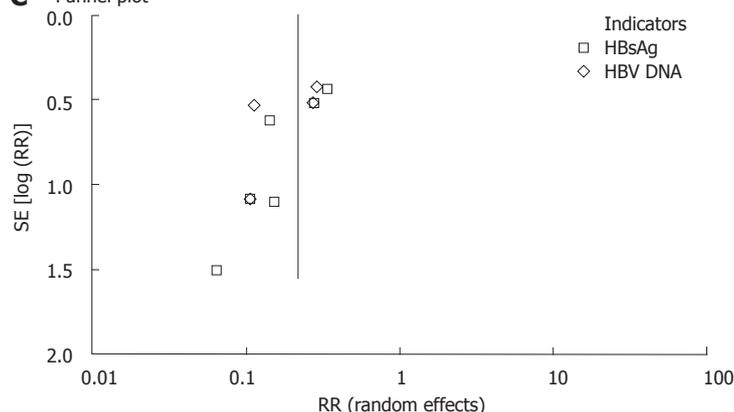


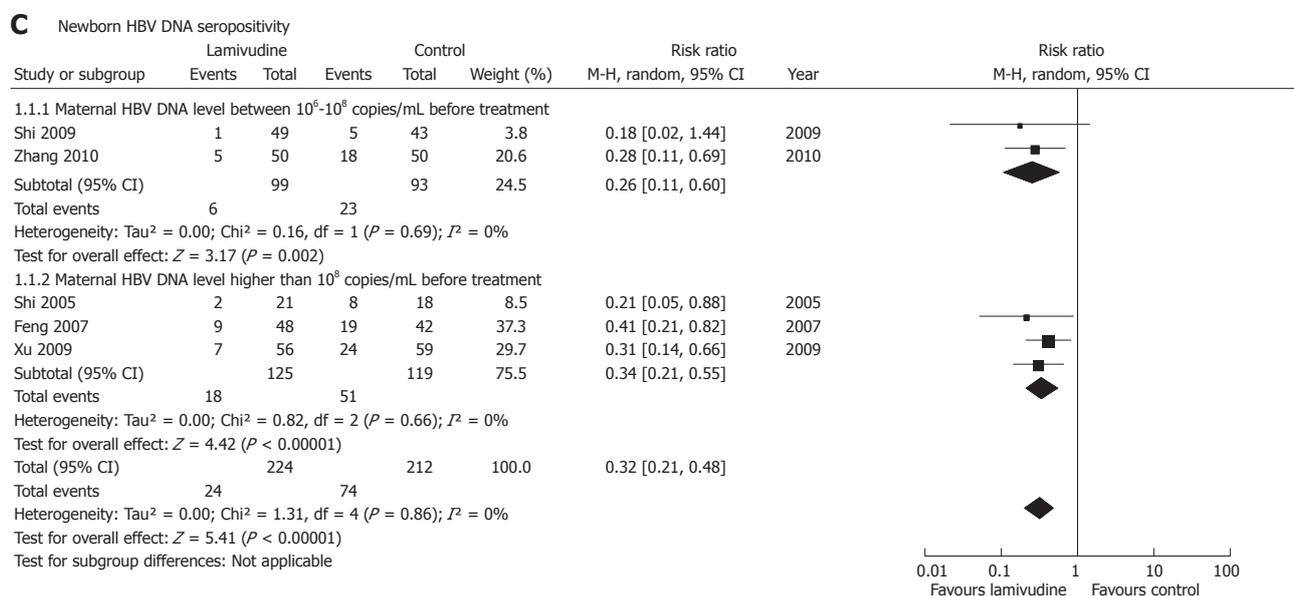
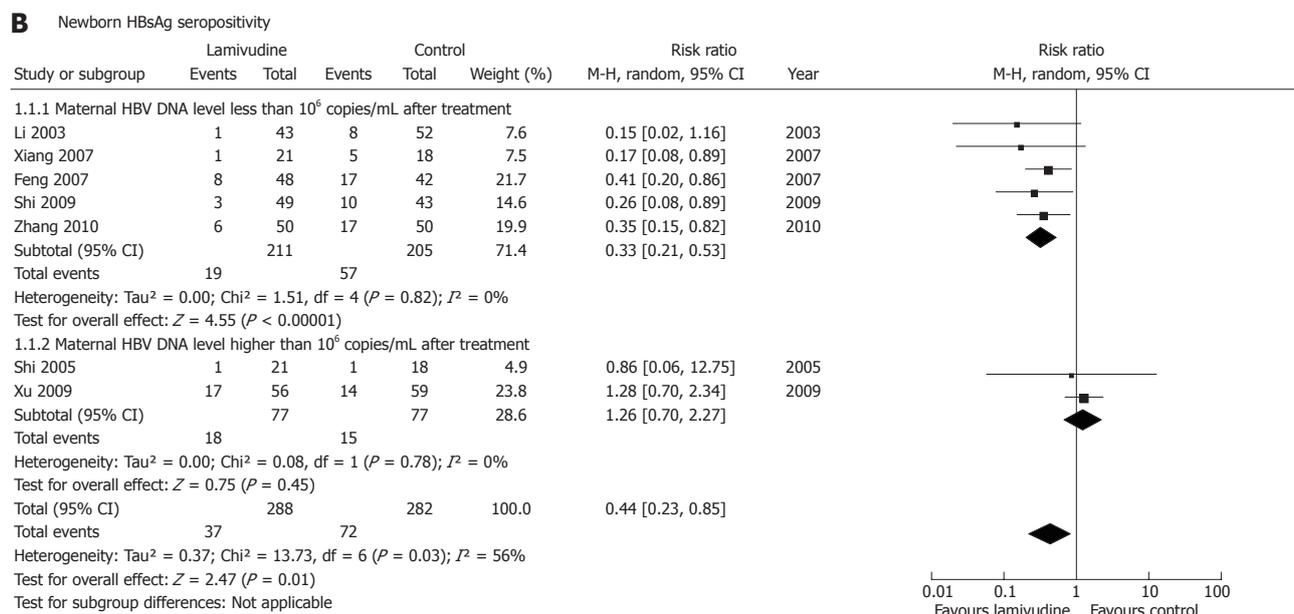
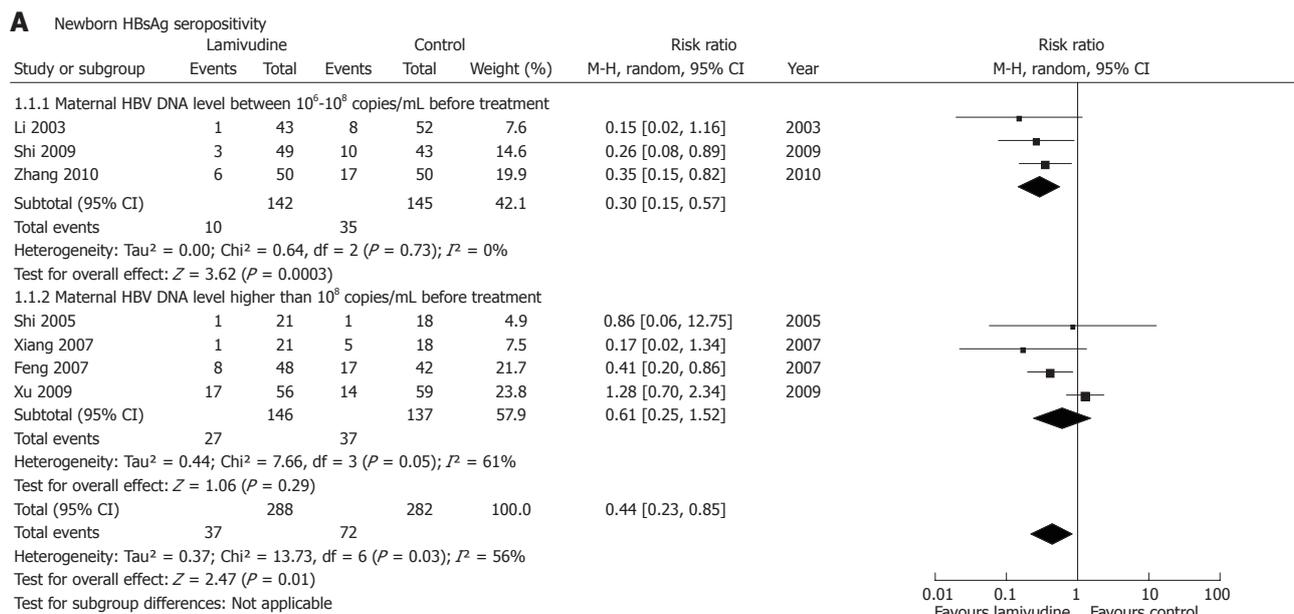
Figure 4 Effects of lamivudine vs control (placebo or no intervention) on interruption of hepatitis B virus mother-to-child transmission as indicated by serum hepatitis B surface antigen or hepatitis B virus DNA of infants 6-12 mo after birth. Vertical line indicates no difference between compared treatment. Horizontal lines show 95% CIs. Squares indicate point estimates, and the size of the squares indicates the weight of each study in the meta-analysis. HBsAg: Hepatitis B surface antigen; M-H random: Mantel-Haenszel random-effects model; CI: Confidence interval; HBV: Hepatitis B virus; RR: Risk ratio.

0.01, with minimum heterogeneity (*P* = 0.33, *I*² = 13%) as shown by serum HBV DNA (Figure 4B). The funnel plots showed possible publication bias (Figure 4C).

Two RCTs evaluated the effect of lamivudine treatment *vs* HBIG in late pregnancy on the interruption of MTCT^[22,23]. The efficacy of lamivudine *vs* HBIG in interruption of MTCT indicated by serum HBsAg or HBV DNA in 2 RCTs was 0.05, 0.01-0.41; *P* < 0.01, with minimum heterogeneity (*P* = 0.95, *I*² = 0); and 0.06, 0.01-0.47; *P* < 0.01, with minimum heterogeneity (*P* = 0.95, *I*² = 0), respectively. Two RCTs evaluated the efficacy of lamivudine plus HBIG *vs* HBIG alone in the interruption of MTCT indicated by serum HBsAg^[24,32]. The corresponding value was 0.28, 0.13-0.65, *P* < 0.01, with minimum heterogeneity (*P* = 0.41, *I*² = 0).

Influence of maternal viral load before or after lamivudine treatment on MTCT indicated by serum HBsAg or HBV DNA of newborns

To determine the effect of viral load of mothers before or after lamivudine treatment on interruption of MTCT, we stratified the included mothers into subgroups with different viral loads and compared with the controls. MTCT was indicated by newborn serum HBsAg. In the mothers with a viral load of 10⁶-10⁸ copies/mL before lamivudine treatment, the efficacy (RR, 95% CI) of lamivudine *vs* controls was 0.30, 0.15-0.57 in 3 RCTs, *P* < 0.01, with minimum heterogeneity (*P* = 0.73, *I*² = 0), however, in the mothers with a viral load > 10⁸ copies/mL before lamivudine treatment, the corresponding value in lamivudine *vs* controls was 0.61, 0.25-1.52 in 4 RCTs, *P* = 0.29, with



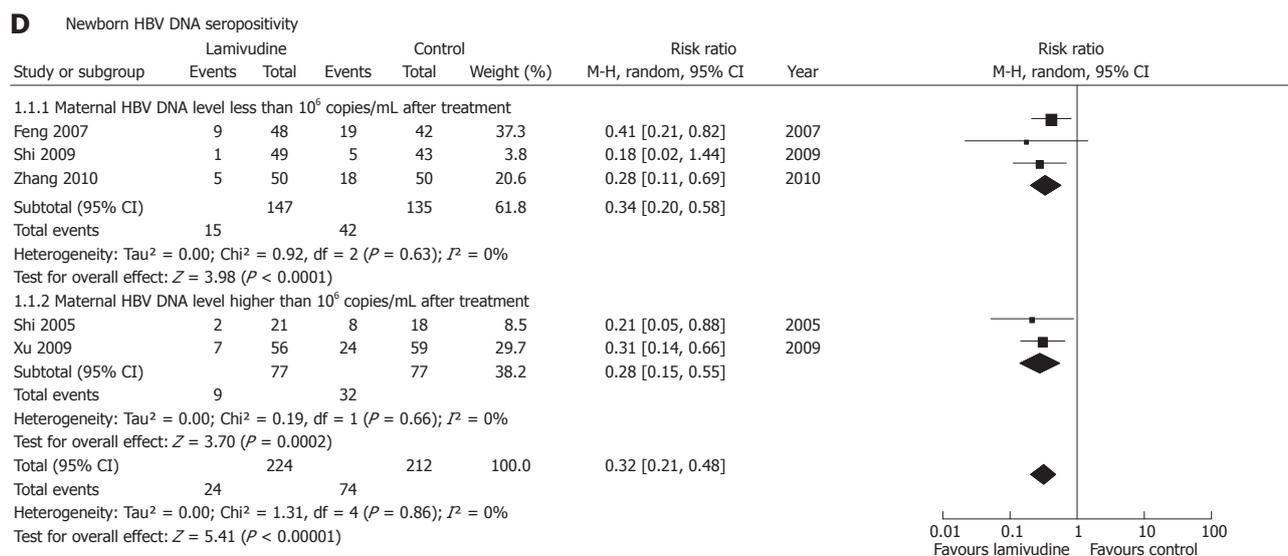


Figure 5 Influence of maternal viral load before or after lamivudine treatment on hepatitis B virus mother-to-child transmission as indicated by serum hepatitis B surface antigen or hepatitis B virus DNA of newborns within 24 h after birth. Vertical line indicates no difference between compared treatments. Horizontal lines show 95% CIs. Squares indicate point estimates, and the size of the squares indicates the weight of each study in the meta-analysis. CI: Confidence interval; HBV: Hepatitis B virus; M-H random: Mantel-Haenszel random-effects model; RR: Risk ratio; HBsAg: Hepatitis B surface antigen.

significant heterogeneity (*P* = 0.05, *I*² = 61%) (Figure 5A). In the mothers with a viral load < 10⁶ copies/mL after lamivudine treatment, the efficacy of lamivudine treatment *vs* controls was 0.33, 0.21-0.53 in 5 RCTs, *P* < 0.01, with minimum heterogeneity (*P* = 0.82, *I*² = 0), however, in the mothers with a viral load > 10⁶ copies/mL after lamivudine treatment, the corresponding value was 1.26, 0.70-2.27 in 2 RCTs, *P* = 0.45, with minimum heterogeneity (*P* = 0.78, *I*² = 0%) (Figure 5B). When MTCT was indicated by newborn serum HBV DNA, lamivudine treatment significantly interrupted MTCT in groups with various maternal viral loads before or after lamivudine treatment (*P* < 0.01) (Figure 5C and D).

Effect of lamivudine treatment starting time on interruption of MTCT indicated by serum HBsAg or HBV DNA of newborns within 24h after birth

Lamivudine treatment was initiated from week 28 of gestation in most of the included studies. The treatment was initiated from week 32 of gestation in one study^[25]. The efficacy of lamivudine treatment initiated at week 28 of gestation *vs* controls in interruption of MTCT indicated by serum HBsAg or HBV DNA (RR, 95% CI) was 0.34, 0.22-0.52 in 7 RCTs; *P* < 0.01, with minimum heterogeneity (*P* = 0.92, *I*² = 0); and 0.33, 0.22-0.50 in 5 RCTs; *P* < 0.01, with minimum heterogeneity (*P* = 0.86, *I*² = 0), respectively. When lamivudine treatment was initiated at week 32 of gestation, MTCT was not significantly interrupted as indicated by serum HBsAg, however, lamivudine significantly interrupt MTCT as shown by serum HBV DNA (Figure 6).

Efficacy of lamivudine treatment in interruption of MTCT indicated by serum HBsAg or HBV DNA of newborns within 24h after birth among different studies

We stratified the included studies into high and low

qualities and evaluated the efficacy of lamivudine in interruption of MTCT. Using pooled data of “low-quality” studies, the efficacy of lamivudine *vs* controls was 0.35, 0.23-0.55 in 6 RCTs; *P* < 0.01, with minimum heterogeneity (*P* = 0.88, *I*² = 0%) indicated by serum HBsAg. It was 0.34, 0.22-0.52 in 4 RCTs; *P* < 0.01, with minimum heterogeneity (*P* = 0.81, *I*² = 0) indicated by serum HBV DNA. Using pooled data of the 2 “high-quality” studies, the corresponding value was 0.63, 0.13-3.04, *P* = 0.57, with significant heterogeneity (*P* = 0.02, *I*² = 81%) as shown by serum HBsAg. However, the corresponding value was 0.29, 0.14-0.59, *P* < 0.01 with minimum heterogeneity (*P* = 0.93, *I*² = 0) as indicated by serum HBV DNA. These results are shown in Figure 7.

Side effects of lamivudine treatment

Three RCTs reported adverse effects of lamivudine in mothers^[25,26,34]. The incidence of adverse effects was not significantly different as compared with the control. Only one trial reported adverse event in the newborns^[25]. Among the ten major adverse events, only one was considered drug-related, with a symptom of jaundice.

DISCUSSION

This meta-analysis included 15 RCTs published up to January 2011, including a total of 1693 HBV-carrier mothers. We demonstrated that lamivudine treatment in the HBV-carrier mothers, as compared with controls, significantly interrupted MTCT as indicated by serum HBsAg or HBV DNA of newborns or infants. And lamivudine treatment *vs* HBIG in the HBV-carrier mothers significantly interrupted MTCT as indicated by serum HBsAg or HBV DNA of newborns or infants. This is also true for lamivudine plus HBIG *vs* HBIG. In a recent meta-analysis, advantage of lamivudine treat-

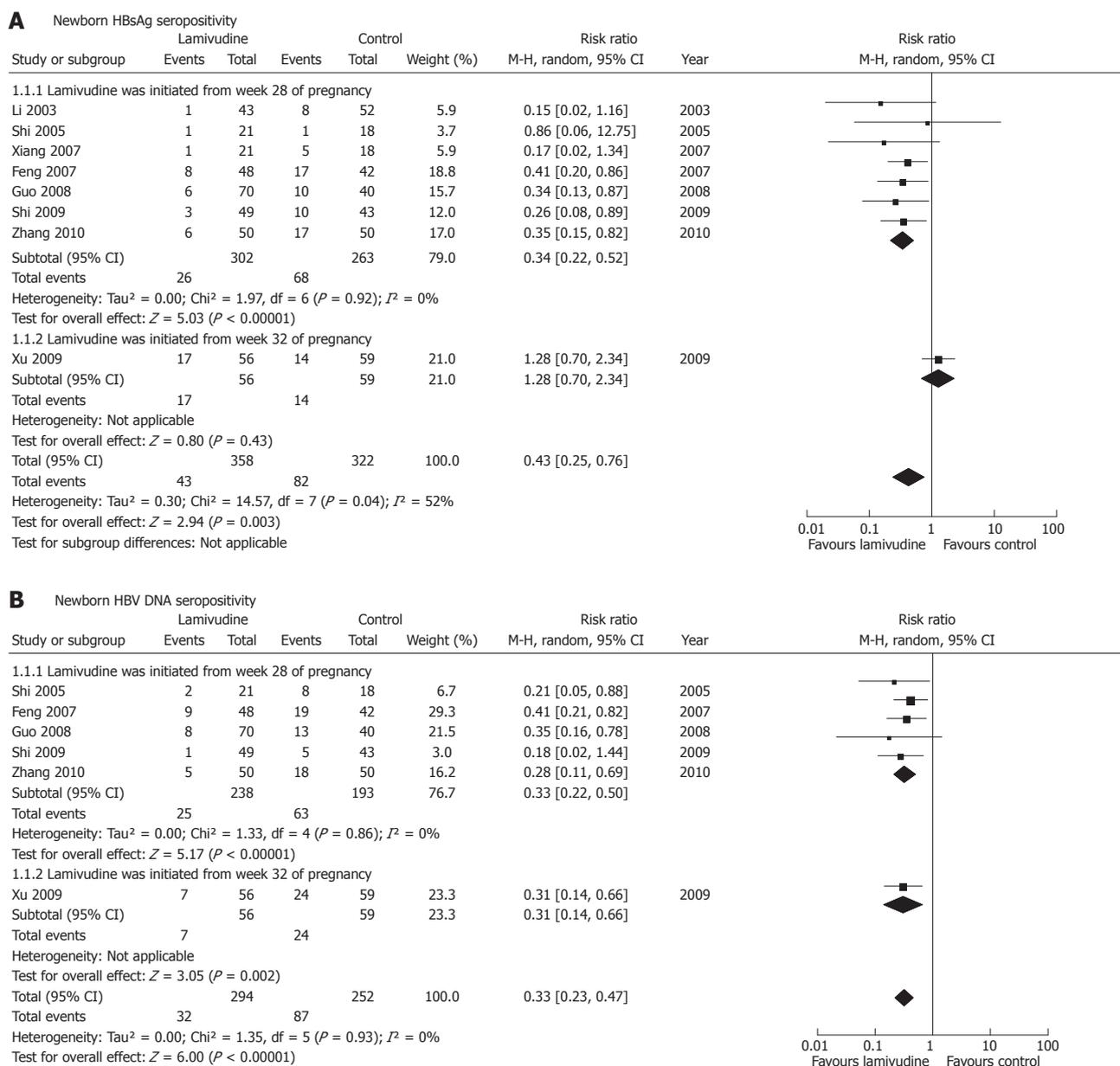


Figure 6 Effect of lamivudine treatment starting time on interruption of mother-to-child transmission indicated by newborn hepatitis B surface antigen or hepatitis B virus DNA. Vertical line indicates no difference between compared treatments. Horizontal lines show 95% CIs. Squares indicate point estimates, and the size of the squares indicates the weight of each study in the meta-analysis. CI: Confidence interval; HBV: Hepatitis B virus; M-H random: Mantel-Haenszel random-effects model; RR: Risk ratio; HBsAg: Hepatitis B surface antigen.

ment was not found over HBIG because two important papers were not included^[15]. This result is quite reasonable because the recommended dose of HBIG might be insufficient to neutralize the huge virus load in HBV-carrier mothers at late pregnancy, although HBIG to HBeAg-seropositive mothers from week 28 of gestation significantly decreased the seropositivity of HBV DNA in newborns^[36]. Thus, lamivudine can be used for the pregnant women with a high degree of infectiousness.

Serum HBsAg, HBeAg, and/or HBV DNA in newborns or infants born to HBV-carrier mothers are routine indicators of MTCT. Of these indicators, HBsAg is a reliable and widely used one. Beasley *et al*^[37] suggested two criteria for HBV perinatal infection: (1) high titers of

HBsAg within 24 h after birth; and (2) after the joint immunoprophylaxis, infants developed into HBsAg carriers. In addition, continuous monitoring of HBeAg and/or HBV DNA is also suggested, because HBeAg from the mother through the placenta will disappear within 7 mo after birth and peripheral blood HBV DNA testing is more reliable and sensitive than other HBV markers^[38,39]. In this study, although the results using HBsAg or HBV DNA as an indicator were mostly consistent, there were some inconsistencies in indicating MTCT. If indicated solely by HBsAg, lamivudine treatment in the mothers with a viral load > 10⁸ copies/mL before the treatment or in those with a viral load > 10⁶ copies/mL after the treatment could not significantly interrupt MTCT. How-

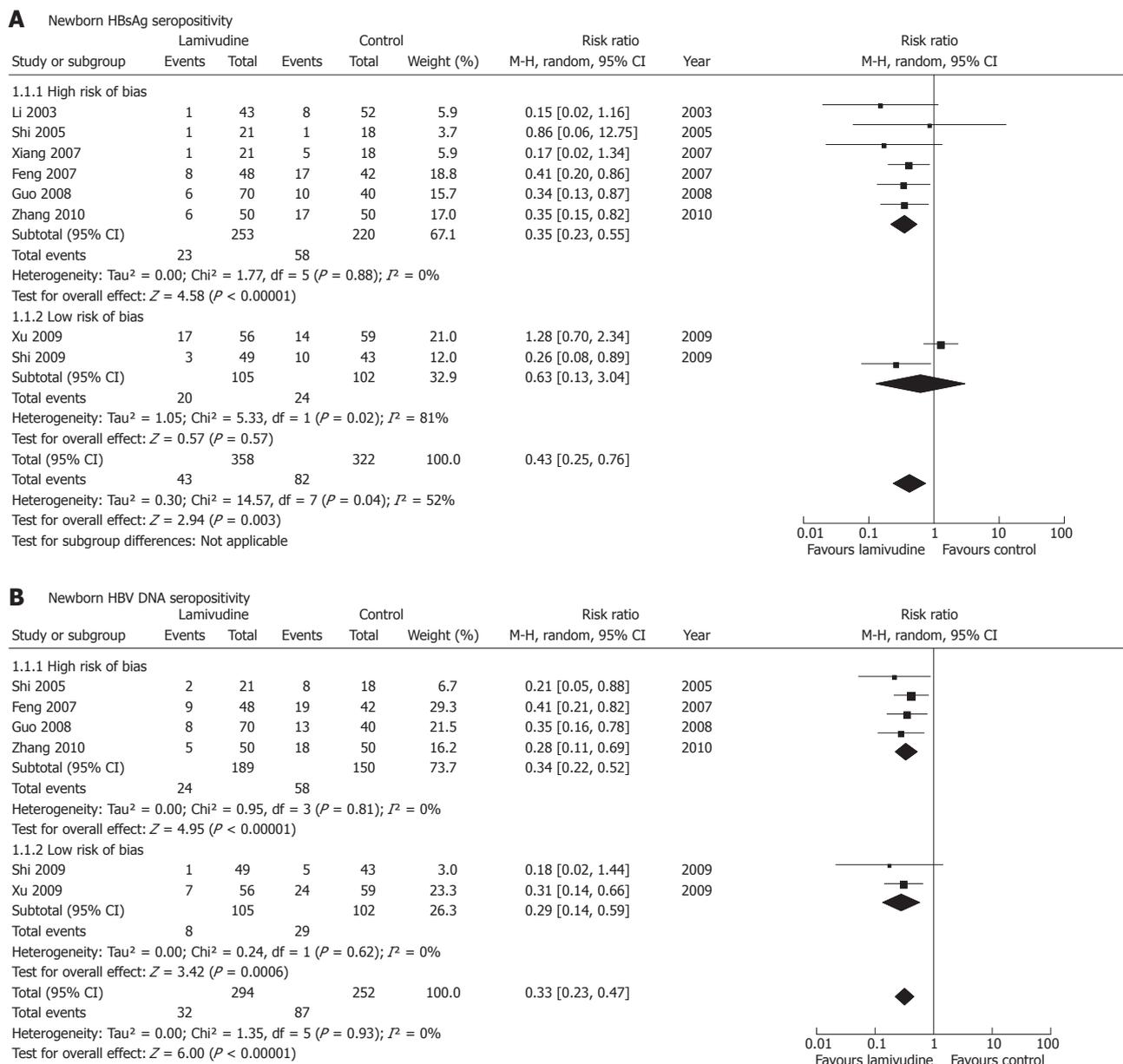


Figure 7 Efficacy of lamivudine treatment in “high-quality” studies or “low-quality” studies in interruption of mother-to-child transmission indicated by serum hepatitis B surface antigen or hepatitis B virus DNA of newborns. Vertical line indicates no difference between compared treatments. Horizontal lines show 95% CIs. Squares indicate point estimates, and the size of the squares indicates the weight of each study in the meta-analysis. CI: Confidence interval; HBV: Hepatitis B virus; M-H random: Mantel-Haenszel random-effects model; RR: Risk ratio; HBsAg: Hepatitis B surface antigen.

ever, if indicated by newborn HBV DNA, lamivudine treatment in the mothers with a viral load > 10⁸ copies/mL before the treatment or in those with a viral load > 10⁶ copies/mL after the treatment significantly interrupted MTCT. The same results were found in the pooled analysis of “low quality” and “high quality” studies. These controversial evidences reflect the validity and reliability of the indicators. In the included studies, HBV DNA was measured by quantitative PCR method. However, a viral load ≤ 5 × 10² copies/mL is usually undetected using commercially available reagents in mainland China. HBV DNA is frequently negative in HBsAg seropositive subjects, especially in asymptomatic HBsAg carriers^[40-42]. Thus, efficacy of lamivudine treatment in

interruption of MTCT might be over-estimated by using newborn HBV DNA alone. Serial examination of HBV DNA and HBsAg from newborns to infants 6-12 mo after birth is highly suggested. In this study, we also found that lamivudine treatment was unable to interrupt transmission of HBeAg from mothers to newborns. HBeAg, a small soluble protein, might pass through the placenta during gestation and disappear within 6-7 mo after birth, indicating that HBeAg is unsuitable for indicating MTCT for newborns, but can be used for the confirmation of MTCT 6-12 mo after birth.

In this study, we confirmed that lamivudine treatment from week 28 of gestation was efficient in interrupting MTCT as indicated by serum HBsAg or HBV DNA of

newborns within 24 h after birth. However, only one study reported that lamivudine treatment from week 32 of gestation was inefficient in interrupting MTCT as indicated by serum HBsAg, although newborn HBV DNA could be significantly decreased. Thus, we suggest that lamivudine treatment should be initiated from week 28 of gestation.

The incidence of adverse effects was not significantly different in HBV carrier mothers with and without lamivudine treatment. Lamivudine treatment in HBV carrier-mothers in late pregnancy has been inversely associated with the complications of HBV-infected pregnant patients^[43]. Thus, lamivudine treatment is well-tolerated and safe for the HBV-carrier mothers at the late stage of pregnancy. However, long-term treatment with lamivudine might generate the treatment-escape mutations like V173L in the B domain and M204V or I substitution in the C domain of the polymerase/reverse transcriptase^[44]. Generation of lamivudine treatment-escape mutations might prevent future treatment of the HBV carrier mothers.

Our meta-analysis has several potential limitations. Firstly, some analysis included few trials so that the subgroup analysis could not be conducted appropriately. Secondly, HBeAg status of mothers was not evaluated because the data was incomplete in the original studies. Thirdly, the majority of included RCTs were of low quality and had high risk of bias in design, and funnel plot showed possible publication bias. The results from this meta-analysis should be discreetly interpreted.

In conclusion, lamivudine treatment in HBV carrier-mothers from 28 wk of gestation efficiently interrupts MTCT as indicated by newborn or infant serum HBsAg or HBV DNA. Lamivudine treatment is safe for the HBV-carrier mothers in late pregnancy and more efficient than HBIG in interrupting MTCT. If maternal viral load is reduced to $< 10^6$ copies/mL by lamivudine treatment, HBV MTCT can be prevented more efficiently as indicated by newborn serum HBsAg.

COMMENTS

Background

Mother-to-child transmission (MTCT) of hepatitis B virus (HBV) is associated with a very high rate of chronicity, especially in countries where HBV is endemic. Prevention of MTCT is the most important strategy in the global eradication of HBV infection. Apart from the joint immunoprophylaxis to infants born to HBV-carrier mothers, lamivudine treatment in late pregnancy has been reported to be effective in interrupting MTCT and safe in pregnant women. However, the sample sizes of these studies were small and results were controversial, which necessitates a meta-analysis by pooling data of more available studies.

Research frontiers

Compared with placebo controls or hepatitis B immunoglobulin (HBIG), lamivudine treatment in late pregnancy significantly interrupted MTCT as indicated by serum hepatitis surface antigen (HBsAg) or HBV DNA of newborns 24h or infants 6-12 mo after birth. In the mothers with viral load $< 10^8$ copies/mL, lamivudine treatment has a clear beneficial effect, as indicated by newborn serum HBsAg. Lamivudine treatment initiated at week 28 of gestation is efficient in interruption of MTCT as indicated by serum HBsAg or HBV DNA of newborns.

Innovations and breakthroughs

A recent meta-analysis of randomized controlled trials using data up to October 2009 has demonstrated that lamivudine treatment in HBV-infected mothers in

late pregnancy effectively prevented MTCT. However, difference in interruption of MTCT between lamivudine treatment and HBIG was not found.

Applications

Lamivudine treatment for HBV carrier mothers should be initiated at week 28 of gestation. For the HBV carrier mothers with viral load $> 10^8$ copies/mL, antiviral treatment with lamivudine alone might be not enough to interrupt MTCT. MTCT might be efficiently interrupted if maternal viral load is decreased to the level of $< 10^6$ copies/mL by lamivudine treatment.

Terminology

MTCT of HBV includes in utero transmission and perinatal transmission of HBV. In East and Southeast Asia, in utero transmission of HBV is rare, whereas perinatal transmission is common.

Peer review

The study determines the effect of lamivudine treatment in hepatitis B virus-carrier mothers in late pregnancy on interruption of MTCT by means of meta-analysis.

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S- Editor Tian L L- Editor Ma JY E- Editor Zhang DN

Sixty-four-slice computed tomography in surgical strategy of portal vein cavernous transformation

Ming-Man Zhang, Cong-Lun Pu, Ying-Cun Li, Chun-Bao Guo

Ming-Man Zhang, Cong-Lun Pu, Ying-Cun Li, Chun-Bao Guo, Department of Hepatobiliary Surgery, Children's hospital, Chongqing Medical University, Chongqing 400014, China
Author contributions: Guo CB designed the research; Pu CL, Zhang MM, Li YC and Guo CB performed the research; Pu CL and Guo CB analyzed the data and were involved in editing the manuscript; Zhang MM and Pu CL contribute equally to this research; and Guo CB wrote the paper, co-ordinated and provided the collection of all the human material as well as provided financial support for this work.

Supported by National Natural Science Foundation of China, No. 30973440 and No. 30770950; and key project of Chongqing Natural Science Foundation (CSTC, 2008BA0021)

Correspondence to: Chun-Bao Guo, MD, PhD, Department of Hepatobiliary Surgery, Children's Hospital of Chongqing Medical University, 136 Zhongshan 2nd Rd, Chongqing 400014, China. gchunbao@yahoo.com.cn

Telephone: +86-23-63893006 Fax: +86-23-63893006

Received: August 13, 2010 Revised: November 23, 2010

Accepted: November 30, 2010

Published online: October 14, 2011

Abstract

AIM: To investigate the role of 64-slice computed tomography (CT) in portal vein cavernous transformation to determine surgical strategy.

METHODS: The site of lesions and extent of collateral circulation in 12 pediatric cases of cavernous transformation of the portal vein with surgical treatment were analyzed.

RESULTS: Eleven of 12 children had esophageal varices and were treated with lower esophageal and gastric devascularization and splenectomy, and the other case was only treated with splenectomy. There were eight cases with spontaneous spleen/stomach-renal shunt, four with Retzius vein opening, which was reserved during surgery. Three cases of lesions involving the intrahepatic portal vein (PV) were treated with living

donor liver transplantation. One patient died from PV thrombosis after liver transplantation, and the rest had no significant complications.

CONCLUSION: The PV, its branches and collateral circulation were clearly seen by 64-slice spiral CT angiography, which helped with preoperative surgical planning.

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Key words: Cavernous transformation; Portal vein; 64-slice computed tomography; Liver transplantation; Portal hypertension; Esophageal devascularization; Gastric devascularization

Peer reviewer: Dr. Furqan Haider Sherazi, King Edward Medical University, House 975, Street 48, Sector G/11-2, Islamabad 44000, Pakistan

Zhang MM, Pu CL, Li YC, Guo CB. Sixty-four-slice computed tomography in surgical strategy of portal vein cavernous transformation. *World J Gastroenterol* 2011; 17(38): 4334-4338 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4334.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4334>

INTRODUCTION

Cavernous transformation of the portal vein (CTPV) is a rare disease in children. The location of the cavernous transformation determines the clinical manifestation of the patients and the treatment procedures^[1]. Computed tomography (CT) can be utilized for angiography of CTPV and has distinct advantages in locating the position and assessing the severity of the cavernous transformation^[2]. Few people have reported using 64-slice CT for the diagnosis of CTPV or its impact on surgical procedures. The current study retrospectively analyzed the angiographic performance of 64-slice CT in 12 cases

and evaluated whether it affects surgical procedures by comparing the corresponding surgical results.

MATERIALS AND METHODS

Clinical information

Eighteen pediatric cases of CTPV were admitted to our department between June 1999 and December 2007. Among these 12 patients (nine male and three female) received 64-slice CT and surgical treatment after 2004. The patient ages were between 3 and 8 years, with an average of 5 years and 3 mo. The clinical records of the patients were thoroughly analyzed, and the location of the thrombus in the portal vein (PV), the pathology of the hepatic lesions, surgical procedures, findings during surgery, and the corresponding treatment were recorded. The symptoms of CTPV were reviewed and analyzed along with the surgical results.

Imaging examination

All the cases in this group were first diagnosed with CTPV and portal hypertension using color Doppler ultrasound. After 2004, they were all further examined with 64-slice CT. The choice of surgical procedures was determined by the range, degree and location of cavernous transformation in the hepatic hilar area, as well as the condition of thrombus in the vein and establishment of collateral circulation in the surrounding tissue.

Selection of surgical procedures

Infants with little bleeding and no obvious symptoms were subjected to conservative treatment. The surgical procedures were determined based on the clinical manifestation, the location of the cavernous transformation and the condition of the varices. Surgical vascular disconnection was performed in the gastric fundus and the lower esophagus for the children with symptoms of CTPV with little bleeding (less than twice). No significant damage of hepatic function, cavernous transformation outside the liver, or varices in the lower esophagus and gastric fundus was observed. Surgery should completely disconnect the variceal veins. Splenectomy was only performed in children who showed splenomegaly and hypersplenism, but who did not have gastrointestinal bleeding or varices in the lower esophagus and gastric fundus. Living-donor liver transplantation was performed in children with cavernous transformation in intrahepatic veins, which was accompanied by liver dysfunction. Patients in this group were younger and had smaller vein diameters and thinner venous walls, which led to difficulties in anastomosis and risk of obstruction at the anastomosis, therefore, we did not perform shunt surgery on these patients.

Follow-up management

Information on clinical, laboratory and imaging examinations, as well as treatment procedures and prognosis, was recorded for all patients. The treatments and their effectiveness were evaluated for each patient. For those

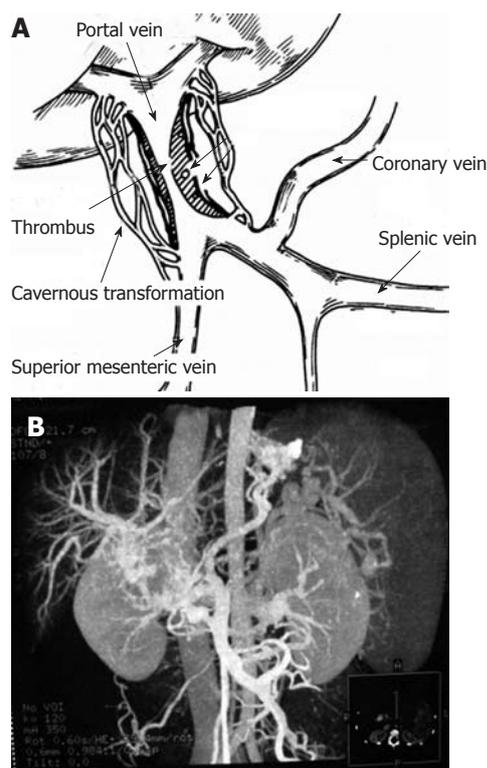


Figure 1 Cavernous transformation of portal vein and sixty-four-slice computed tomography angiography. A: Illustration of cavernous transformation of the portal vein (PV); B: Sixty-four-slice computed tomography angiography of cavernous transformation in the extrahepatic PV indicates varices in the esophagus and gastrosplenic area.

patients treated with surgery, the results were considered good if the clinical symptoms disappeared after surgery. The longest follow-up time was 5 years. The follow-up content included the inquiry of the incidence of hematemesis and melena after surgery, as well as liver function examination and type B ultrasound examination.

RESULTS

Location of CTPV

For all 12 cases of CTPV, a large amount of collateral circulation was found around the PV. The normal PV structure disappeared at the hepatic hilar area, and nodular vessels, with anfractuosity, hemangiectasis and different diameters were instead detected on the angiogram and were accompanied by an apparently enhanced venous phase. The patients exhibited typical symptoms of cavernous transformation. Occlusion in the PV trunk was found in nine cases, and complete occlusion was found in three. Four cases had occlusion in the PV trunk, in combination with occlusion of the superior mesenteric vein or proximal splenic vein. Five cases had occlusion of the large part of the superior mesenteric vein and splenic vein (Figure 1). Three cases had liver laceration with obvious narrowing or occlusion, or unclear angiograms in both the left and right branches of the intrahepatic PV. Cavernous transformation in the intrahepatic PV frequently compressed the biliary system and led to occlusion, infec-

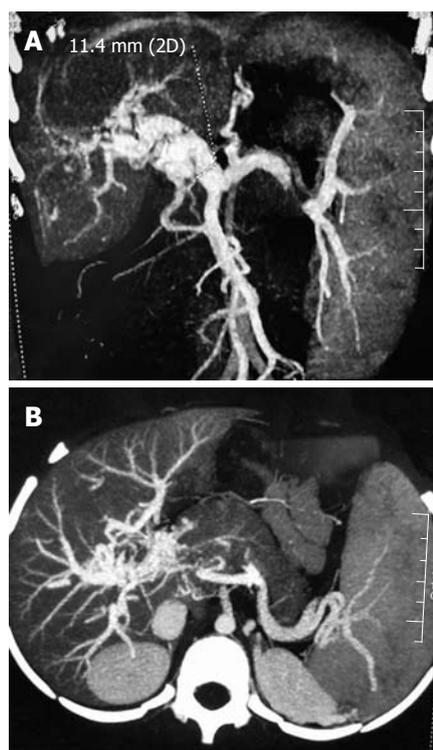


Figure 2 Dilated and tortuous portal vein. A: Splenic vein; B: Left gastric vein.

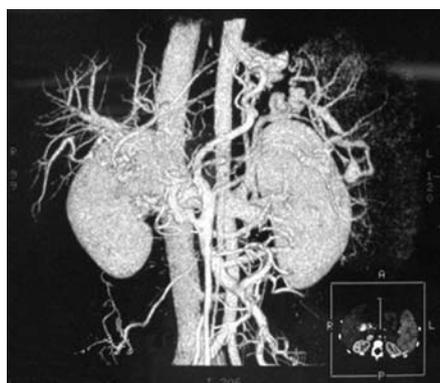


Figure 3 Splenorenal shunt.

tion and other complications, resulting in liver dysfunction, which is an indication for liver transplantation.

Portal hypertension

Among the 12 pediatric patients with CTPV, nine had splenomegaly and six had ascites. The major branches of the PV showed different degrees of dilation (Figure 2). Five cases exhibited severe dilation, showing lumpy, tortuous, dilated blood vessels in the lower esophagus and under the gastric fundic mucosa, narrowing of the lumen of the lower esophagus and lumpy protuberance in the gastric cavity (Figure 1). Eight cases had spontaneous splenorenal/gastrorenal shunt, showing highly tortuous, dilated blood vessel structure in disorderly directions. The blood vessels originated from the short gastric vein, posterior gastric vein or blood vessels at the splenic hilum,

and connected to the left renal vein through the tortuous, dilated retroperitoneal venous plexus (Figure 3). Seven cases had paraumbilical vein patefaction, showing tortuous dilation with spiral-shaped changes in the ligamentum teres hepatis. The blood vessels were in a radial pattern at the upper and lower end of the structure and connected to chest wall veins as well as the deep and superficial veins of the abdominal wall. Four cases had open retroperitoneal communicating branches (venous plexus of Retzius), showing tortuous disordered retroperitoneal blood vessels, which were dilated in a bundle shape or cirroid shape or connected to the inferior vena cava in a radial shape. Except for the collateral circulation in the lower esophagus and gastric fundal varices, which can lead to bleeding in the digestive tract, collateral circulation formed at other locations can help to reduce PV pressure and were thus preserved during surgery. When selecting surgical procedures, collateral circulation formation should be considered, which can help estimate prognosis on postoperative recurrence of gastrointestinal bleeding.

Impact of severity of CTPV on surgical procedure

Surgical procedure selection was based on the severity of vascular dilation in the lower esophagus and gastric fundic mucosa, whether CTPV was located inside the liver and the extent of liver dysfunction. Surgical vascular disconnection in the gastric fundus and lower esophagus in combination with splenectomy was performed in 5 cases with severe dilation in the lower esophagus and gastric fundic mucosa. Among them, surgical removal of the thrombus and end-to-end anastomosis of the PV were performed in one case with occlusion of the PV trunk. Splenectomy was performed for four cases without obvious lumpy, tortuous dilation of the veins in the lower esophagus and gastric fundus, but with apparent splenomegaly. Two of these four cases had occlusion of the PV trunk, and surgical removal of the thrombus and end-to-end anastomosis of the PV were performed along with splenectomy. The findings from intraoperative exploration were completely consistent with the results from the preoperative angiography using 64-slice CT. Three children were diagnosed with cavernous transformation involving the inside of the intrahepatic portal vein using 64-slice CT, and all three patients had damaged liver function. Therefore, living-donor liver transplantation was performed on these patients. Due to the lack of collateral vessels, the great saphenous vein of the donor was used as the vascular bypass graft for anastomosis between the PV and the superior mesenteric vein in one case (Figure 4). The findings from the intraoperative exploration were consistent with the preoperative imaging results.

Postoperative recovery and follow-up

The average follow-up time was 2 years (from 3 mo to 5 years). During the follow-up, no recurrence of severe hematemesis or obvious black tarry feces was observed in the 9 pediatric patients who received gastric fundic and lower esophageal vascular disconnection and splenec-

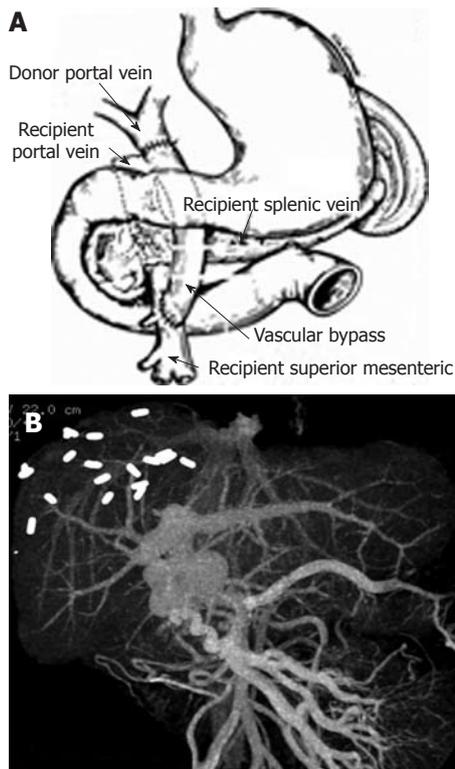


Figure 4 Vascular bypass and sixty-four-slice computed tomography angiography. A: Illustration of vascular bypass between the donor portal vein and the recipient superior mesenteric vein for liver transplantation; B: Sixty-four-slice computed tomography angiography after liver transplantation.

tomy. Three of the patients had liver transplantation; one of them died of portal thrombosis 5 d after surgery. The other two patients did not experience more hematemesis, melena or lower esophageal stenosis, and their liver function completely recovered. One of the patients was already back in elementary school with good performance, and the other went to kindergarten with normal body development (body weight and height) compared to the average values of the children of the same age. Two wk after surgery, one patient had adhesive ileus, which was cured after another operation. Six months after surgery, barium meal examination showed that esophageal varices disappeared in 10 cases, were relieved in one, and one patient was lost to follow-up.

DISCUSSION

The degree and size of CTPV have an apparent impact on the difficulty level, operative duration and incidence of postoperative complications. Therefore, preoperative understanding of the condition of the CTPV is very important for the surgical process^[5]. Ultrasound is a routine clinical examination for CTPV. However, color Doppler ultrasound cannot provide sufficient information on the formation of collateral circulation. Apart from B-type ultrasound, magnetic resonance angiography and 64-slice CT, portal angiography is the ideal examination method for angiogram. However, the former is limited by breath-

holding time and it cannot finish a comprehensive examination for the abdominal wall, paraumbilical area and retroperitoneal varices at one time. Because 64-slice CT can clearly demonstrate the anatomy of the PV system and collateral circulation, sub-millimeter thin-layer volume scan was performed with 64-slice spiral CT for the patients in this study, with 3D reconstitution to display in high resolution the anatomical morphology of the PV system in three dimensions and a clear relationship with the adjacent structures. Sixty-four-slice CT has a positive influence on surgical planning, locating blood vessels and preventing intraoperative injuries. Sixty-four-slice CT can also visualize the veins surrounding and those adjacent to the esophagus. The former are the smaller veins attached to the outer membrane of the esophagus, and the latter are the larger veins that are separated from the outer membrane of the esophagus. Previously, examination of these veins has required left gastric angiography or esophageal ultrasound examination. Collateral vessels in the patients in the present study were mainly located in the lower esophagus and gastric fundus, which was consistent with the clinical symptoms of upper gastrointestinal bleeding such as hematemesis and melena. All five cases were treated with surgical venous disconnection in the gastric fundus and esophagus and did not experience any recurrence during postoperative follow-up. Vascular disconnection was performed for the four cases in this study that did not show obvious gastric fundic and esophageal varices. Only splenectomy was carried out in these patients to reduce PV pressure and relieve reduce esophageal and gastric fundic varices. The findings from intraoperative exploration were completely consistent with the results from the preoperative imaging examination. No gastrointestinal bleeding occurred in these patients for 5 years after surgery.

Angiography of the left gastric vein indicates the existence of coronary venous reflux. Coronary venous reflux is an important pathological symptom of portal hypertension. It is also an indicator of esophageal variceal rupture and bleeding. Measures should be taken to prevent this bleeding^[4,5]. In the present study, the patients with the most severe symptoms all had dilated and tortuous left gastric veins in 64-slice CT, showing stiffness and irregularity in the running direction of the blood vessels. In addition, to reduce the pressure of the gastric coronary vein, part of the blood in the PV with hypertension can shunt from the open umbilical vein and splenorenal vein to the superior and inferior vena cava. Upper gastrointestinal bleeding is diagnosed based on analysis of the open extent of RVs using 64-slice spiral CT for PV angiography^[6]. In the present study, open RV was found in four cases, and spontaneous portosystemic shunt was found in five, which were all conserved during surgery. None of the patients displayed upper gastrointestinal bleeding after surgery. This is similar to reports from other countries^[7]. Cavernous transformation in the intrahepatic PV frequently compresses the biliary system and leads to occlusion, infection and other complications, resulting in damage to

liver function, which is an indication for liver transplantation. In the present study, three cases had deformed blood vessels and hemangioma-like symptoms in the intrahepatic portal system, which were accompanied by damaged liver function and hypersplenism. All of these patients were at the late stage of portal hypertension. Studies have shown that intrahepatic CTPV is one of the indications for liver transplantation^[8]. Three of our patients received liver transplantation. Due to the difficulties in anastomosis resulting from a large lesioned area, the great saphenous vein of the donor was used as a vascular bypass graft for anastomosis between the PV and superior mesenteric vein in one case. The findings from intraoperative exploration were consistent with the preoperative imaging results. From the follow-up, the symptoms of portal hypertension in the three patients were all remarkably relieved, demonstrating excellent short-term clinical results. However, liver transplantation has not been widely used to treat CTPV. Long-term follow-up is still necessary for the assessment of its long-term therapeutic effects and to compare it with other surgical procedures.

In summary, many changes occur in the portal venous system following CTPV. Sixty-four-slice spiral CT portal angiography and the 3D reconstitution technique can effectively demonstrate the pathological changes in the PV system, clearly showing the major branches of the PV as well as the running direction and distribution of collateral circulation, precisely locating the pathological lesions, demonstrating the space relationship between the lesions and the blood vessels and providing accurate information for clinical assessment and surgery planning. Sixty-four-slice CT is an optimal diagnostic method. However, it requires corresponding equipment and facilities. In addition, its cost is relatively high, which not all young patients can afford.

ACKNOWLEDGMENTS

We wish to express our gratitude to all our transplant coordinators, nursing staff, and administrative personnel, without whom this work would not have been possible. In particular, Lin Mo, Zhi-Mei Ren, Yuan Shi, Lin Bo, Ying-Liang Li, Qi-Lin Li, Yi Tang and Qiao Wang. We would also like to thank our previous and current transplant fellows for their diligent and tireless work: Xiao-Ke Dai, Qiang Xiong, Kai Chen and Xiao-Mei Zhu.

COMMENTS

Background

Portal venography by 64-slice computed tomography (CT) can depict the anatomical characteristics of portosystemic collateral vessels in pediatric patients with cavernous transformation of the portal vein (CTPV). Sixty-four slice CT has been used widely in collateral circulation studies of esophageal and gastric varices. The drainage veins of esophageal varices can be clearly displayed by 64-slice CT.

Research frontiers

Diagnosing and treatment of this complicated disease is problematic mostly due

to the absence of more accurate data on the position and severity of the disease features in such patients. Recent technical advances offer us increasingly greater imaging clarity of CTPV for its diagnosis. Few people have reported the impact of 64-slice CT on surgical strategy planning. This study focused on the utilization of 64-slice CT to provide referable information for clinical management selection and prognosis evaluation.

Innovations and breakthroughs

Recent reports have highlighted the importance of 64-slice CT in the diagnosis of CTPV or its impact on prognosis evaluation. However, this is the first study to report that 64-slice CT is also valuable in the selection of surgical procedures. This studies suggest that 64-slice CT is a useful method for the assessment of therapeutic effect following treatment of gastric varices. In the majority of cases in the present study, the collateral circulation pattern and the morphological characteristics of CTPV were revealed as a result of the high spatial resolution images of 64-slice CT and the appropriate images post-processing.

Applications

Displaying the morphological characteristics of the PV system and collateral circulation, 64-slice CT portal angiography may represent a future strategy for therapeutic management of patients with CTPV. It would seem that 64-slice CT has a valuable role in this situation.

Terminology

Faster scanning with 64-slice CT, combined with rapid intravenous administration of contrast material, allows visualization of the more distal branches of the portosystemic vessels. CTPV is a relatively rare condition resulting from extrahepatic PV obstruction with recanalization or collateral vein formation to bypass the obstruction. Paraesophageal varices are the varices that exist outside the esophagus. The subphrenic vein is the bilateral vessel that ends up at the inferior vena cava at the diaphragm level.

Peer review

The authors demonstrated that 64-slice CT portal angiography could provide accurate information for clinical assessment, especially surgical planning. This is a good paper with excellent images. The results are interesting and may represent the optimal selection of clinical therapy and evaluation of prognosis.

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S- Editor Wu X L- Editor Kerr C E- Editor Xiong L

Comparison of laparoscopic and open surgery for pyogenic liver abscess with biliary pathology

Jin-Fu Tu, Xiu-Fang Huang, Ru-Ying Hu, He-Yi You, Xiao-Feng Zheng, Fei-Zhao Jiang

Jin-Fu Tu, Xiu-Fang Huang, Ru-Ying Hu, He-Yi You, Xiao-Feng Zheng, Fei-Zhao Jiang, Department of Laparoscopic Surgery, the First Affiliated Hospital of Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, China

Author contributions: Tu JF wrote the paper; Huang XF and Hu RY collected and analyzed the data; Jiang FZ, Tu JF and You HY performed the laparoscopic operation; Zheng XF revised the manuscript.

Correspondence to: Dr. Jin-Fu Tu, Department of Laparoscopic Surgery, the First Affiliated Hospital of Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, China. tujinfu@sina.com

Telephone: +86-577-88069206 Fax: +86-577-88069555

Received: February 26, 2011 Revised: May 19, 2011

Accepted: May 26, 2011

Published online: October 14, 2011

Abstract

AIM: To investigate the feasibility and therapeutic effect of laparoscopic surgery for pyogenic liver abscess (PLA) with biliary pathology.

METHODS: From January 2004 to October 2010, 31 patients with PLA combined with biliary pathology meeting entry criteria received surgical management in our hospital. Of the 31 patients, 13 underwent laparoscopic surgery (LS group) and 18 underwent open surgery (OS group). Clinical data including operation time, intraoperative blood loss, postoperative complication rate, length of postoperative hospital stay, and abscess recurrence rate were retrospectively analyzed and compared between the two groups.

RESULTS: All patients received systemic antibiotic therapy. Four patients underwent ultrasound-guided percutaneous catheter drainage before operation. Postoperative complications occurred in 5 patients (16.1%, 5/31) including 2 in the LS group and 3 in the OS group. One patient had retained calculus in the common bile duct and another had liver abscess recurrence in the OS group. No retained calculus and liver abscess

recurrence occurred in the LS group. In the two groups, there was no mortality during the perioperative period. There were no significant differences in operation time, intraoperative blood loss and transfusion, postoperative complication rate and abscess recurrence rate between the two groups. Oral intake was earlier (1.9 ± 0.4 d vs 3.1 ± 0.7 d, $P < 0.05$) and length of postoperative hospital stay was shorter (11.3 ± 2.9 d vs 14.5 ± 3.7 d, $P < 0.05$) in the LS group than in the OS group.

CONCLUSION: Laparoscopic surgery for simultaneous treatment of PLA and biliary pathology is feasible in selected patients and the therapeutic effect is similar to that of open surgery.

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Key words: Liver abscess; Biliary; Laparoscopy; Surgery; Therapeutic effect

Peer reviewer: Jon C Gould, MD, FACS, Associate Professor of Surgery, University of Wisconsin School of Medicine and Public Health, 600 Highland Avenue, H4/726, Madison, WI 53792, United States

Tu JF, Huang XF, Hu RY, You HY, Zheng XF, Jiang FZ. Comparison of laparoscopic and open surgery for pyogenic liver abscess with biliary pathology. *World J Gastroenterol* 2011; 17(38): 4339-4343 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4339.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4339>

INTRODUCTION

Pyogenic liver abscess (PLA) is a potentially fatal disease. Before the 1970s open surgical drainage was often adopted for the treatment of PLA. With the development of imaging techniques, ultrasound or computed tomography (CT)-guided percutaneous catheter drainage combined with systemic antibiotics has become the preferred treatment, and mortality has significantly decreased^[1-3]. In

some conditions such as percutaneous drainage failure or underlying biliary pathology, surgery is still required^[4-6]. With the development of laparoscopic instruments and techniques, laparoscopic liver abscess drainage has become popular^[7-11]. Compared with conventional open liver abscess drainage, laparoscopic drainage has some advantages in operation time, postoperative recovery and length of hospital stay^[10,11]. However, little research has been done on laparoscopic surgery (LS) for simultaneous treatment of both PLA and biliary pathology. The purpose of this study was to explore the safety and feasibility of laparoscopic or open surgery (OS) for simultaneous treatment of both PLA and biliary pathology, and to compare the therapeutic effects of the two methods through retrospective analysis of 31 patients with PLA meeting entry criteria.

MATERIALS AND METHODS

Patients

From January 2004 to October 2010, 348 patients with PLA were treated in our hospital. Of the 348 patients, 31 met entry criteria. The entry criteria included (1) ultrasound, CT, surgery or pathogen culture-diagnosed PLA combined with biliary pathology including gallstone, choledocholith, hepatolith, biliary stricture and biliary tract neoplasms; (2) simultaneous surgical treatment for both PLA and underlying biliary pathology. Patients undergoing drainage of liver abscess alone were excluded. Of the 31 patients with PLA, 13 underwent LS (LS group) and 18 OS (OS group). In the 13 patients in the LS group, 5 patients were men and 8 women, with a mean age of 57.5 years (range 37-69). In the 18 patients in the OS group, 8 patients were men and 10 women, with a mean age of 55.8 years (range 35-73). Fever and/or chills were common, then abdominal pain. Most patients had right upper quadrant tenderness. Symptoms and signs of patients are shown in Table 1.

Routine blood analysis, liver function tests and blood coagulation assays were performed in all patients. Increased white blood cell count was the most common laboratory abnormality in these patients (Table 2). Abdominal ultrasound examination was performed in 31 patients, and indicated liver abscess in 30 patients and biliary pathology in 28 patients. CT scan and contrast-enhanced CT scan were performed in 30 patients, and indicated liver abscess in 30 patients and biliary pathology in 26 patients. Magnetic resonance imaging (MRI) was performed in 5 patients, and indicated liver abscess in 5 patients, choledocholith in 3 patients and cholecystolithiasis in 2 patients. In the 13 patients from the LS group, 5 patients had right liver abscess, 7 left liver abscess and one both right and left liver abscess; 11 patients had single liver abscess and 2 multiple liver abscesses; and 5 patients had > 5 cm diameter liver abscess. In the 18 patients from the OS group, 9 patients had right liver abscess, 7 left liver abscess and 2 both right and left liver abscess; 15 patients had single liver abscess and 3 multiple liver abscesses; and 10 patients had > 5 cm-diameter liver abscess. In the 13

patients from the LS group, 6 patients had cholecystolithiasis combined with acute cholecystitis; 3 patients had choledocholith cholangitis (2 also had cholecystolithiasis); 3 patients had left multiple hepatolithiasis (one also had choledocholith); one patient had left intrahepatic biliary stricture. In the 18 patients from the OS group, 7 patients had cholecystolithiasis combined with acute cholecystitis (one also had primary gallbladder cancer); 5 patients had choledocholith cholangitis (2 also had cholecystolithiasis and one right hepatolithiasis); 4 patients had left multiple hepatolithiasis (one also had choledocholith); one patient had left intrahepatic biliary stricture; and another one had intrahepatic bile duct cystadenoma combined with hepatolithiasis (Table 3). Among the 31 patients, aerobic bacterium culture was positive in 21 patients. The common bacteria were colibacillus, then *Klebsiella pneumoniae*. Three of the 13 patients in the LS group had diabetes mellitus. Four of the 18 patients in the OS group had diabetes mellitus, 2 patients had hypertensive disease, one patient had a history of biliary surgery and another patient had a history of two-time biliary surgery. There were no statistical differences in age, sex, clinical manifestations, characteristics of liver abscess and underlying biliary pathology between the two groups.

Operative techniques

Laparoscopic liver abscess drainage: After general anesthesia, a small cut below the umbilicus was made followed by establishing pneumoperitoneum and placing a laparoscope into the peritoneal cavity. The pore site positions were determined according to the size and position of liver abscess. Under laparoscopy, the adhesions of the liver with the abdominal wall or diaphragm were separated using electrocautery. In large and superficial liver abscess, the surface of the liver was locally elevated with gray-and-white or yellowish-white color. After aspirating pus from the liver abscess through paracentesis, a small hole was made on the elevated or thinnest surface of the liver abscess. The abscess cavity was then unroofed using electrocautery, and samples were routinely obtained for bacterial culture and drug sensitivity testing. Debridement and irrigation of the abscess cavity were performed. Laparoscopic cholecystectomy or common bile duct exploration for calculus removed was routinely carried out. Laparoscopic left hepatectomy was performed as previously described^[12]. Thick latex drainage tubes were left in the abscess cavity and subhepatic space, respectively. When B-ultrasonic image or CT confirmed abscess cavity collapse or closure 6-9 d after operation, and 24-hour drainage liquid was less than 20 mL, drainage tubes might be removed.

Open surgery: A right subcostal incision or a superior median abdominal incision was made to explore abdominal lesions. The position of the liver abscess was determined through paracentesis, and then hemostatic forceps entered the abscess cavity along the paracentetic needle to remove pus and separate fibrous septa. A latex drainage tube was left. At the same time, biliary pathology was also treated.

Clinical data including operation time, intraoperative

Table 1 Clinical manifestation of patients with pyogenic liver abscesses

Variables	LS group (n = 13)	OS group (n = 18)
Fever/chills	11	17
Abdominal pain	10	14
Vomiting	8	10
Jaundice	5	8
Septic shock	1	1
RUQ tenderness	10	15
Murphy's sign	6	7
Hepatomegally	4	6

LS: Laparoscopic surgery; OS: Open surgery; RUQ: Right upper quadrant.

Table 2 Initial laboratory values for pyogenic liver abscesses

Parameter	LS group (n = 13)	OS group (n = 18)
WBC count (> 10 000/mL)	12	18
Serum albumin (< 35 g/L)	8	11
Total bilirubin (> 20 μmol/L)	7	10
AST (> 60 U/L)	6	9
Serum creatinine (> 80 μmol/L)	2	3
PT (> 14.8 s)	2	2

LS: Laparoscopic surgery; OS: Open surgery; WBC: White blood cell; AST: Aspartate aminotransferase; PT: Prothrombin time.

Table 3 Origin of pyogenic liver abscesses

Variables	LS group (n = 13)	OS group (n = 18)
Cholelithiasis	6	7
Choledocholithiasis	3	5
Hepatolithiasis	3	4
Intrahepatic biliary stricture	1	1
Biliary cystadenoma	0	1

LS: Laparoscopic surgery; OS: Open surgery.

blood loss, postoperative complication rate, length of postoperative hospital stay, and abscess recurrence rate were compared between the two groups.

Statistical analysis

Categorical parameters in each group were compared by the chi-square test, and continuous parameters were compared using independent sample *t* test. All analyses were performed using SPSS 12.0, and *P* < 0.05 was considered statistically significant.

RESULTS

All patients received systemic antibiotic therapy. Four patients (one patient in the LS group and 3 patients in the OS group) underwent ultrasound-guided percutaneous catheter drainage before surgery. Preoperative drainage lasted 2-8 d. After percutaneous catheter drainage, diameters of liver abscesses were decreased by 2-5 cm in 3 patients, while in another patient, the diameter of

Table 4 Operative procedures performed on the patients

Operative procedures	LS group (n = 13)	OS group (n = 18)
Drainage of abscess with cholecystectomy	6	7
Drainage of abscess with CBD exploration/and cholecystectomy	3	5
Left lateral segmentectomy	2	4
Left hemihepatectomy	2	2

LS: Laparoscopic surgery; OS: Open surgery; CBD: Common bile duct.

liver abscess was unchanged due to inadequate drainage. In 7 patients with diabetes mellitus, blood glucose levels were all controlled under 10 mmol/L by administration of insulin. General anesthesia was performed in 31 patients through endotracheal intubation. In the LS group, 6/13 patients received laparoscopic liver abscess drainage and cholecystectomy; 3 patients received liver abscess drainage, cholecystectomy, common bile duct exploration and T tube drainage; 2 patients with hepatolithiasis limited to the left lateral segment combined with liver abscess received left lateral segmentectomy, and of these 2 patients, one also received cholecystectomy, common bile duct exploration and T tube drainage; one patient with left hepatic duct stenostomia and another patient with hepatolithiasis limited in left hepatic lobe received laparoscopic left hemihepatectomy. In the OS group, 7/18 patients received laparoscopic liver abscess drainage and cholecystectomy, and of these 7 patients, one with gallbladder cancer also received radical cholecystectomy; 5 patients received liver abscess drainage and common bile duct exploration, and of these 5 patients, primary closure of the bile duct was performed in 2 patients and T tube drainage in 3 patients, and 4 patients also received cholecystectomy; 4 patients received left lateral segmentectomy, and of these 4 patients, two also received cholecystectomy, common bile duct exploration and T tube drainage; 2 patients received hemihepatectomy, and one of these patients also received cholecystectomy, common bile duct exploration and T tube drainage (Table 4).

Postoperative complications occurred in 5 patients (2 patients in the LS group and 3 in the OS group, 16.1%). In the LS group, one patient had biliary leakage (100-200 mL of bile per day), and it automatically healed 7 d after drainage; another patient had right hydrothorax, and it was relieved 4 d after closed drainage. In the OS group, early postoperative inflammatory ileus occurred in one patient who recovered 10 d after conservative treatment; subphrenic abscess occurred in one patient and was relieved 8 d after ultrasound-guided puncture; and incision infection occurred in one patient who showed second-class healing after changing dressings. In the OS group, one patient had retained calculus in the common bile duct and left hospital with T tube, the retained calculus were removed using fibercholedochoscope 58 d after operation; liver abscess recurrence occurred in one patient 20 d after surgery and were relieved a week after systemic use of antibiotics and ultrasound-guided percutaneous cath-

Table 5 Comparison of results in laparoscopic surgery group and open surgery group

Variables	LS group (n = 13)	OS group (n = 18)
Operating time (min)	117 ± 27	112 ± 31
Intraoperative blood loss (mL)	139 ± 51	146 ± 47
Intraoperative blood transfusion (%)	1 (7.7)	1 (5.6)
Commencement of oral intake (d)	1.9 ± 0.4	3.1 ± 0.7
Postoperative complications (%)	2 (15.4)	3 (16.7)
Postoperative hospital stay (d)	11.3 ± 2.9	14.5 ± 3.7
Intermediate residual stone (%)	0 (0)	1 (5.6)
Abscess recurrence (%)	0 (0)	1 (5.6)
Perioperative mortality (%)	0 (0)	0 (0)

LS: Laparoscopic surgery; OS: Open surgery.

eter drainage. In the LS group, no retained calculus and liver abscess recurrence occurred. In the LS group, one patient was given 2U of concentrated red cells intravenously while in the OS group, one patient was given 4U of concentrated red cell intravenously. In the two groups, there was no mortality during the perioperative period. There were no significant differences in operation time, intraoperative blood loss and transfusion, postoperative complication rate and abscess recurrence rate between the two groups. Oral intake was earlier (1.9 ± 0.4 d *vs* 3.1 ± 0.7 d, $P < 0.05$) and length of postoperative hospital stay was shorter (11.3 ± 2.9 d *vs* 14.5 ± 3.7 d, $P < 0.05$) in the LS group than in the OS group (Table 5).

DISCUSSION

In the early 20th century, PLA was commonly secondary to pyelophlebitis caused by acute appendicitis. Since the mid 20th century, PLA has been mainly due to benign or malignant biliary pathology, accounting for about 40%-65%^[1,13,14]. These common biliary lesions include cholecystolithiasis, intrahepatic and extrahepatic cholangiolithiasis, ascariasis of the biliary tract, biliary stricture and biliary tumor. When these biliary lesions lead to acute suppurative cholecystitis, acute suppurative cholangitis, and intrahepatic and extrahepatic cholangitis, bacteria may enter intrahepatic bile ducts and cholangioles to cause PLA. Perforation of gallbladder may also result in PLA^[15]. Mezhir *et al*^[5] have reported that 88% of patients with PLA have a history of malignant tumor including pancreatic cancer (36%), cholangiocarcinoma (17%), colon carcinoma (12%) and gallbladder cancer (10%) in 58 patients between 1998 and 2009. However, in India^[14] and China^[16], the main cause of PLA is still biliary calculi. Since the 1980s, the incidence of hepatolithiasis has been decreased and the incidence of cholecystolithiasis has been significantly increased. In China, cholecystolithiasis is increasingly becoming a main cause of PLA. In this study, the main causes of PLA were cholecystolithiasis and cholangiolithiasis.

Before the 1970s, conservative treatment or open surgical drainage was mainly adopted for the treatment of PLA, but the mortality rate was as high as 65%^[1]. With the development of imaging techniques and effective

broad-spectrum antibiotics, image-guided percutaneous catheter drainage combined with systemic antibiotics have become preferred for the treatment of PLA, and the mortality rate is under 10%^[2,3,17]. Image-guided percutaneous catheter drainage is suitable not only to unilocular abscess, but also to multiple unilocular abscesses and multiloculated abscess^[2], and has some advantages including simple procedures, low cost and good therapeutic effect. However, percutaneous catheter drainage has some disadvantages. For example, multiple percutaneous drainages are required due to drainage tube block or inadequate drainage; it has the possibility of hepatic hemorrhage or pneumothorax; and it cannot simultaneously treat PLA with underlying hepatobiliary pathology.

Liver abscess surgical drainage and percutaneous drainage are complementary techniques. In this study, 4 patients received percutaneous drainage before operation, and following improvement of pathogenetic condition, underwent surgical management. Liver abscess open surgical drainage is suitable after percutaneous drainage failure or for patients having primary diseases such as biliary PLA, abscess rupture and so on^[4-6]. Surgical drainage has some advantages including positioning accuracy, and simultaneous treatment of both abscess and primary diseases^[4,14,18]. In this study, as well as liver abscess drainage laparoscopic cholecystectomy was also performed in 6 patients, open cholecystectomy was performed in 7 patients, laparoscopic common bile duct exploration for calculus removed was performed in 3 patients and open common bile duct exploration for calculus removed was performed in 5 patients. Hepatolobectomy is suitable for hepatolithiasis or hepatobiliary tumor combined with PLA^[6,19]. In this study, 7 patients with hepatolithiasis combined with PLA underwent left lateral segmentectomy or left hemihepatectomy. Hepatolobectomy can achieve radical treatment results, because of removal of not only the abscess but also biliary stones, biliary stricture and hepatic lesions. Moreover, hepatolobectomy for treatment of hepatolithiasis combined with PLA is conducive to long-term prevention of biliary carcinogenesis. With the development of laparoscopic techniques, laparoscopic drainage may replace traditional open drainage in the treatment of PLA. A laparoscopic drainage group is better than an open drainage group in operation time, blood loss and length of hospital stay, and laparoscopic drainage is safe and feasible in patients who have no response to conservative treatment^[10,11]. In this study, LS or OS were used to treat PLA combined with biliary pathology in 31 patients with a postoperative complication rate of 16.1%. In the two groups, there was no mortality during the perioperative period. There were no significant differences in operation time, intraoperative blood loss and transfusion, postoperative complication rate and abscess recurrence rate between the two groups. Oral intake was earlier and length of postoperative hospital stay was shorter in the LS group than in the OS group.

LS or OS for the concomitant treatment of both PLA and biliary pathology is suitable for (1) cholecystolithiasis, common bile duct calculi, ascariasis of biliary

tract, biliary stricture or biliary tumor-caused PLA; (2) vital signs stable, and tolerable anesthesia and surgery for the important organs such as heart, lung, liver and kidney. In patients with diabetes mellitus, the preoperative blood glucose level should be controlled under 10 mmol/L and sensitive broad-spectrum antibiotics should be given before and after operation. Preoperative ultrasound, CT and MRI indicate the conditions including abscess liquefaction, size of abscess cavity, pus volume, fibrous septa, abscess number and biliary pathology. If preoperative images indicate that the abscess cavity is deep, in order to prevent pus spill, fine needle aspiration is performed first, and thick needle puncture is done to remove pus. Radial incision from the porta hepatic may avoid the damage to intrahepatic bile ducts and blood vessels. If aspirated pus is significantly less than expected pus, there may be fibrous septa in the abscess and the incision should be extended to explore abscess cavities. During separation of fibrous septa of the abscess cavity, the hepatic frame structure cannot be transected and the separated stick should not enter normal tissue through the abscess cavity wall. Pulse-like arterial hemorrhage can be stopped by occlusion with titanium clips. Since adhesions are often severe in the cystohepatic triangle, cholecystectomy requires careful separation to avoid damage to the bile ducts. The key of laparoscopic hepatectomy is to prevent and control intraoperative hemorrhage^[12].

In summary, for the treatment of most PLA, ultrasound-guided percutaneous catheter drainage combined with systemic antibiotics is preferred. In selected patients with biliary PLA, laparoscopic or open surgery for simultaneous treatment of PLA with underlying biliary pathology is safe and feasible. Laparoscopic surgery has advantages in postoperative recovery of gastrointestinal function and length of postoperative hospital stay.

COMMENTS

Background

Pyogenic liver abscess (PLA) is a potentially fatal disease. Before the 1970s, open surgical drainage was often adopted for the treatment of PLA. With the development of imaging techniques, ultrasound or computed tomography-guided percutaneous catheter drainage combined with systemic antibiotics has become preferred, and the mortality has been significantly decreased. In some conditions such as percutaneous drainage failure or underlying biliary pathology, surgery is still required. With the development of laparoscopic instruments and techniques, laparoscopic liver abscess drainage has become popular. Compared with conventional open liver abscess drainage, laparoscopic drainage has some advantages in operation time, postoperative recovery and length of hospital stay. However, little research has been done on laparoscopic surgery for simultaneous treatment of both PLA and biliary pathology.

Research frontiers

The feasibility and therapeutic effect of laparoscopic surgery for simultaneous treatment of both PLA and biliary pathology is a hotspot.

Innovations and breakthroughs

This study was performed to explore the safety and feasibility of laparoscopic or open surgery for simultaneous treatment of both PLA and biliary pathology, and to compare the therapeutic effects of the two methods through retrospective analysis of 31 patients with PLA meeting entry criteria

Applications

In selected patients with biliary PLA, laparoscopic or open surgery for simultaneous treatment of PLA and biliary pathology is safe and feasible. Laparoscopic surgery has advantages in postoperative recovery of gastrointestinal function

and length of postoperative hospital stay.

Terminology

PLA is the result of bacterial infection of the liver parenchyma, with subsequent infiltration by inflammatory cells and formation of a collection of pus.

Peer review

This is a nice case series. The numbers of patients are obviously small, and meaningful comparisons between the 2 groups are difficult.

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Sudden blindness in a child with Crohn's disease

Arrigo Vittorio Barabino, Paolo Gandullia, Angela Calvi, Silvia Vignola, Serena Arrigo, Riccardo De Marco

Arrigo Vittorio Barabino, Paolo Gandullia, Angela Calvi, Silvia Vignola, Serena Arrigo, Gastroenterology and Endoscopy Unit G. Gaslini Institute for Children, L.go G. Gaslini 5, 16148 Genoa, Italy

Riccardo De Marco, Ophthalmology Department G. Gaslini Institute for Children, L.go G. Gaslini 5, 16148 Genoa, Italy

Author contributions: Barabino AV, Gandullia P, Calvi A, Vignola S, Arrigo S and De Marco R contributed equally to this work; Calvi A and Vignola S designed the research; Calvi A, Vignola S and Gandullia P performed the research; Barabino A and Arrigo S wrote the paper.

Correspondence to: Arrigo Vittorio Barabino, MD, Consultant Pediatric Gastroenterologist, Chief of Gastroenterology and Endoscopy Unit G. Gaslini Institute for Children, L.go G. Gaslini 5, 16148 Genoa, Italy. arrigobarabino@ospedale-gaslini.ge.it

Telephone: +39-010-5636711 Fax: +39-010-383688

Received: March 10, 2011 Revised: April 27, 2011

Accepted: May 4, 2011

Published online: October 14, 2011

Key words: Crohn's disease; Extraintestinal manifestations; Optic neuritis; Demyelinating disease

Peer reviewers: Walter Fries, MD, Department Medicina Interna and Terapia Medica, UOS Malattie Intestinali Croniche, Policlinico Messina, 98125 Messina, Italy; Andrew S Day, MB, ChB, MD, FRACP, AGAF, A/Professor, Paediatric Gastroenterology, Christchurch Hospital, and Associate Professor, Head of Department, Department of Paediatrics, University of Otago, Christchurch, PO Box 8140, Christchurch, New Zealand

Barabino AV, Gandullia P, Calvi A, Vignola S, Arrigo S, De Marco R. Sudden blindness in a child with Crohn's disease. *World J Gastroenterol* 2011; 17(38): 4344-4346 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4344.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4344>

Abstract

Inflammatory bowel disease (IBD) is often associated with extraintestinal manifestations (EIMs) such as optic neuritis (ON), although this has been described in only a few adult patients so far, all of whom were affected with Crohn's disease (CD). Furthermore, ON and demyelinating diseases have been demonstrated to be more frequent in IBD patients than in control populations. In our current case report, we describe a child with active CD who developed sudden blindness due to bilateral ON that was not related to any known cause, and that promptly responded to a high dose of steroids. Investigations and a clinical follow-up have so far ruled out the development of demyelinating diseases in this patient. To our knowledge, this is the first report of ON in a pediatric patient with CD. Possible explanations for this case include an episodic EIM of an active bowel disease, an associated autoimmune disorder such as a recurrent isolated ON, the first manifestation of multiple sclerosis, or another demyelinating disease that could appear in a later follow-up.

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INTRODUCTION

Joint, skin, eye and biliary tract disorders are frequently associated with inflammatory bowel disease (IBD) as an extraintestinal manifestation (EIM), although nearly every organ may be involved in this disorder. Some EIMs are clearly related to intestinal disease activity, whereas others occur independently. Ophthalmologic complications are independent of the extent of bowel involvement, usually occurring during the early years of IBD. In rare cases, eye manifestations precede the IBD diagnosis and their course tends to parallel that of the underlying bowel disease. Several ophthalmologic manifestations from the anterior and the posterior segment have now been described^[1]. Among the known posterior segment manifestations, optic neuritis (ON) may be present in up to 4% of adult IBD patients^[2,3]. When ON is diagnosed, a thorough differential diagnosis has to be applied as it can be an isolated condition or a manifestation of neurologic or systemic disease. In particular, ON may be the sign of an autoimmune demyelinating disease.

A possible association between multiple sclerosis (MS) and IBD has been hypothesized for decades and

an approximately 3-fold increased risk of MS in IBD patients has been suggested^[4]. On the other hand anti-tumor necrosis factor (TNF)- α therapies may trigger the new onset of MS, ON and other demyelinating diseases in IBD patients. However, because of the small number of controlled clinical trials with anti-TNF- α medications conducted to date, it is not possible to state with certainty whether a causal association exists between these drugs and demyelinating disorders. Nevertheless, a recent study conducted in the era before anti-TNF therapies^[4], has demonstrated that patients with Crohn's disease (CD) and ulcerative colitis were 54% and 75% more likely, respectively, than community controls to have been diagnosed with MS, ON or other demyelinating conditions^[5]. The results of this study have given credence to the emerging concept that patients with immune-mediated conditions are more likely than the general population to have another autoimmune disease. Thus, ON in IBD patients can signify an EIM of the underlying bowel disease or a demyelinating condition that seems more frequent in this population.

CASE REPORT

An 11-year-old boy presented with a recent history of severe and bloody diarrhea, abdominal pain and vomiting. The abdomen was meteoric and tender. His weight was 26 kg and height was 138 cm, which were, respectively, less than the 5th and the 10th-25th percentile for his age. Laboratory tests revealed hemoglobin 11 g/dL, platelets 507 000/m³, erythrocyte sedimentation rate 38 mm/h, C-reactive protein 9.5 mg/dL (normal < 0.45 mg/dL), and albumin 2.4 g/dL (normal > 3.5 g/dL). Thorough stool investigations ruled out infection or infestation. An abdominal ultrasonogram demonstrated thickened (9 mm) and hyperemic colonic walls. The patient was given iv albumin, metronidazole, ampicillin + sulbactam, and omeprazole without subsequent improvement. Pancolonoscopy with a terminal ileoscopy showed skip areas of deep ulcerations with a cobblestone pattern in the transverse and descending colon, and focal aphthous ulcers in the rectum. Esophagogastroduodenoscopy revealed patchy duodenal erosions and deep, wide ulcerations of the stomach. Owing to endoscopic findings that were consistent with severe CD fasting, a regimen of total parenteral nutrition (TPN) and iv methylprednisolone (20 mg bid) was started, and resulted in a slow clinical improvement. On histology, a chronic-active, patchy, erosive-ulcerative inflammation of the colon, stomach and duodenum, without evidence of granulomata, was seen. A barium meal and follow-through did not reveal small bowel lesions. On hospitalization day 20, at 14 d after the commencement of this treatment, steroids were tapered (5 mg/wk) and the antibiotics and TPN were discontinued.

On hospitalization day 25, on which 35 mg/d of iv steroids was given, the patient suddenly complained of bilateral visual loss. Diffuse and severe bilateral papilledema was detected (Figure 1). Magnetic resonance imaging (MRI) of the head, with paramagnetic iv contrast, and cytologi-



Figure 1 Papilledema of the right eye.

cal and chemical analysis of the cerebrospinal fluid (CSF) showed no abnormalities. A diagnosis of ON was made and three iv pulses of methylprednisolone (30 mg/kg on alternate days) were administered. At the end of this treatment course, visual acuity completely recovered and the optic disc appearance improved. Steroid treatment was prolonged orally. MRI of the spine, visual and brainstem auditory and somatosensory-evoked potentials, motor and sensory nerve conduction velocity, blood and CSF polymerase chain reaction analysis for viruses, CSF monoclonal band and the B₁₂ blood level showed normal or negative results. On hospitalization day 40, the child was eventually discharged and was in clinical remission on prednisone (25 mg/d to be tapered to 5 mg weekly until discontinuation) and azathioprine (1 mg/kg per day). Eighteen days after the ON diagnosis, the optic nerve edema had resolved and optical coherence tomography revealed decreased thickness of the retinal nerve fiber layer (RNFL) in the temporal superior quadrant of both eyes. The child was then regularly followed-up and at 20 mo after diagnosis is still in clinical remission with normal growth.

DISCUSSION

IBDs are often associated with EIMs which occur in approximately one third of patients^[6]. Little has been published, however, on their frequency in pediatric patients and most of the current data are from studies in adults^[7,8]. The incidence of ocular manifestations ranges from 3.5% to 43% according to previous reports^[1-3,9-11] and seems to occur more frequently in colonic CD cases. The most common complications of IBDs are episcleritis, scleritis, and uveitis occurring in up to 29%, 18% and 17% of patients, respectively. The reported incidence of posterior segment manifestations ranges between less than 1% to 30% depending upon the series^[1,2,12]. ON as an ophthalmologic manifestation of IBD has been described in only a few adult patients so far^[1,12,13], all affected with CD. The term ON is used to describe any inflammation, demyelination, or degeneration of the optic nerve with attendant impairment of function^[14]. The disease process for ON is usually acute, with a rapid and progressive loss of vision, and may be unilateral or bilateral. In childhood, ON may

occur as an isolated condition or as a manifestation of a neurologic or systemic disease. ON may also be secondary to inflammatory disease, infections, toxic causes or a vitamin B₁₂ deficiency and may signify a demyelinating disease of childhood such as MS, Devic disease or acute diffuse encephalomyelitis. Patients who experience an isolated episode of ON can develop other symptoms later on associated with a demyelinating disorder. High-dose iv methylprednisolone may help to speed the visual recovery in young adults^[14].

We here present a case report of a child with severe acute gastrointestinal symptoms in which our investigations, in accordance with the Porto criteria^[15], led to the diagnosis of diffuse CD. This disease was difficult to treat and required iv steroids, antibiotics and TPN. The patient subsequently developed a sudden loss of visual acuity consistent with a bilateral ON and not due to any known cause. This vision loss was promptly responsive to a high dose of steroids. Nevertheless after resolution, a residual segmental decrease of RNFL thickness was detected in both eyes. An extensive diagnostic work-up was required and investigations and clinical follow-up, that have not shown any recurrence, have ruled out demyelinating diseases thus far in this patient.

Reports of MS, demyelination (D), and ON associated with anti-TNF- α therapy have resulted in warnings in the instructions for prescribing of infliximab and adalimumab. However, the underlying relationship between IBD and these neurologic conditions has not been established. A previous retrospective cohort and retrospective cross-sectional study, performed in the era before TNF- α blockers were in clinical use, has reported that the incidence and prevalence of MS/D/ON is higher in patients with IBD compared with their matched controls. In particular, ON was recorded in 6 of 7988 CD patients (0.08%) and in 17 of 12 185 ulcerative colitis patients (0.14%) in this study, in comparison with 50 of 80 666 controls (0.06%)^[4].

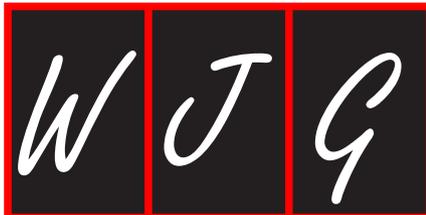
To our knowledge, our current report represents the first description of ON in a pediatric patient with CD. A correlation between ON and CD is possible in this case but, taking into account the current literature, a clear relationship cannot yet be argued. Our current case may represent an episodic EIM of the active bowel disease or an associated autoimmune disorder, such as recurrent isolated ON. It could also be a first manifestation of MS or other demyelinating diseases that are more frequent in IBD patients and could be clarified in a later follow-up of our current patient. We conclude from this case study

that, although extremely rare, ON should be considered by pediatric gastroenterologists and ophthalmologists as a possible IBD complication. In patients with IBD presenting with ON, a thorough diagnostic work-up and strict long-term follow-up are recommended.

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S- Editor Tian L L- Editor Cant MR E- Editor Li JY



Regional lymphadenectomy strongly recommended in T1b gallbladder cancer

Ulrich Klaus Fetzner, Arnulf H Hölscher, Dirk L Stippel

Ulrich Klaus Fetzner, Arnulf H Hölscher, Dirk L Stippel, Department of General, Visceral and Cancer Surgery, University Hospital of Cologne, D-50937 Cologne, Germany

Author contributions: Fetzner UK, Hölscher AH and Stippel DL all together, contributed equally to this work.

Correspondence to: Dr. Ulrich Klaus Fetzner, Department of General, Visceral and Cancer Surgery, University Hospital of Cologne, Kerpener Strasse 62, D-50937 Cologne, Germany. ulrich.fetzner@uk-koeln.de

Telephone: +49-221-4784801 Fax: +49-221-4784843

Received: March 3, 2011 Revised: June 24, 2011

Accepted: July 1, 2011

Published online: October 14, 2011

Abstract

This article discusses the adequate treatment of early gallbladder cancer (T1a, T1b) and is based on published studies extending over nearly 3 decades. Randomized studies and meta analyses comparing different surgical treatments do not exist. The literature shows that in up to 20% of patients lymph node metastasis are found in T1b gallbladder cancer. Due to high malignancy with early angiolymphatic spread and resistance to chemotherapy and radiation on the one hand, and the relative low operative risk of extended cholecystectomy (cholecystectomy and regional lymphadenectomy) on the other hand, we believe that this procedure is mandatory in early gallbladder cancer.

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Key words: Gallbladder cancer; Long-term survival; Lymphadenectomy; Surgical treatment

Peer reviewer: Clifford S Cho, MD, Assistant Professor, Department of Surgery, Section of Surgical Oncology, University of Wisconsin School of Medicine and Public Health, H4/724 Clinical Sciences Center, 600 Highland Avenue, Madison, WI 53792-7375, United States

Fetzner UK, Hölscher AH, Stippel DL. Regional lymphadenectomy strongly recommended in T1b gallbladder cancer. *World J Gastroenterol* 2011; 17(38): 4347-4348 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i38/4347.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i38.4347>

TO THE EDITOR

We read with interest the systematic review by Lee and colleagues, which compares the results of surgical treatment of T1a and T1b gallbladder cancer with cholecystectomy alone or by extended cholecystectomy^[1]. In this study, the published results of 1266 patients were evaluated. The authors conclude that there is no evidence to show that extended cholecystectomy is advantageous over simple cholecystectomy for T1b cancer. However, they recommend performing regional lymphadenectomy in T1b gallbladder cancer. The argument for extended cholecystectomy was mentioned at the end of the discussion: via lymphadenectomy material can be obtained for correct staging which is the basis for possible re-resection.

In fact, the debate on adequate treatment of so-called early gallbladder cancer has extended over nearly 3 decades^[2]. Randomized controlled studies and consecutive meta-analyses comparing different surgical treatment strategies for T1a and T1b gallbladder cancer do not exist.

The general accepted opinion of visceral surgeons - as the leading physicians for this disease- is to perform regional lymphadenectomy in patients with T1b status on^[3].

In their retrospective evaluation, Lee and colleagues found that 10.8% of patients had lymph node metastasis in T1b gallbladder cancer, and 9.3% of patients with T1b gallbladder cancer died due to tumour recurrence. 12.5% of T1b patients had recurrence after simple cholecystectomy, and only 2.7% had recurrence after extended cholecystectomy. These data are consistent with recent studies.

In contrast, the rate of postoperative morbidity was 28% and postoperative mortality was 1.5% after extended cholecystectomy in the evaluation by Lee *et al*^[1] which, in our opinion, may not be representative^[4].

The detection rate of early gallbladder carcinoma has increased in recent years, due to the high frequency of laparoscopic cholecystectomy. Currently, the detection rate of T1a and T1b tumours exceeds more than the 10% as cited by Lee and colleagues, who evaluated data from 1991 on. The high rate of open cholecystectomy (e.g., 54.4% in T1a gallbladder carcinoma) argues for a past era in the treatment of benign gallbladder disease.

In the debate on surgical strategies for early gallbladder cancer, an exact comparison of operating procedures is mandatory as there is a wide variation in the procedures carried out by surgeons during “regional lymphadenectomy”.

Regional lymphadenectomy in our group consists of lymphadenectomy in the hepatoduodenal ligament, pericholedochal, periportal and along the celiac axis.

When considering current experience in the interdisciplinary treatment of solid gastrointestinal cancer, abandonment of lymphadenectomy in submucosal T1b-esophageal-, gastric-, and colorectal cancer is no longer defensible^[3].

The characteristics of gallbladder cancer are high malignancy with aggressive direct, lymphatic and hemato-

gen-venous spread, and extensive resistance to chemotherapy and radiation^[6]. Combined with the low morbidity and mortality of regional lymphadenectomy, this procedure is mandatory in T1b cancer of the gallbladder^[4].

The study by Lee and colleagues is important and commendable. It shows that oncosurgical treatment recommendations for patients with highly malignant cancer are not only based on literature studies.

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S- Editor Tian L L- Editor Webster JR E- Editor Zhang DN

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.

Luis Bujanda, PhD, Professor, Department of Gastroenterology, CIBEREHD, University of Country Basque, Donostia Hospital, Paseo Dr. Beguiristain s/n, 20014 San Sebastián, Spain

Marcelo A Beltran, MD, Chairman of Surgery, Hospital La Serena, P.O. BOX 912, La Serena, IV REGION, Chile

Dr. Herwig R Cerwenka, Professor, Department of Surgery, Medical University of Graz, Auenbruggerplatz 29, A-8036 Graz, Austria

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Mark D Gorrell, PhD, Professor, Centenary Institute of Cancer Medicine and Cell Biology, Locked bag No. 6, Newtown, NSW 2042, Australia

Eric R Kallwitz, MD, Assistant Professor, Department of Medicine, University of Illinois, 840 S Wood Street, 7th Floor, MC 787, Chicago, IL 60612, United States

Selin Kapan, Dr., Associate Professor of General Surgery, Dr. Sadi Konuk Training and Research Hospital, Department of General Surgery, Kucukcekmece, Istanbul 34150, Turkey

Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Nobuyuki Matsuhashi, MD, Department of Gastroenterology, Kanto Medical Center, NTT East, 5-9-22 Higashi-gotanda, Shinagawa-ku, Tokyo 141-8625, Japan

Utaro Motosugi, Assistant Professor, Department of Radiology, University of Yamanashi, 1110 Shimokato, Chuo-shi, Yamanashi 409-3898, Japan

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

Ferenc Sipos, MD, PhD, Cell Analysis Laboratory, 2nd Department of Internal Medicine, Semmelweis University, Szentkirályi u. 46., Budapest 1088, Hungary

Francis Seow-Choen, MBBS, FRCSEd, FAMS, Professor, Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10, 228510, Singapore

Yukihiko Shimizu, MD, PhD, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyō, Kyoto 615-8256, Japan

A M El-Tawil, MSc, MRCS, PhD, Department of Surgery, University Hospital of Birmingham, East Corridor, Ground Floor, Birmingham, B15 2TH, United Kingdom

Jian Wu, Associate Professor of Medicine, Internal Medicine/Transplant Research Program, University of California, Davis Medical Center, 4635 2nd Ave. Suite 1001, Sacramento CA 95817, United States

Mitsunori Yamakawa, Professor, Department of Pathological Diagnostics, Yamagata University, Faculty of Medicine, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan

Chunqing Zhang, Professor, Department of Gastroenterology, Shandong Provincial Hospital, Jinan 250021, Shandong Province, China

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

Events Calendar 2011

- January 14-15, 2011
 AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States
- January 20-22, 2011
 Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States
- January 27-28, 2011
 Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany
- January 28-29, 2011
 9. Gastro Forum München, Munich, Germany
- February 4-5, 2011
 13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany
- February 13-27, 2011
 Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia
- February 17-20, 2011
 APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver Bangkok, Thailand
- February 22, 2011-March 04, 2011
 Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada
- February 24-26, 2011
 Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland
- February 24-26, 2011
 2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil
- February 24-26, 2011
 International Colorectal Disease Symposium 2011, Hong Kong, China
- February 26-March 1, 2011
 Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada
- February 28-March 1, 2011
 Childhood & Adolescent Obesity:
- A whole-system strategic approach, Abu Dhabi, United Arab Emirates
- March 3-5, 2011
 42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States
- March 7-11, 2011
 Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States
- March 14-17, 2011
 British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom
- March 17-19, 2011
 41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany
- March 17-20, 2011
 Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States
- March 18, 2011
 UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States
- March 25-27, 2011
 MedicRes IC 2011 Good Medical Research, Istanbul, Turkey
- March 26-27, 2011
 26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States
- April 6-7, 2011
 IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States
- April 7-9, 2011
 International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy
- April 15-16, 2011
 Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany
- April 18-22, 2011
 Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States
- April 20-23, 2011
 9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea
- April 25-27, 2011
 The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia
- April 25-29, 2011
 Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States
- April 28-30, 2011
 4th Central European Congress of Surgery, Budapest, Hungary
- May 7-10, 2011
 Digestive Disease Week, Chicago, IL 60446, United States
- May 12-13, 2011
 2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom
- May 19-22, 2011
 1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain
- May 21-24, 2011
 22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venice, Italy
- May 25-28, 2011
 4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina
- June 11-12, 2011
 The International Digestive Disease Forum 2011, Hong Kong, China
- June 13-16, 2011
 Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy
- June 14-16, 2011
 International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia
- June 22-25, 2011
 ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain
- June 29-2, 2011
 XI Congreso Interamericano de Pediatría "Monterrey 2011", Monterrey, Mexico
- September 2-3, 2011
 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany
- September 10-11, 2011
 New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States
- September 10-14, 2011
 ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States
- September 30-October 1, 2011
 Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium
- October 19-29, 2011
 Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia
- October 22-26, 2011
 19th United European Gastroenterology Week, Stockholm, Sweden
- October 28-November 2, 2011
 ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States
- November 11-12, 2011
 Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan
- December 1-4, 2011
 2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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.

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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 Identifier, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 Gastroenterology and Hepatology).

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Baishideng Publishing Group Co., Limited

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 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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Volume 17 Number 39
October 21, 2011



Published by Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building,
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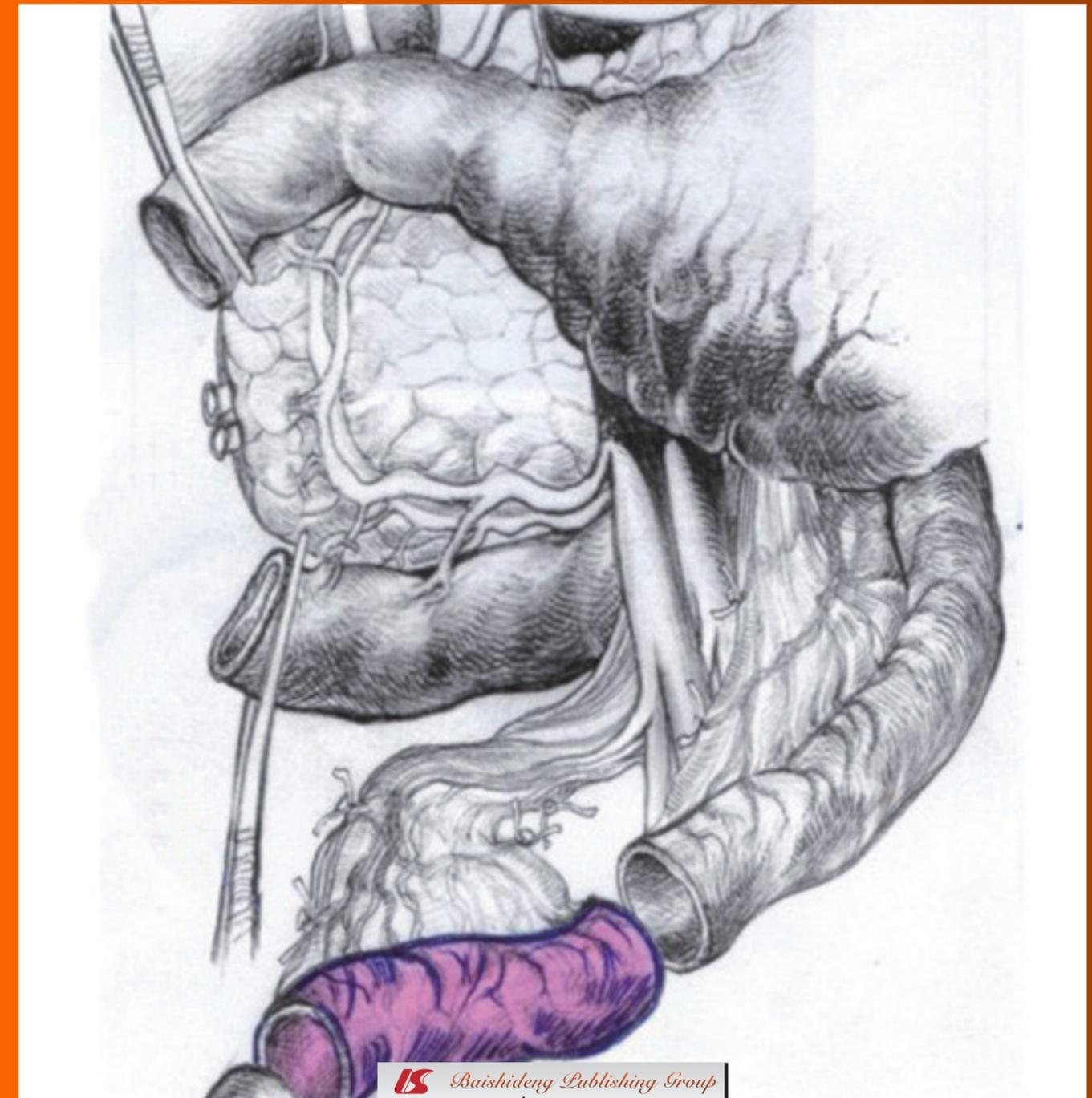
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ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

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

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October 21, 2011

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

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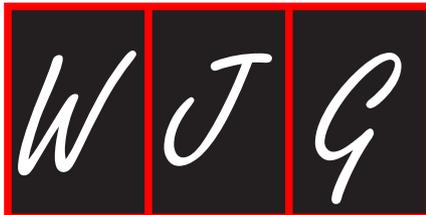
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Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
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Cystic dystrophy of the duodenal wall is not always associated with chronic pancreatitis

Raffaele Pezzilli, Donatella Santini, Lucia Calculli, Riccardo Casadei, Antonio Maria Morselli-Labate, Andrea Imbrogno, Dario Fabbri, Giovanni Taffurelli, Claudio Ricci, Roberto Corinaldesi

Raffaele Pezzilli, Antonio Maria Morselli-Labate, Andrea Imbrogno, Dario Fabbri, Roberto Corinaldesi, Pancreas Unit, Department of Digestive Diseases and Internal Medicine, Sant'Orsola-Malpighi Hospital, 40138 Bologna, Italy
Donatella Santini, Department of Pathology, Sant'Orsola-Malpighi Hospital, University of Bologna, 40138 Bologna, Italy
Lucia Calculli, Department of Radiology, Sant'Orsola-Malpighi Hospital, University of Bologna, 40138 Bologna, Italy
Riccardo Casadei, Giovanni Taffurelli, Claudio Ricci, Department of Surgery, Sant'Orsola-Malpighi Hospital, University of Bologna, 40138 Bologna, Italy

Author contributions: Pezzilli R, Santini D, Calculli L, Casadei R, Taffurelli G and Ricci C followed the patients clinically; Imbrogno A, Fabbri D and Morselli-Labate AM collected the literature data; Morselli-Labate AM and Pezzilli R analyzed the data and interpreted the results; Pezzilli R and Corinaldesi R coordinated and collected all the patient information; Pezzilli R designed the study and wrote the manuscript.

Correspondence to: Dr. Raffaele Pezzilli, Pancreas Unit, Department of Digestive Diseases and Internal Medicine, Sant'Orsola-Malpighi Hospital, 40138 Bologna, Italy. raffaele.pezzilli@aosp.bo.it

Telephone: +39-051-6364148 Fax: +39-051-6364148

Received: February 18, 2011 Revised: March 31, 2011

Accepted: April 7, 2011

Published online: October 21, 2011

Abstract

Cystic dystrophy of the duodenal wall is a rare form of the disease which was described in 1970 by French authors who reported the presence of focal pancreatic disease localized in an area comprising the C-loop of the duodenum and the head of the pancreas. German authors have defined this area as a "groove". We report our recent experience on cystic dystrophy of the paraduodenal space and systematically review the data in the literature regarding the alterations of this space. A MEDLINE search of papers published between 1966 and 2010 was carried out and 59 papers

were considered for the present study; there were 19 cohort studies and 40 case reports. The majority of patients having groove pancreatitis were middle aged. Mean age was significantly higher in patients having groove carcinoma. The diagnosis of cystic dystrophy of the duodenal wall can now be assessed by multi-detector computer tomography, magnetic resonance imaging and endoscopic ultrasonography. These latter two techniques may also add more information on the involvement of the remaining pancreatic gland not involved by the duodenal malformation and they may help in differentiating "groove pancreatitis" from "groove adenocarcinoma". In conclusion, chronic pancreatitis involving the entire pancreatic gland was present in half of the patients with cystic dystrophy of the duodenal wall and, in the majority of them, the pancreatitis had calcifications.

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Key words: Pancreatitis; Cystic dystrophy of duodenal wall; Therapy; Outcome

Peer reviewer: José Julián calvo Andrés, Department of Physiology and Pharmacology, University of Salamanca, Edificio Departamentl, Plaza de los Doctores de la Reina, Campus Miguel de Unamuno. 37007 Salamanca, Spain

Pezzilli R, Santini D, Calculli L, Casadei R, Morselli-Labate AM, Imbrogno A, Fabbri D, Taffurelli G, Ricci C, Corinaldesi R. Cystic dystrophy of the duodenal wall is not always associated with chronic pancreatitis. *World J Gastroenterol* 2011; 17(39): 4349-4364 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4349.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4349>

INTRODUCTION

Cystic dystrophy of the duodenal wall is a rare form of the

disease which was described for the first time in 1970 by Potet and Duclert^[1]. Potet and Duclert and other French authors^[2,3] described the presence of focal pancreatic disease localized in an area comprising the C-loop of the duodenum and the head of the pancreas (Figure 1A). In 1991, Becker and Mischke^[4] defined this area as a “groove” and pointed out that it serves as a bed for the large vessels, lymph nodes, common bile duct (CBD) and main pancreatic duct. These authors also reported that pancreatitis can be found in this area and they suggested the term “groove pancreatitis” which was well received. They also classified groove pancreatitis as “pure groove pancreatitis” (Figure 1B), **segmental pancreatitis of the head and chronic pancreatitis with groove involvement** (Figure 1C). **In addition, in recent years, Adsay and Zamboni^[5] proposed the term “paraduodenal pancreatitis” in patients classified as having “cystic dystrophy of the heterotopic pancreas” or “paraduodenal wall cyst” or “groove pancreatitis”; they also recognized two types of pancreatitis: one characterized by cystic changes and the other characterized by solid lesions. These authors pointed out that the latter type of pancreatitis is difficult to distinguish from an adenocarcinoma originating in this area. Finally, the presence of cystic dystrophy of the duodeno-pancreatic space together with chronic pancreatitis of the remaining pancreas is not always true because there is also the possibility of disease limited to the CBD^[6]. Thus, in this review, we report our recent experience on cystic dystrophy of the space from the C-loop of the duodenum and the pancreas by reporting three cases observed in the last year, and also systematically review and discuss the data in the literature on the alteration of the groove space.**

OUR EXPERIENCE ON THREE RECENT OBSERVED CASES OF CYSTIC DYSTROPHY OF THE DUODENAL WALL

We report our experience on three recently observed cases of cystic dystrophy of duodenal wall. Patients were one female and two males aged 49-65 years having persistent abdominal pain and weight loss. One male patient was a drinker and the diagnosis in all 3 patients was confirmed at laparotomy. The pathological examination in two cases confirmed cystic dystrophy of duodenal wall associated with chronic pancreatitis in one case and autoimmune pancreatitis and pancreatic carcinoma in the remaining one.

Case 1

A 65-year-old female was admitted to our department in April 2009 for persistent abdominal discomfort and progressive weight loss (about 5 kg in two months). Before this admission, she had had a one-year history of recurrent epigastric pain; an ultrasonographic (US) examination showed gallstones and the patient had been cholecystectomized in another hospital. After surgery, she

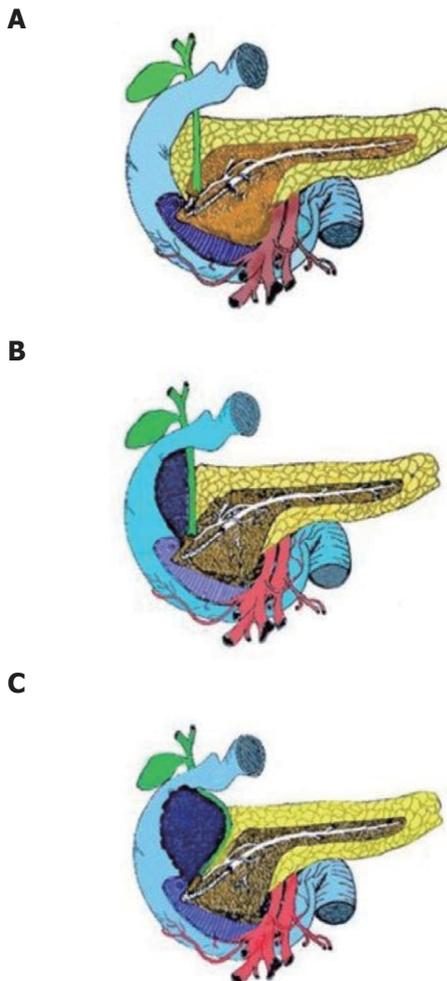


Figure 1 Classification of the various types of groove pancreatitis. A: Typical finding of groove pancreatitis (purple area); B: Segmental head pancreatitis: the scar tissue (dark blue) expands towards the duodenum; C: Pancreatitis of the head: the scar tissue (dark blue) expands to the duodenal area, determining duodenal stenosis and displacement of the common bile duct.

continued to have recurrent and frequent episodes of epigastric pain; US showed a dilation of the CBD and, two months after surgery, she underwent an endoscopic sphincterotomy. One month after this procedure, epigastric pain reappeared and, due to the presence of scleral jaundice (total bilirubin 3.2 mg/dL), the patient underwent another endoscopic retrograde cholangiopancreatography (ERCP). The papilla of Vater was substenotic and another sphincterotomy was carried out without any clinical improvement. On admission to our department, physical examination was unremarkable as was a routine blood examination; her body temperature was 37.2 °C, her arterial pressure was 110/60 mmHg and her cardiac rate was 73 bpm. Contrast-enhanced multidetector computer tomography (MDCT) was carried out. This examination showed the presence of multiple hypodense lesions in the liver (Figure 2); a US fine needle biopsy of one of these lesions was carried out and the pathological specimen was compatible with an abscess; the liver tissue was also cultured and the patient was treated with a spe-

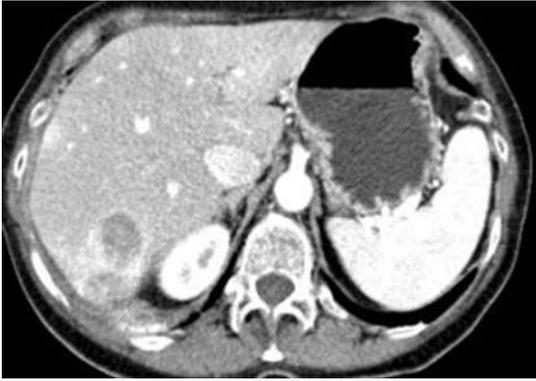


Figure 2 Case No. 1 computer tomography liver evaluation. Liver multiple hypodense lesions compatible with abscesses.

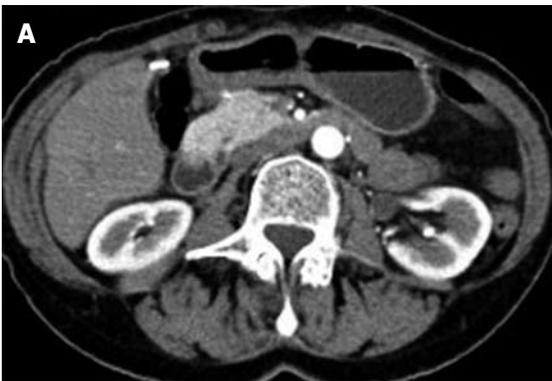


Figure 3 Case No. 1 computer tomography duodenal and pancreatic gland evaluation. A: Presence of duodenal bulging; B: Normal appearance of the pancreatic gland.

cific antibiotic. At computer tomography (CT) examination, there was the presence of biliary sludge and a dilation of the left intrahepatic biliary tree. There was also the presence of duodenal bulging (Figure 3A) while the pancreatic gland was normal (Figure 3B). An endoscopic US (EUS) was finally carried out. It confirmed the presence of duodenal bulging (Figure 4A) and showed CBD sludge; in addition, cysts in the duodenal wall were seen (Figure 4B) and a diagnosis of cystic dystrophy of duodenal wall was made. The patient refused surgery, and conservative treatment with ursodeoxycholic acid was carried out. Twenty months after discharge, the patient



Figure 4 Case No. 1 computer tomography duodenal and pancreatic gland evaluation. A: Presence of duodenal bulging; B: Presence of cysts in the duodenal wall.

was free of abdominal discomfort and regained her lost weight.

Case 2

A 49-year-old male patient with a history of chronic alcoholic consumption (about 40 g of pure alcohol intake per day) was admitted to our Department in May 2010 with persistent epigastric pain of seven months duration associated with nausea and biliary vomiting; there was also weight loss of 13 kg. The following biochemical tests were carried out: Hb 11.9 g/dL, MCV 85.8; amylase 156 U/L (upper reference value 100), CA 19-9: 52 U/mL (upper reference value 37). The patient underwent an upper gastrointestinal endoscopy which was normal. Ultrasonographic examination did not show alterations of the abdominal parenchyma. MDCT showed an enlarged pancreatic head and the presence of multiple cysts between the enlarged pancreatic head and the duodenum (Figure 5A); the remaining pancreas was normal as was demonstrated by magnetic resonance imaging (MRI) (Figure 5B). The patient was operated on and a pancreatic head resection was performed. The pathology of the resected specimen showed cystic dystrophy of the duodenal wall with hypertrophy of the Brunner glands and the presence of an ectopic pancreas (Figure 6A), showing chronic pancreatitis (Figure 6B). Seven months after surgery, the patient was symptom free and in good general health.

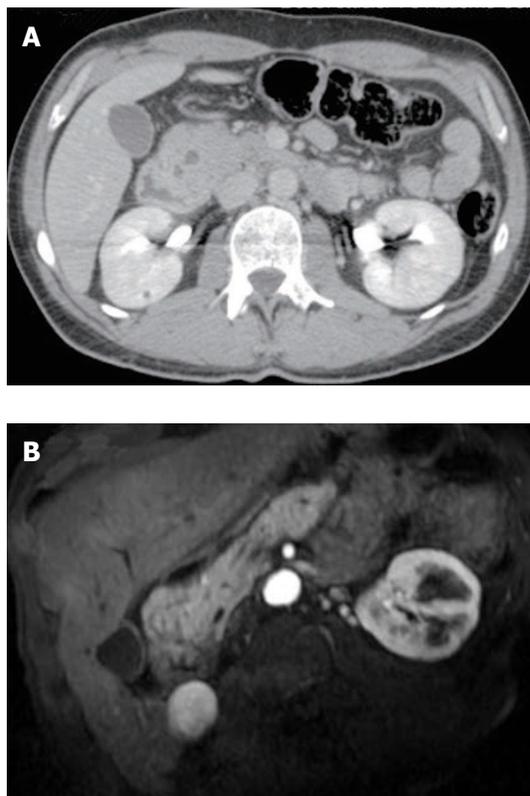


Figure 5 Case No. 2 computer tomography duodenal and pancreatic gland evaluation. A: Enlarged pancreatic head and the presence of multiple cysts between the enlarged pancreatic head and the duodenum (multidetector computer tomography); B: The remaining pancreas was normal as demonstrated by the magnetic resonance imaging.

Case 3

A 56-year-old male affected by Crohn's disease was seen in August 2010 with persistent epigastric pain of one month duration associated with jaundice, weight loss, nausea and intermittent vomiting. The patient was not an alcohol drinker. The following biochemical tests were carried out: total bilirubin, 25.4 mg/dL, AST, 63 U/L (upper normal limit 38), ALT, 66 U/L (upper normal limit 40), alkaline phosphatases, 1105 U/L (normal value 98-280), amylase, 108 U/L (upper normal limit 100), lipase, 293 U/L (upper normal limit 60), CA, 19-9 2345 U/mL (upper reference value 37). The patient underwent US which showed a dilated CBD and a mass of 2.5 cm in the head of the pancreas. The MDCT showed the pancreatic head focally enlarged with a 2.5 cm heterogeneous area extending to and involving the wall of the posterior bulbar duodenum. The main pancreatic duct was uniformly dilated in caliber and appearance with no changes in the pancreatic body or tail. The patient underwent a pancreaticoduodenectomy and surgical pathology showed the presence of cystic dystrophy of the duodenal wall (Figure 7A) with aspects of chronic pancreatitis in the heterotopic pancreas (Figure 7A), aspects of autoimmune pancreatitis (Figure 7B) and, finally, groove adenocarcinoma extending to the pancreatic head (Figure 7C). At present, the patient is still alive and is in adjuvant chemotherapy

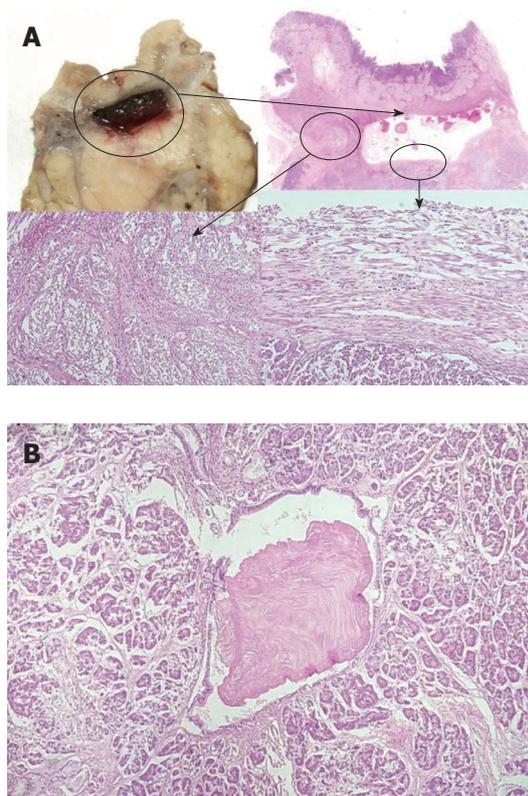


Figure 6 Case No. 2 pancreatic and duodenal surgical specimens. A: Resected specimen showing cystic dystrophy of the duodenal wall with hyperplasia of the Brunner glands and the presence of an ectopic pancreas (arrows); B: Chronic pancreatitis in the remaining pancreas together with cystic dystrophy of the duodenal wall.

with gemcitabine.

CLINICAL CONSIDERATIONS AND AIMS

The present report involving three cases of cystic dystrophy of the duodenal wall represents one of the few case series published concerning this rare entity. All of our patients presented with symptoms consistent with chronic pancreatitis; however, pancreatic diseases were found in two and these two patients improved dramatically after surgical head pancreatic resection while one is symptom free after medical treatment. It is important to diagnose the pathological involvement of the proximal duodenum in order to detect the presence of malignancy and to evaluate the prognosis of these subjects. In order to better establish the features of this rare entity we also undertook a systematic review of the literature.

LITERATURE SEARCH AND DATA EXTRACTION

A search was carried out on December 18, 2010 using the MEDLINE/PubMed database (United States National Library of Medicine National Institutes of Health) in order to select the data existing in the literature under the headings of pancreatitis and groove

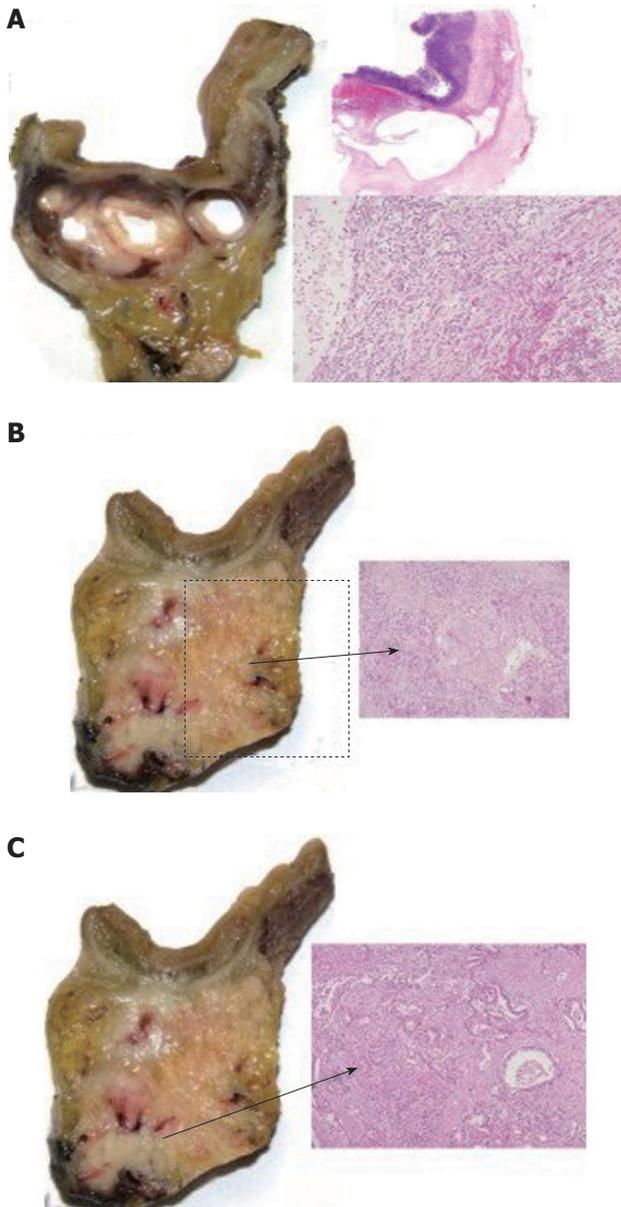


Figure 7 Case No. 3 pancreatic and duodenal pathological specimens. A: Cystic dystrophy of the duodenal wall with aspects of chronic pancreatitis in the heterotopic pancreas; B: Aspects of autoimmune pancreatitis (arrow); C: Groove adenocarcinoma extending to the pancreatic head (arrow).

pancreatitis. The terms used were “groove pancreatitis” or “duodenal cystic dystrophy” (explanatory variables) and “pancreatic diseases” (outcome variable). The search was limited to human studies written in English. We identified additional studies by means of a hand search of the bibliographies from the primary studies, review articles and key journals. A total of 70 citations were found in MEDLINE/PubMed^[4-73]. Four investigators (Pezzilli R, Morselli-Labate AM, Fabbri D, and Imbrogno A) independently screened all articles for those meeting the broad inclusion criteria. Of the 70 papers, 4 were excluded because they contained data regarding diseases other than those searched for^[8,15,48,71]. Of the remaining 66 papers, 10 were excluded because they

were review articles not containing data useful for the analyses^[4,5,24,41,42,45,56,58,60,68] and one because it was a comment on an article^[19] without new data/cases; therefore, 55 papers with available data remained. Of these 55 papers, 4 were also excluded for the following reasons: 1 because it was a duplicated publication^[55] and 3 because it was not possible to extract useful data^[49,57,73]. Eight papers were added to these 51 papers because they were extracted from the references^[74-81]. Thus, 59 papers were considered for the present study; there were 19 cohort studies^[6,7,10-13,16,18,23,30,36,37,39,40,46,47,52,63,81] and 40 case reports^[9,14,17,20-22,25-29,31-35,38,43,44,50,51,53,54,59,61,62,64-67,69,70,72,74-80].

For each study, the following information was recorded: gender, mean age for the cohort studies or age of the subjects studied in the case reports, interval time from the appearance of the symptoms to diagnosis, alcoholism, the presence of clinical variables (such as abdominal pain, weight loss and jaundice, hyperamylasemia, CBD stenosis, duodenal stenosis), the need for surgery and the type of surgery, the possible presence of chronic pancreatitis in the pancreas together with cystic dystrophy as well as the presence of pseudocysts, the possible presence of pancreatic neoplasms, the time of follow-up and death.

Data are presented as absolute numbers and relative frequencies, mean \pm SD, medians, ranges, and interquartile ranges (IQR); follow-up data are also presented as crude survival.

EVALUATION OF THE SELECTED STUDIES

Due to the low frequency of diseases, such as groove pancreatitis and groove carcinomas, there is a limited number of cohort studies (No. 19) and a large number of case reports (No. 40). All the cohort studies were retrospective and patients were enrolled from 1959^[11] to 2008^[6]. Thus, the changes in diagnostic techniques with the appearance of MRI and EUS in clinical practice render the studies not comparable as to what is the best technique for diagnosing groove diseases. Furthermore, the mean follow-ups vary greatly and the longest follow-up is about 8 years which is that reported by Casetti *et al.*^[63]. As shown in Tables 1-8, we found no substantial differences between the data reported in the cohort studies and those we calculated when grouping the series of case reports by gender, age at diagnosis, alcoholism, presence of pain, weight loss, jaundice, hyperamylasemia, CBD stenosis, duodenal stenosis and the need for surgery. The presence of chronic pancreatitis and deaths were more frequently reported in the cohort studies than in the case reports while associated adenocarcinoma and pseudocysts were more frequently reported in the case reports than in the cohort studies.

EPIDEMIOLOGY

We have no epidemiological data regarding the preva-

Table 1 Epidemiological and clinical characteristics of patients in the 18 retrospective studies involving patients with a benign cystic duodenal wall

Author ^[Ref.] yr	Time interval of patient enrollment	n (%)			Age (yr)	Alcohol drinkers
		Total	Males	Females	Mean (range)	n (%)
Stolte <i>et al</i> ^[7] 1982	NR	30	30 (100)	-	41.3 (NR)	22 (73.3)
Yamaguchi <i>et al</i> ^[10] 1992	1983-1989	8	8 (100)	-	58.0 (33-70)	4 (50.0)
Fléjou <i>et al</i> ^[11] 1993	1959-1991	10	10 (100)	-	41.0 (31-56)	2 (20.0)
Itoh <i>et al</i> ^[12] 1994	NR	4	3 (75.0)	1 (25.0)	43.0 (37-53)	NR
Fékété <i>et al</i> ^[13] 1996	1989-1993	6	6 (100)	-	40.0 (35-46)	4 (66.7)
Procacci <i>et al</i> ^[16] 1997	1992-1996	10	10 (100)	-	41.0 (32-59)	9 (90.0)
Irie <i>et al</i> ^[18] 1998	1995-1996	5	5 (100)	-	41.0 (33-46)	2 (40.0)
Vullierme <i>et al</i> ^[23] 2000	1988-1998	20	18 (90.0)	2 (10.0)	44.0 (36-56)	NR
Aoun <i>et al</i> ^[81] 2005	NR	4	2 (50.0)	2 (50.0)	69.0 (66-71)	NR
Pessaux <i>et al</i> ^[36] 2006	1990-2004	12	11 (91.7)	1 (8.3)	42.4 (34-54)	9 (75.0)
Jouannaud <i>et al</i> ^[37] 2006	1990-2002	23	20 (87.0)	3 (13.0)	45.0 (30-66)	23 (100)
Tison <i>et al</i> ^[39] 2007	1983-2001	9	8 (88.9)	1 (11.1)	48.0 (37-63)	8 (88.9)
Rebours <i>et al</i> ^[40] 2007	1995-2004	105	96 (91.4)	9 (8.6)	46.0 (24-75)	86 (81.9)
Rahman <i>et al</i> ^[46] 2007	2000-2005	11	10 (90.9)	1 (9.1)	48.0 (35-61)	10 (90.9)
Castell-Monsalve <i>et al</i> ^[47] 2008	NR	5	4 (80.0)	1 (20.0)	47.0 (40-53)	4 (80.0)
Jovanovic <i>et al</i> ^[52] 2008	1996-2006	13	10 (76.9)	3 (23.1)	41.5 (17-60)	6 (6.2)
Casetti <i>et al</i> ^[63] 2009	1990-2006	58	54 (93.1)	4 (6.9)	44.7 (IQR 36.8-51.8)	57 (98.3)
Ishigami <i>et al</i> ^[6] 2010	2001-2008	15	14 (93.3)	1 (6.7)	48.0 (31-64)	NR
Overall	-	348	319 (91.70)	29 (8.30)	-	246/305 (80.70)

IQR: Interquartile range; NR: Not reported.

Table 2 Epidemiological and clinical characteristics of patients in the 18 retrospective studies involving patients with a benign cystic duodenal wall (continues from Table 1) n (%)

Author ^[Ref.] yr	Time interval from the symptoms to the diagnosis	Abdominal pain		Weight loss	Jaundice	Hyperamylasemia
		No. of cases	Type			
Stolte <i>et al</i> ^[7] 1982	NR	NR	NR	30 (100)	NR	NR
Yamaguchi <i>et al</i> ^[10] 1992	NR	3 (37.5)	NR	0	2 (25.0)	NR
Fléjou <i>et al</i> ^[11] 1993	NR	7 (70.0)	Persistent	9 (90.0)	4 (40.0)	NR
Itoh <i>et al</i> ^[12] 1994	NR	3 (75.0)	NR	NR	NR	3 (75.0)
Fékété <i>et al</i> ^[13] 1996	NR	6 (100)	Recurrent	6 (100)	0	6 (100)
Procacci <i>et al</i> ^[16] 1997	4.5 yr (1-9)	10 (100)	Recurrent	4 (40.0)	1 (10.0)	NR
Irie <i>et al</i> ^[18] 1998	NR	4 (80.0)	NR	0	0	NR
Vullierme <i>et al</i> ^[23] 2000	41.5 d (1-140)	NR	NR	NR	NR	NR
Aoun <i>et al</i> ^[81] 2005	NR	3 (75.0)	NR	0	1 (25.0)	NR
Pessaux <i>et al</i> ^[36] 2006	NR	9 (75.0)	Persistent in 4 (44.4)	12 (100)	2 (16.7)	NR
Jouannaud <i>et al</i> ^[37] 2006	NR	22 (95.7)	NR	16 (69.6)	0	NR
Tison <i>et al</i> ^[39] 2007	NR	9 (100)	NR	9 (100)	2 (22.2)	NR
Rebours <i>et al</i> ^[40] 2007	1 yr (0-24)	91 (86.7)	Continuous in 35 (38.4); occasional in 56 (61.5)	73 (69.6)	13 (12.4)	NR
Rahman <i>et al</i> ^[46] 2007	NR	11 (100)	Recurrent in 8 (72.7)	10 (90.9)	0	2 (18.2)
Castell-Monsalve <i>et al</i> ^[47] 2008	NR	5 (100)	Persistent	NR	NR	5 (100)
Jovanovic <i>et al</i> ^[52] 2008	7.5 mo (0.5-36)	12 (92.3)	NR	4 (30.8)	4 (30.8)	NR
Casetti <i>et al</i> ^[63] 2009	NR	46 (79.3)	Persistent	NR	3 (5.2)	NR
Ishigami <i>et al</i> ^[6] 2010	NR	NR	NR	NR	NR	NR
Overall	-	241/283 (85.20)	-	173/246 (70.30)	32/274 (11.70)	16/26 (61.50)

NR: Not reported.

lence and incidence of cystic dystrophy of the duodenal wall in the general population. The data regarding this anomaly mainly describes patients with associated chronic pancreatitis. A recent Italian survey which reviewed the data on chronic pancreatitis in Italy in mixed medical/surgical cases from 2000 to 2005^[57] reported that the frequency of groove pancreatitis was 6.2% (55

out of 893 patients) with a higher frequency in males (7.6%, 50/660) than in females (2.1%, 5/233). In a surgical setting, groove pancreatitis ranges from 2.7% to 24.5%^[4,7,10,63]; in these cases, the frequency in males is also higher than that in females. We have no epidemiological data regarding groove carcinomas or biliary involvement without pancreatitis or pancreatic adenocar-

Table 3 Epidemiological and clinical characteristics of patients in the 18 retrospective studies involving patients with a benign cystic duodenal wall (continues from Table 2) *n* (%)

Author ^[Ref.] yr	Imaging	Duodenal findings	CBD stenosis	Duodenal stenosis
Stolte <i>et al</i> ^[7] 1982	NR	Brunner hyperplasia in 25	15 (50.0)	NR
Yamaguchi <i>et al</i> ^[10] 1992	US, CT, ERCP, PTC	Edema and nodular appearance; Brunner hyperplasia	4 (50.0)	5 (62.5)
Fléjou <i>et al</i> ^[11] 1993	ERCP, EUS	Edema and congestion of the mucosa	0	7 (70.0)
Itoh <i>et al</i> ^[12] 1994	CT	NR	NR	NR
Fékété <i>et al</i> ^[13] 1996	CT, ERCP, EUS	Edema and congestion of the mucosa	0	5 (83.3)
Procacci <i>et al</i> ^[16] 1997	CT, ERCP, EUS	Inflammation in 8	2 (20.0)	2 (20.0)
Irie <i>et al</i> ^[18] 1998	MRI	Brunner hyperplasia in 3	2 (40.0)	3 (60.0)
Vullierme <i>et al</i> ^[23] 2000	CT	NR	3 (15.0)	20 (100)
Aoun <i>et al</i> ^[81] 2005	US, CT, ERCP, EUS	NR	4 (100)	NR
Pessaux <i>et al</i> ^[36] 2006	US, EUS, CT, ERCP, MRI	NR	NR	NR
Jouannaud <i>et al</i> ^[37] 2006	EUS, CT	Inflammation in 3	NR	8 (34.8)
Tison <i>et al</i> ^[39] 2007	US, CT, MRI, angiography	Non specific inflammation in 9	5 (55.6)	9 (100)
Rebours <i>et al</i> ^[40] 2007	CT, EUS	Brunner hyperplasia in 61	26 (24.8)	50 (47.6)
Rahman <i>et al</i> ^[46] 2007	CT, MRI, EUS	Brunner hyperplasia	0	5 (45.5)
Castell-Monsalve <i>et al</i> ^[47] 2008	MRI, EUS	Duodenal stenosis in 3	3 (60.0)	3 (60.0)
Jovanovic <i>et al</i> ^[52] 2008	US, CT, MRI, EUS	NR	6 (46.2)	NR
Casetti <i>et al</i> ^[63] 2009	US, CT, MRI, EUS	NR	3 (5.2)	NR
Ishigami <i>et al</i> ^[6] 2010	CT, MRI	NR	9 (60.0)	NR
Overall	-	-	82/309 (26.50)	117/212 (55.20)

CBD: Common bile duct; NR: Not reported; US: Transabdominal ultrasonography; CT: Computer tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography; PTC: Percutaneous transhepatic cholangiography.

Table 4 Epidemiological and clinical characteristics of patients in the 18 retrospective studies involving patients with a benign cystic duodenal wall (continues from Table 3) *n* (%)

Author ^[Ref.] yr	Surgery		Associated chronic pancreatitis	Associated neoplasms	Pseudocyst	Follow-up		
	No. of cases	Type				Mean (range)	Death	Lost
Stolte <i>et al</i> ^[7] 1982	30 (100)	PD	4 (13.3) (all with calcification)	No	5 (16.7)	NR	NR	NR
Yamaguchi <i>et al</i> ^[10] 1992	8 (100)	PD	NR	No	No	2 yr	1 (12.5)	NR
Fléjou <i>et al</i> ^[11] 1993	10 (100)	WP in 8; derivative in 2	0	No	No	1-5 yr	1 (10.0)	4 (40.0)
Itoh <i>et al</i> ^[12] 1994	3 (75.0)	PD	NR	No	No	No	NR	NR
Fékété <i>et al</i> ^[13] 1996	6 (100)	PD in 5; antrectomy in 1	NR	No	No	32 mo (18-64)	No	NR
Procacci <i>et al</i> ^[16] 1997	10 (100)	PD	7 (70.0) (calcifications in 5)	No	5 (head) (50.0)	NR	NR	NR
Irie <i>et al</i> ^[18] 1998	3 (60.0)	PD	2 (40.0) (all with calcifications)	No	No	NR	NR	NR
Vullierme <i>et al</i> ^[23] 2000	20 (100)	PD	9 (45.0) (calcifications in 5)	No	No	NR	NR	NR
Aoun <i>et al</i> ^[81] 2005	4 (100)	PD	NR	NR	NR	NR	NR	NR
Pessaux <i>et al</i> ^[36] 2006	12 (100)	PD	8 (66.7) (calcification in 2)	No	No	64 mo (6-158)	1 (8.3)	1 (8.3)
Jouannaud <i>et al</i> ^[37] 2006	14 (60.9)	PD in 11; derivative in 3	17 (73.9) (calcification in 10)	No	No	47 mo	1 (4.3)	NR
Tison <i>et al</i> ^[39] 2007	9 (100)	PD	5 (55.6)	No	No	72 mo	4 (44.4)	NR
Rebours <i>et al</i> ^[40] 2007	29 (27.6)	PD in 17; digestive and biliary by pass in 12	97 (92.4) (calcification in 96)	No	No	15 mo (0-243)	NR	NR
Rahman <i>et al</i> ^[46] 2007	11 (100)	PD	0	No	No	NR	NR	NR
Castell-Monsalve <i>et al</i> ^[47] 2008	4 (80.0)	WP in 3; 1 laparotomy	3 (60.0)	No	No	NR (13-36 mo)	No	NR
Jovanovic <i>et al</i> ^[52] 2008	13 (100)	PD	6 (46.2)	No	No	NR	NR	NR
Casetti <i>et al</i> ^[63] 2009	58 (100)	PD	NR	Neuroendocrine in 1	No	93.6 mo (IQR 59.7-129.7)	NR	NR
Ishigami <i>et al</i> ^[6] 2010	6 (40.0)	PD in 3, derivative surgery in 3	NR	NR	NR	NR	NR	NR
Overall	250/348 (71.8)	-	158/253 (62.5)	1/329 (0.3%)	10/329 (3.0)	-	8/73 (11.0)	5/22 (22.7)

IQR: Interquartile range; NR: Not reported; PD: Pancreaticoduodenectomy; WP: Whipple procedure.

cinoma. In all these studies, the patients having groove pancreatitis were middle aged (about 45 years of age), having a wide range from 20 mo^[76] to 75 years of age^[40]. Only two of the patients described were children (a

20-mo-old girl and a 15-year-old boy)^[51,76]. Mean age was significantly higher in patients having groove carcinoma than in those having groove pancreatitis, namely 70 years of age (range 57 to 80 years)^[51].

Table 5 Epidemiological and clinical characteristics of patients in the 38 case report papers involving 46 subjects with a benign cystic duodenal wall (a paper may report more than one patient), the three cases reported in the present paper are also shown

Author ^[Ref.] yr	Gender	Age (yr)	Alcohol drinker
Bill <i>et al</i> ^[74] 1982	Male	64	Yes
Holstege <i>et al</i> ^[75] 1985	Male	44	Yes
Tio <i>et al</i> ^[9] 1991	Male	48	NR
Tio <i>et al</i> ^[9] 1991	Male	53	NR
Flaherty <i>et al</i> ^[75] 1992	Female	20 mo	No
Izbicki <i>et al</i> ^[77] 1994	Male	25	NR
Fujita <i>et al</i> ^[14] 1997	Male	42	Yes
Shudo <i>et al</i> ^[17] 1998	Male	66	Yes
Wu <i>et al</i> ^[78] 1998	Male	39	NR
Babál <i>et al</i> ^[79] 1998	Female	70	NR
Rubay <i>et al</i> ^[21] 1999	Male	46	Yes
Balachandar <i>et al</i> ^[22] 1999	Male	18	NR
Mohl <i>et al</i> ^[25] 2001	Male	44	Yes
Mohl <i>et al</i> ^[25] 2001	Male	42	Yes
Munthali Lovemore <i>et al</i> ^[26] 2001	Male	24	No
Indinnimeo <i>et al</i> ^[27] 2001	Male	46	Yes
Shudo <i>et al</i> ^[28] 2002	Male	53	Yes
Glaser <i>et al</i> ^[29] 2002	Male	51	Yes
Hwang <i>et al</i> ^[31] 2003	Male	46	Yes
Jovanovic <i>et al</i> ^[32] 2004	Male	38	No
McFaul <i>et al</i> ^[80] 2004	Male	29	Yes
McFaul <i>et al</i> ^[80] 2004	Male	62	Yes
Isayama <i>et al</i> ^[33] 2005	Male	56	Yes
Chatelain <i>et al</i> ^[34] 2005	Male	47	Yes
Chatelain <i>et al</i> ^[34] 2005	Female	44	Yes
Balzan <i>et al</i> ^[35] 2005	Male	47	NR
Sanada <i>et al</i> ^[43] 2007	Male	81	No
Balakrishnan <i>et al</i> ^[44] 2007	Male	40	Yes
de Tejada <i>et al</i> ^[50] 2008	Male	47	Yes
Stefanescu <i>et al</i> ^[51] 2008	Male	15	No
Varma <i>et al</i> ^[53] 2008	Female	23	NR
Galloro <i>et al</i> ^[54] 2008	Male	44	Yes
Thomas <i>et al</i> ^[59] 2009	Male	43	NR
Levenick <i>et al</i> ^[61] 2009	Female	35	Yes
Levenick <i>et al</i> ^[61] 2009	Male	47	Yes
Levenick <i>et al</i> ^[61] 2009	Female	36	Yes
Levenick <i>et al</i> ^[61] 2009	Female	54	NR
Yoshida <i>et al</i> ^[62] 2009	Male	63	Yes
Meesiri ^[64] 2009	Male	44	Yes
Funamizu <i>et al</i> ^[65] 2009	Female	54	NR
Viñolo Ubiña <i>et al</i> ^[66] 2010	Male	40	Yes
Tezuka <i>et al</i> ^[67] 2010	Male	55	Yes
Lee <i>et al</i> ^[69] 2010	Male	75	NR
Egorov <i>et al</i> ^[70] 2010	Male	32	Yes
Egorov <i>et al</i> ^[70] 2010	Male	43	NR
German <i>et al</i> ^[72] 2010	Male	34	Yes
Pezzilli 2011 Present paper	Female	65	No
Pezzilli 2011 Present paper	Male	49	Yes
Pezzilli 2011 Present paper	Male	56	No
Overall	Males: 40 (81.6%) Females: 9 (18.4%)	45.3 ± 15.2	29/36 (80.5%)

NR: Not reported.

CLINICAL AND BIOCHEMICAL FEATURES

As shown in Tables 1-8, the main symptoms of cystic dystrophy of the duodenal wall were epigastric pain, weight loss and jaundice. These symptoms were similar in those patients having associated chronic groove pancreatitis and in those patients having groove carcinoma. All these symptoms can be present, further complicating

the differential diagnosis with ampullary and periampullary cancers. Pain may be persistent or recurrent, and nausea and vomiting are usually present as accompanying symptoms. The majority of these patients are heavy alcohol drinkers (275/341, 80.6%), and this may explain the fact that most of the patients with groove pancreatitis are males. In addition, in the 18 patients with groove adenocarcinoma, the majority of cases were males (11/18, 61.1%) (Tables 9-12).

Regarding the laboratory examinations, serum amylase activity was usually abnormally high in these patients (38/59, 64.4%) (Tables 1-8), but the magnitude of this elevation varied greatly. An increase in bilirubin may have also been present, along with an increase in alkaline phosphatases in patients with jaundice. Finally, it has also been reported in the literature that tumor markers, such as serum CA 19-9, are usually within the normal limits^[10,58].

ASSOCIATED DISEASES

The majority of patients with cystic dystrophy of the duodenal wall have been reported to have chronic groove pancreatitis or groove carcinoma. However, the lesions in the remaining pancreatic gland not affected by groove pancreatitis have not been fully evaluated. As shown in Tables 1-12, in patients with groove pancreatitis as well as in those with groove carcinoma, the pancreatic gland above the groove lesion is generally not affected by chronic pancreatitis. Chronic pancreatitis of the entire pancreas was reported in 166 of the 302 (55.1%) patients and there were pancreatic calcifications in 125 of these 166 patients (75.3%) (Tables 1-8). The presence of pancreatic pseudocysts was usually rare (13 out of 378, 3.4%) (Tables 1-8), and, in most cases, they were localized in the head of the pancreas (7/13, 53.8%). In addition, some authors have reported that groove pancreatitis is associated with the occasional findings of neuroendocrine tumors^[63] or pancreatic cystadenoma^[54].

IMAGING ASSESSMENT

As shown in Tables 3, 7, 8 and 11, the imaging diagnosis of dystrophy of the duodenal wall is rarely assessed using a single radiological modality. Even if US is the first line imaging modality in these patients, it is rarely diagnostic. ERCP, which was frequently used in the past, is feasible and in typical cases it demonstrates smooth tubular stenosis at the distal part of the CBD without abnormality of the main pancreatic duct or, occasionally, with only slight irregularities^[44,65]. ERCP may also demonstrate irregularity, tapering obstruction or dilatation of the Santorini duct and its branches, sometimes with intraductal stones or protein plugs^[44]. At present, ERCP is used mainly for endoscopic therapy^[33]; in fact, successful treatment for groove pancreatitis by endoscopic drainage *via* the minor papilla was carried out in only one patient^[33].

Table 6 Epidemiological and clinical characteristics of patients in the 38 case report papers involving 46 subjects with a benign cystic duodenal wall (a paper may report more than one patient), the three cases reported in the present paper are also shown (continues from Table 5)

Author ^[Ref.] yr	Time interval from the onset of symptoms to diagnosis	Abdominal pain	Weight loss	Jaundice	Hyperamylasemia
Bill <i>et al</i> ^[74] 1982	NR	Yes (Persistent)	Yes	No	No
Holstege <i>et al</i> ^[75] 1985	6 mo	Yes (Persistent)	Yes	No	Yes
Tio <i>et al</i> ^[9] 1991	NR	Yes (NR)	No	No	NR
Tio <i>et al</i> ^[9] 1991	NR	Yes (NR)	No	Yes	NR
Flaherty <i>et al</i> ^[76] 1992	NR	Yes (NR)	No	No	NR
Izbicki <i>et al</i> ^[77] 1994	NR	Yes (Recurrent)	No	No	No
Fujita <i>et al</i> ^[14] 1997	NR	Yes (Recurrent)	Yes	No	No
Shudo <i>et al</i> ^[17] 1998	NR	Yes (Persistent)	No	No	Yes
Wu <i>et al</i> ^[78] 1998	10 yr	Yes (Recurrent)	Yes	No	NR
Babál <i>et al</i> ^[79] 1998	NR	No	No	No	NR
Rubay <i>et al</i> ^[21] 1999	7 yr	Yes (Recurrent)	Yes	No	Yes
Balachandar <i>et al</i> ^[22] 1999	NR	No	No	Yes	No
Mohl <i>et al</i> ^[25] 2001	1 yr	Yes (Recurrent)	Yes	No	NR
Mohl <i>et al</i> ^[25] 2001	1 yr	Yes (Persistent)	Yes	No	NR
Munthali Lovemore <i>et al</i> ^[26] 2001	NR	Yes (Persistent)	NR	Yes	Yes
Indinnimeo <i>et al</i> ^[27] 2001	10 yr	Yes (Recurrent)	No	No	Yes
Shudo <i>et al</i> ^[28] 2002	NR	Yes (Persistent)	No	No	Yes
Glaser <i>et al</i> ^[29] 2002	NR	Yes (Persistent)	Yes	No	No
Hwang <i>et al</i> ^[31] 2003	NR	Yes (Persistent)	Yes	No	Yes
Jovanovic <i>et al</i> ^[32] 2004	NR	Yes (Persistent)	Yes	No	Yes
McFaul <i>et al</i> ^[80] 2004	13 mo	Yes (Recurrent)	Yes	No	NR
McFaul <i>et al</i> ^[80] 2004	2 yr	Yes (Recurrent)	Yes	Yes	NR
Isayama <i>et al</i> ^[33] 2005	2 yr	Yes (Persistent)	Yes	No	No
Chatelain <i>et al</i> ^[34] 2005	1 yr	Yes (Recurrent)	Yes	No	No
Chatelain <i>et al</i> ^[34] 2005	NR	Yes (Persistent)	Yes	No	No
Balzan <i>et al</i> ^[35] 2005	2 yr	Yes (Persistent)	No	No	Yes
Sanada <i>et al</i> ^[43] 2007	NR	Yes (Persistent)	No	No	Yes
Balakrishnan <i>et al</i> ^[44] 2007	NR	Yes (Persistent)	Yes	No	Yes
de Tejada <i>et al</i> ^[50] 2008	2 mo	Yes (Persistent)	Yes	No	NR
Stefanescu <i>et al</i> ^[51] 2008	5 mo	Yes (Persistent)	Yes	No	NR
Varma <i>et al</i> ^[53] 2008	3 mo	Yes (Persistent)	Yes	No	NR
Galloro <i>et al</i> ^[54] 2008	NR	Yes (Recurrent)	Yes	No	Yes
Thomas <i>et al</i> ^[59] 2009	NR	Yes (NR)	Yes	No	NR
Levenick <i>et al</i> ^[61] 2009	NR	Yes (Recurrent)	NR	No	NR
Levenick <i>et al</i> ^[61] 2009	NR	Yes (Recurrent)	Yes	No	NR
Levenick <i>et al</i> ^[61] 2009	NR	Yes (Recurrent)	Yes	No	NR
Levenick <i>et al</i> ^[61] 2009	NR	No	Yes	No	NR
Yoshida <i>et al</i> ^[62] 2009	NR	Yes (Persistent)	No	No	Yes
Meesiri ^[64] 2009	NR	Yes (Recurrent)	No	No	Yes
Funamizu <i>et al</i> ^[65] 2009	NR	Yes (Persistent)	No	Yes	Yes
Viñolo Ubiña <i>et al</i> ^[66] 2010	3 mo	Yes (Persistent)	No	No	Yes
Tezuka <i>et al</i> ^[67] 2010	NR	Yes (NR)	No	No	Yes
Lee <i>et al</i> ^[69] 2010	NR	Yes (Recurrent)	No	No	Yes
Egorov <i>et al</i> ^[70] 2010	2 mo	Yes (Persistent)	Yes	No	Yes
Egorov <i>et al</i> ^[70] 2010	1 yr	Yes (Persistent)	Yes	Yes	No
German <i>et al</i> ^[72] 2010	NR	Yes (Recurrent)	Yes	No	Yes
Pezzilli 2011 Present paper	1 yr	Yes (Recurrent)	Yes	Yes	No
Pezzilli 2011 Present paper	7 mo	Yes (Persistent)	Yes	No	Yes
Pezzilli 2011 Present paper	1 mo	Yes (Persistent)	Yes	Yes	Yes
Overall	2.1 ± 3.1 yr	46/49 (93.90%)	30/47 (63.80%)	8/49 (16.30%)	22/33 (66.70%)

CBD: Common bile duct; NR: not reported.

For many years, CT has been an excellent imaging modality for diagnosing chronic pancreatitis or adenocarcinoma associated with cystic dystrophy of the duodenal wall^[6,16]. In the pure form of groove pancreatitis, it may be visualized as a poorly enhancing hypodense lesion between the pancreatic head and the duodenum, near the minor papilla, reflecting the pathological characteristics of the mass. The delayed enhancement is mainly

due to delayed blood circulation caused by fibrous tissue proliferation and artery constriction^[12]. In addition, CT may reveal the presence of duodenal stenosis with wall thickening and cystic lesions in the duodenal wall or in the groove area. The cysts may be tiny even if multilocular cystic lesions may be observed. The main pancreatic duct may be mildly dilated above the lesion while, in the pure form, paraduodenal pancreatitis can be expected.

Table 7 Epidemiological and clinical characteristics of patients in the 38 case report papers involving 46 subjects with a benign cystic duodenal wall (a paper may report more than one patient), the three cases reported in the present paper are also shown (continues from Table 6)

Author ^[Ref.] yr	Imaging	Duodenal findings	CBD stenosis	Duodenal stenosis
Bill <i>et al</i> ^[74] 1982	US, ERCP, angiography	NR	Yes	No
Holstege <i>et al</i> ^[75] 1985	US, CT, ERCP	Severe erosive gastritis + bulging of the duodenum	No	Yes
Tio <i>et al</i> ^[9] 1991	ERCP, EUS, US	Polypoid lesion	Yes	Yes
Tio <i>et al</i> ^[9] 1991	ERCP, EUS, US	NR	No	Yes
Flaherty <i>et al</i> ^[76] 1992	US	No	No	No
Izbicki <i>et al</i> ^[77] 1994	US, angiography, ERCP	NR	Yes	Yes
Fujita <i>et al</i> ^[14] 1997	US, CT, ERCP	Inflammation	No	Yes
Shudo <i>et al</i> ^[17] 1998	CT, US, ERCP, EUS, celiac angiography	Edema duodenal wall. Brunner hyperplasia	No	Yes
Wu <i>et al</i> ^[78] 1998	CT	NR	No	No
Babál <i>et al</i> ^[79] 1998	NR	NR	No	No
Rubay <i>et al</i> ^[21] 1999	CT, ERCP, MRI, EUS	No alterations	NR	Yes
Balachandar <i>et al</i> ^[22] 1999	CT, ERCP	No duodenal alteration	Yes	No
Mohl <i>et al</i> ^[25] 2001	CT	Stenosis	No	Yes
Mohl <i>et al</i> ^[25] 2001	US, CT, ERCP	Normal duodenal mucosa	No	No
Munthali Lovemore <i>et al</i> ^[26] 2001	US, CT, ERCP	NR	Yes	No
Indinnimeo <i>et al</i> ^[27] 2001	CT, MRI, EUS	No alterations	No	No
Shudo <i>et al</i> ^[28] 2002	CT, US, ERCP, EUS, celiac angiography	Irregular polypoid bulging; inflammation of the mucosa	NR	Yes
Glaser <i>et al</i> ^[29] 2002	US	Severe deformation + inflammatory changes	No	Yes
Hwang <i>et al</i> ^[31] 2003	US, CT, MRI	Duodenal inflammation, duodenal stenosis	NR	Yes
Jovanovic <i>et al</i> ^[32] 2004	US, CT, EUS, MRI	Stenosis	No	Yes
McFaul <i>et al</i> ^[80] 2004	US, CT, MRI	Brunner hyperplasia	Yes	Yes
McFaul <i>et al</i> ^[80] 2004	US, PET-CT	Brunner hyperplasia	No	No
Isayama <i>et al</i> ^[33] 2005	CT, EUS, MRCP, ERCP	NR	No	Yes
Chatelain <i>et al</i> ^[34] 2005	EUS, CT	Duodenal stenosis, inflammation	No	Yes
Chatelain <i>et al</i> ^[34] 2005	EUS, CT	Duodenal stenosis	No	Yes
Balzan <i>et al</i> ^[35] 2005	US, MRI, CT	NR	NR	NR
Sanada <i>et al</i> ^[43] 2007	CT, ERCP	Edema duodenal wall. Brunner hyperplasia	Yes	No
Balakrishnan <i>et al</i> ^[44] 2007	CT, ERCP, EUS	Edematous, shiny, reddish raise mucosa with polypoid appearance; Brunner hyperplasia	No	No
de Tejada <i>et al</i> ^[50] 2008	MRI, EUS	Bulging, Brunner hyperplasia	No	No
Stefanescu <i>et al</i> ^[51] 2008	CT, EUS	NR	No	Yes
Varma <i>et al</i> ^[53] 2008	US, CT	Brunner hyperplasia	No	No
Galloro <i>et al</i> ^[54] 2008	US, CT, EUS	Duodenal stenosis	No	Yes
Thomas <i>et al</i> ^[59] 2009	US, CT, EUS, octreotide scan	Brunner hyperplasia	No	Yes
Levenick <i>et al</i> ^[61] 2009	EUS, MRCP	Duodenal stenosis	No	Yes
Levenick <i>et al</i> ^[61] 2009	CT, EUS	Duodenal inflammation, duodenal stenosis	NR	Yes
Levenick <i>et al</i> ^[61] 2009	CT, EUS	Edema with acute and chronic inflammation	No	Yes
Levenick <i>et al</i> ^[61] 2009	CT, EUS, ERCP	NR	Yes	No
Yoshida <i>et al</i> ^[62] 2009	CT, MRCP	Normal mucosa	No	Yes
Meesiri ^[64] 2009	US, CT, MRI	Edema and hemorrhagic mucosa with inflammation	NR	No
Funamizu <i>et al</i> ^[65] 2009	ERCP, CT, angiography	NR	Yes	No
Viñolo Ubiña <i>et al</i> ^[66] 2010	CT	Stenosis	NR	Yes
Tezuka <i>et al</i> ^[67] 2010	CT, ERCP	Edema duodenal wall	No	Yes
Lee <i>et al</i> ^[69] 2010	CT, MRCP	Active ulcer	Yes	No
Egorov <i>et al</i> ^[70] 2010	US, CT, EUS	Deformation, infiltration and ulcer; Inflammation	No	Yes
Egorov <i>et al</i> ^[70] 2010	US, CT, MRI, EUS	NR	Yes	Yes
German <i>et al</i> ^[72] 2010	US, CT, MRI	Edema duodenal wall; Brunner hyperplasia	Yes	Yes
Pezzilli 2011 Present paper	US, CT, EUS, ERCP	No	Yes	No
Pezzilli 2011 Present paper	US, CT, MRI	Hypertrophy of the Brunner glands	No	No
Pezzilli 2011 Present paper	US, CT	No	Yes	No
Overall	-	-	14/42 (33.30%)	28/48 (58.30%)

CBD: Common bile duct; NR: Not reported; US: Transabdominal ultrasonography; CT: Computer tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography; PET-CT: Positron emission tomography with associated computer tomography.

Table 8 Epidemiological and clinical characteristics of patients in the 38 case report papers involving 46 subjects with a benign cystic duodenal wall (a paper may report more than one patient), The three cases reported in the present paper are also shown (continues from Table 7)

Author ^[Ref.] yr	Surgery	Type of surgery	Endoscopic treatment	Associated chronic pancreatitis	Associated neoplasms	Pseudocyst	Follow-up	Death
Bill <i>et al</i> ^[74] 1982	Yes	PD	No	NR	No	No	No	NR
Holstege <i>et al</i> ^[75] 1985	Yes	WP	No	No	No	No	No	NR
Tio <i>et al</i> ^[9] 1991	No		No	No	No	No	7 yr	No
Tio <i>et al</i> ^[9] 1991	Yes	Derivative surgery	No	No	No	No	6 mo	NR
Flaherty <i>et al</i> ^[75] 1992	Yes	PD	No	No	No	No	9 mo	No
Izbicki <i>et al</i> ^[77] 1994	Yes	PD	No	No	No	No	6 yr	No
Fujita <i>et al</i> ^[14] 1997	Yes	PD	No	No	No	No	3 yr	No
Shudo <i>et al</i> ^[17] 1998	Yes	PD	No	No	No	No	NR	NR
Wu <i>et al</i> ^[78] 1998	Yes	WP	No	No	No	No	9 mo	No
Babál <i>et al</i> ^[79] 1998	No		No	No	No	No	NR	During hospitalization
Rubay <i>et al</i> ^[21] 1999	Yes	PD	No	No	No	No	2 mo	No
Balachandar <i>et al</i> ^[22] 1999	Yes	Derivative	No	Yes	No	No	NR	NR
Mohl <i>et al</i> ^[25] 2001	Yes	PD	No	No	No	No	No	NR
Mohl <i>et al</i> ^[25] 2001	Yes	PD	No	No	No	No	4 wk after surgery	No
Munthali Lovemore <i>et al</i> ^[26] 2001	Yes	Derivative CBD	No	No	No	No	No	NR
Indinnimeo <i>et al</i> ^[27] 2001	Yes	PD	No	No	No	No	2 yr	No
Shudo <i>et al</i> ^[28] 2002	Yes	PD	No	No	No	No	NR	NR
Glaser <i>et al</i> ^[29] 2002	No		No	No	No	No	No	NR
Hwang <i>et al</i> ^[31] 2003	No		No	No	No	No	NR	NR
Jovanovic <i>et al</i> ^[32] 2004	Yes	PD	No	No	No	No	No	NR
McFaul <i>et al</i> ^[80] 2004	Yes	PD	No	Yes	No	No	2 yr	No
McFaul <i>et al</i> ^[80] 2004	Yes	WP	No	Yes	No	No	NR	No
Isayama <i>et al</i> ^[33] 2005	No		Yes	No	No	No	12 mo	No
Chatelain <i>et al</i> ^[34] 2005	Yes	PD	No	No	No	No	6 mo	No
Chatelain <i>et al</i> ^[34] 2005	Yes	PD	No	No	No	No	12 mo	No
Balzan <i>et al</i> ^[35] 2005	Yes	PD	No	Yes	No	Yes (head)	No	NR
Sanada <i>et al</i> ^[43] 2007	Yes	PD	No	No	No	Yes (head)	No	NR
Balakrishnan <i>et al</i> ^[44] 2007	Yes	Laparotomy	No	Yes	No	No	NR	NR
de Tejada <i>et al</i> ^[50] 2008	Yes	WP	No	No	No	No	3 mo	No
Stefanescu <i>et al</i> ^[51] 2008	Yes	Derivative	No	No	No	No	8 mo	No
Varma <i>et al</i> ^[53] 2008	Yes	WP	No	No	No	No	9 mo	No
Galloro <i>et al</i> ^[54] 2008	Yes	WP	No	Yes (with calcifications)	Cystadenoma	Yes	14 mo	No
Thomas <i>et al</i> ^[59] 2009	Yes	PD	No	No	No	No	NR	NR
Levenick <i>et al</i> ^[61] 2009	Yes	PD	No	No	No	No	3 yr	No
Levenick <i>et al</i> ^[61] 2009	Yes	PD	No	Yes	No	No	NR	NR
Levenick <i>et al</i> ^[61] 2009	Yes	PD	No	No	No	No	NR	NR
Levenick <i>et al</i> ^[61] 2009	Yes	PD	No	No	No	No	NR	NR
Yoshida <i>et al</i> ^[62] 2009	Yes	PD	No	No	No	No	Yes (time NR)	No
Meesiri ^[64] 2009	No		No	No	No	No	Yes (time NR)	No
Funamizu <i>et al</i> ^[65] 2009	Yes	PD	No	No	Yes	No	15 mo	No
Viñolo Ubiña <i>et al</i> ^[66] 2010	Yes	PD	No	No	No	No	NR	No
Tezuka <i>et al</i> ^[67] 2010	Yes	PD	No	No	No	No	NR	No
Lee <i>et al</i> ^[69] 2010	No		No	No	No	No	NR	NR
Egorov <i>et al</i> ^[70] 2010	Yes	Pancreas-preserving duodenal resection	No	No	No	No	6 mo	No
Egorov <i>et al</i> ^[70] 2010	Yes	Pancreas-preserving duodenal resection	No	No	No	No	5 mo	No
German <i>et al</i> ^[72] 2010	Yes	PD	No	No	No	No	2 mo	NR
Pezzilli 2011 Present paper	No		Yes	No	No	No	20 mo	No
Pezzilli 2011 Present paper	Yes	PD	No	No	No	No	7 mo	No
Pezzilli 2011 Present paper	Yes	PD	No	Autoimmune pancreatitis	Yes	No	4 mo	No
Overall	41/49 (83.70%)	-	2/49 (4.10%)	8/48 (16.70%)	3/49 (6.10%)	3/49 (6.10%)	17.9 ± 20.6 mo	1/28 (3.60%)

CBD: Common bile duct; NR: Not reported; PD: Pancreaticoduodenectomy; WP: Whipple procedure.

In groove pancreatitis and in groove carcinoma, the CBD may be stenosed in its distal part and a dilation of the extra- and intra-hepatic biliary system can be observed^[6,16].

The same CT findings can also be observed when utilizing MRI which may reveal a mass between the head of the pancreas and the duodenum associated with duodenal wall thickening. The mass visualized in the groove

Table 9 Epidemiological and clinical characteristics of patients in the two retrospective studies and two case report papers involving two subjects with groove adenocarcinoma

Author ^[Ref.] yr	Type of study	Time interval of patient enrollment	No. of patients			Age (yr) Mean (range)	Alcohol drinkers
			Total	Males	Females		
Suehara <i>et al</i> ^[20] 1998	Case report	1995	1	1	-	61	Yes
Gabata <i>et al</i> ^[30] 2003	Retrospective	1998-2001	9	4 (44.4%)	5 (55.6%)	72 (56-87)	NR
Tan <i>et al</i> ^[38] 2006	Case report	NR	1	-	1	69	NR
Ishigami <i>et al</i> ^[6] 2010	Retrospective	2001-2008	7	6 (85.7%)	1 (14.3%)	70 (57-80)	NR

NR: Not reported.

Table 10 Epidemiological and clinical characteristics of patients in the two retrospective studies and two case report papers involving two subjects with groove adenocarcinoma (continues from Table 9)

Author ^[Ref.] yr	Abdominal pain	Weight loss	Jaundice	Hyperamylasemia
Suehara <i>et al</i> ^[20] 1998	Yes (Persistent)	No	Yes	Yes
Gabata <i>et al</i> ^[30] 2003	NR	NR	NR	NR
Tan <i>et al</i> ^[38] 2006	Yes (Persistent)	Yes	Yes	Yes
Ishigami <i>et al</i> ^[6] 2010	NR	NR	NR	NR

NR: Not reported.

Table 11 Epidemiological and clinical characteristics of patients in the two retrospective studies and two case report papers involving two subjects with groove adenocarcinoma (continues from Table 10)

Author ^[Ref.] yr	Imaging	Duodenal findings	CBD stenosis	Duodenal stenosis
Suehara <i>et al</i> ^[20] 1998	US, EUS, CT, MRI, angiography	NR	Yes	No
Gabata <i>et al</i> ^[30] 2003	CT, RMI, ERCP, angiography	Edema with erosions	9 (100%)	9 (100%)
Tan <i>et al</i> ^[38] 2006	US, MRI, ERCP	NR	Yes	No
Ishigami <i>et al</i> ^[6] 2010	CT, MRI	NR	7 (100%)	NR

CBD: Common bile duct; NR: Not reported; US: Transabdominal ultrasonography; CT: Computer tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography.

Table 12 Epidemiological and clinical characteristics of patients in the two retrospective studies and two case report papers involving two subjects with groove adenocarcinoma (continues from Table 11)

Author ^[Ref.] yr	Surgery		Associated chronic pancreatitis	Pseudocyst	Follow-up
	No. of cases	Type			
Suehara <i>et al</i> ^[20] 1998	Yes	PD	No	No	NR
Gabata <i>et al</i> ^[30] 2003	9 (100%)	PD in 7; derivative in 2	No	No	NR
Tan <i>et al</i> ^[38] 2006	Yes	By-pass surgery	No	No	NR
Ishigami <i>et al</i> ^[6] 2010	6 (85.7%)	PD in 5; derivative in 1	No	NR	NR

NR: Not reported; PD: Pancreaticoduodenectomy.

and/or in the adjacent head of the gland is hypointense

to the pancreatic parenchyma. Delayed enhancement may also be seen in the thickened duodenal wall. These imaging features reflect the fibrous involvement of the lesions of groove pancreatitis. Cysts, which may be present in the groove area and the duodenal wall, have high signal intensity. An important diagnostic aspect of MRI, which cannot be evaluated by CT, is the fact that MRI can be followed by magnetic resonance cholangiopancreatography (MRCP); this additional evaluation provides images similar to those of ERCP without the morbidity of this latter technique. In addition, MRCP may visualize those lesions which are not seen in ERCP in the case of serrated duodenal stenosis^[18]. The diagnostic value of MRI is superior to CT in evaluating biliary ducts in paraduodenal pancreatitis as well as in groove carcinomas. The stricture, or narrowing of the CBD, may be better approached by using MRCP rather than CT and/or ERCP. The dilation of the space comprising the main pancreatic duct, the CBD and the duodenum is another sign which can be observed in patients with groove pancreatitis or groove carcinoma when using MRCP^[47].

In the last few years, EUS has emerged as a useful technique for diagnosing pancreatic diseases because of the accurate evaluation of the biliopancreatic structures through the gastro-duodenal lumen without interference of the abdominal wall or other organs^[82]. EUS can easily demonstrate the hypoechoic area between the duodenal wall and the pancreatic parenchyma, narrowing of the duodenal lumen and stenosis of the CBD and/or pancreatic duct in both groove pancreatitis and groove carcinomas^[50]. Furthermore, the diagnosis can be confirmed by EUS-guided fine-needle aspiration of the mass visualized.

PATHOLOGY

Macroscopically, groove pancreatitis is associated with an absent or narrow Santorini duct or the presence of pancreas divisum^[17], and the difficult outflow of pancreatic fluid may be hypothesized for lesions of the groove similar to those of chronic pancreatitis^[17]. The duodenal wall contains dilated ducts, in the majority of cases with thickened secretions, pseudocystic changes as well as adjacent stromal reactions, foreign-body type giant cell reaction engulfing mucoprotein material and myofibroblastic proliferation. Brunner gland hyperplasia is usually present as is dense myoid stromal proliferation, with intervening rounded lobules of pancreatic acinar

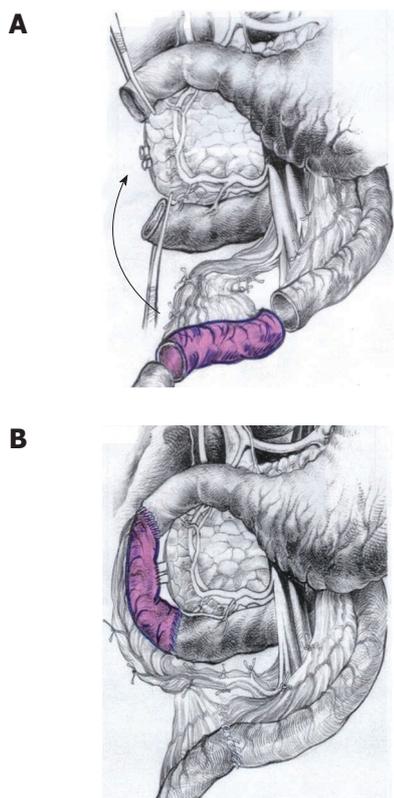


Figure 8 New surgical option for patients having cystic dystrophy of duodenal wall. A: Scheme of the pancreas-preserving resection of the second portion of the duodenum. The second part of the duodenum, including the main papilla, is removed and the segment of the proximal jejunum supplied by the artery and vein is cut out and prepared for transposition between the 1st and 3rd portions of the duodenum; B: The shifted segment is interposed between the 1st and the 3rd parts of the duodenum. Jejunum-jejunum- and duodeno-jejunum-anastomoses are performed. The bile and the pancreatic ducts were implanted in the neoduodenum 4 cm below the proximal duodeno-jejunum-anastomosis (from Egorov *et al*^[70] with the kind permission of the authors).

tissue. Fibrosis into the adjacent pancreas and soft tissue occurs, especially in the groove area which involves the CBD^[5].

In groove carcinoma, the macroscopic pathology is similar to that of groove pancreatitis while the pancreatic tissue has the same histology as that of pancreatic adenocarcinoma^[6,20,30,38].

TREATMENT

Conservative treatment is the main option in the acute phase of the disease, including analgesia and parenteral nutrition. In some patients, enteral nutrition is not always possible due to the presence of duodenal stenosis^[17]. The main therapeutic option for these patients is a surgical approach in benign as well as in malignant diseases of the groove, as shown in Tables 1-12. **The most frequent** surgical approach is a pylorus-preserving pancreaticoduodenectomy or a Whipple procedure; in a limited number of patients, a gastrointestinal by-pass, with or without biliary by-pass, has been carried out. More recently, a new approach has been reported by Egorov *et al*^[70];

these authors have described a new surgical approach carried out on two patients who were successfully treated by two modifications of a pancreas-preserving duodenal resection with reimplantation of the bile and pancreatic ducts into the neoduodenum (Figure 8). The authors have claimed that these two cases are a good example of a pancreas-preserving approach to duodenal dystrophy treatment and that the technique may be an alternative to the Whipple procedure in cases of mild changes of the orthotopic gland.

Only in a few cases was a medical approach carried out (see Case 1 of our three patients), mainly because the patients refused surgery, and also in one patient in whom successful treatment for groove pancreatitis was carried out by endoscopic drainage *via* the minor papilla^[33].

THE FATE OF PATIENTS

The first important question arising from the studies analyzed is the extreme length of time necessary from the onset of the symptoms to reach a diagnosis in patients with groove pancreatitis: it varies from a few days to ten years (Tables 1-8). **In one of the larger studies in this field**, such as that of Rebours *et al*^[40], the mean time from the appearance of the symptoms and the diagnosis is 1 year with a range of 0 to 24 mo. This long time period is similar to that previously reported in chronic pancreatitis^[83]. In patients with groove adenocarcinoma, we have no information on time to diagnosis. The perioperative mortality rate seems to be negligible, the only death being reported by Babál *et al*^[79]. In the only study reporting this information (Tables 1-8), **the mortality rate was 8.9%** (9/101) in the follow-up period in patients with benign disease. However, this information should be taken with caution because, as previously stated, the follow-up period is not quite as long in the majority of studies.

CONCLUSION

The diagnosis of cystic dystrophy of the duodenal wall can be easily assessed by MDCT, MRI and EUS. These latter two techniques may also add more information on the involvement of the part of the pancreatic gland not involved in the duodenal malformation.

Chronic pancreatitis involving the entire pancreatic gland is present in half the patients with cystic dystrophy of the duodenal wall, and the pancreatitis has calcifications in the majority of them. We have no information about exocrine function in these patients and this topic requires additional study. In subjects without pancreatitis, the patients with cystic dystrophy of the duodenal wall are usually in satisfactory general condition after surgical treatment and they regain weight after surgery.

The fact that only two children have been reported to have cystic dystrophy of the duodenal wall confirms the hypothesis that pancreatic and biliary diseases develop over a long period of time.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Paolo Bassi of the Surgical Department, Dr. Maurizio Zani and Dr. Maurizio Iorio of the University of Bologna Clinical Library at Sant'Orsola-Malpighi Hospital for their technical assistance.

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S- Editor Tian L L- Editor O'Neill M E- Editor Zhang DN

Extracorporeal shock wave lithotripsy for pancreatic and large common bile duct stones

Manu Tandan, D Nageshwar Reddy

Manu Tandan, D Nageshwar Reddy, Asian Institute of Gastroenterology, 6-3-652, Somajiguda, Hyderabad 500 082, India
Author contributions: Manu T and Reddy DN contributed equally to this work, both being involved in drafting and final revision of the article.

Correspondence to: Dr. D Nageshwar Reddy, Chairman, Asian Institute of Gastroenterology, 6-3-661 Somajiguda, Hyderabad 500 082, India. aigindia@yahoo.co.in

Telephone: +91-40-23378888 Fax: +91-40-23324255

Received: January 11, 2011 Revised: April 11, 2011

Accepted: April 18, 2011

Published online: October 21, 2011

Abstract

Extraction of large pancreatic and common bile duct (CBD) calculi has always challenged the therapeutic endoscopist. Extracorporeal shockwave lithotripsy (ESWL) is an excellent tool for patients with large pancreatic and CBD calculi that are not amenable to routine endotherapy. Pancreatic calculi in the head and body are targeted by ESWL, with an aim to fragment them to < 3 mm diameter so that they can be extracted by subsequent endoscopic retrograde cholangiopancreatography (ERCP). In our experience, complete clearance of the pancreatic duct was achieved in 76% and partial clearance in 17% of 1006 patients. Short-term pain relief with reduction in the number of analgesics ingested was seen in 84% of these patients. For large CBD calculi, a nasobiliary tube is placed to help target the calculi, as well as bathe the calculi in saline - a simple maneuver which helps to facilitate fragmentation. The aim is to fragment calculi to < 5 mm size and clear the same during ERCP. Complete clearance of the CBD was achieved in 84.4% of and partial clearance in 12.3% of 283 patients. More than 90% of the patients with pancreatic and biliary calculi needed three or fewer sessions of ESWL with 5000 shocks being delivered at each session. The use of epidural anesthesia helped in reducing patient movement. This, together with the better focus achieved with newer third-gen-

eration lithotripters, prevents collateral tissue damage and minimizes the complications. Complications in our experience with nearly 1300 patients were minimal, and no extension of hospital stay was required. Similar rates of clearance of pancreatic and biliary calculi with minimal adverse effects have been reported from the centers where ESWL is performed regularly. In view of its high efficiency, non-invasive nature and low complication rates, ESWL can be offered as the first-line therapy for selected patients with large pancreatic and CBD calculi.

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Key words: Pancreatic calculi; Extracorporeal shock-wave lithotripsy; Common bile duct calculi

Peer reviewer: Pete Muscarella, MD, Division of Gastrointestinal Surgery, The Ohio State University, N711 Doan Hall, 410 W. 10th Ave, Columbus, OH 43210, United States

Tandan M, Reddy DN. Extracorporeal shock wave lithotripsy for pancreatic and large common bile duct stones. *World J Gastroenterol* 2011; 17(39): 4365-4371 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4365.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4365>

INTRODUCTION

Extracorporeal shock wave lithotripsy (ESWL) was first introduced in the 1980s for the fragmentation of renal and ureteric calculi^[1]. Its application was quickly extended to include large biliary and pancreatic calculi. Over the past three decades, it has been utilized at many centers worldwide for fragmentation of biliary and pancreatic calculi that are not amenable to routine endotherapy^[2-16]. In this review, we briefly highlight the principles of ESWL as well as its place in therapy of biliary and pancreatic calculi.

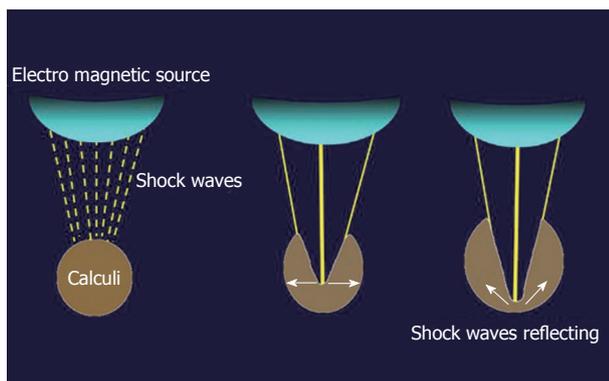


Figure 1 Principle of extracorporeal shockwave lithotripsy. Shockwaves from the source are targeted on the calculi and these induce fragmentation.

PRINCIPLES OF ESWL

ESWL is based on the principle of shock wave energy. Whenever energy is abruptly released in an enclosed space, shock waves are generated. The passage of these shock waves through substances of different acoustic impedance generates compressive stress on the boundary surface. This stress eventually overcomes the tensile strength of the object (in the present case, biliary and pancreatic calculi) and the anterior surface of the calculi crumbles as a result. The shock waves cross to the posterior surface of the calculi and some of them are reflected back and cause further fragmentation^[7] (Figure 1). Modern lithotripsy machines consist of the following basic components.

Shock wave generator

The earlier generation lithotripter utilized electrohydraulic energy or piezoelectric crystals for shock wave generation. The newer third-generation lithotripter utilizes the principle of electromagnetic shock wave generation from an electromagnetic coil. These shock waves are focused on a target (calculi) using an acoustic lens or cylindrical reflector.

Focusing system

Shock waves are focused to the focal point or target in the body. This focal path is conical in shape and all the waves are concentrated at the apex of the cone, which is called the focal point. During ESWL, the focal point targets the calculi. Targeted focusing reduces collateral tissue damage and minimizes the complications.

Localization

Localization of the calculi is basically done by fluoroscopy or ultrasound. All the newer lithotripters are equipped with both these facilities.

Coupling device

The generated shockwaves are transmitted *via* a coupling device, to the skin surface and then through the body tissue to the calculi. The earlier lithotripters used a “water

bath” for this purpose. The newer machines use a small water-filled cushion covered with a silicone membrane to transmit the shock waves to the patient’s skin.

ESWL FOR PANCREATIC CALCULI

Chronic calcific pancreatitis (CCP) is a disease of varied etiology that is associated with the development of pancreatic ductal calculi, which result in upstream hypertension, increased parenchymal pressure, and ischemia. Pain is the dominant feature of both alcoholic and non-alcoholic CCP. Decompression of the duct by clearing the stones leads to relief of pain in many patients. Small pancreatic duct (PD) stones can be extracted by the routine technique of endoscopic pancreatic sphincterotomy and basketing. Stones > 5 mm in diameter are often impacted in the main pancreatic duct and require fragmentation to facilitate their expulsion^[15]. ESWL has been successfully used at many centers for fragmentation of large PD calculi followed by spontaneous or endoscopic clearance with resultant relief in pain^[7-15].

Indications and contraindications

ESWL is indicated in all patients of CCP with large PD calculi (> 5 mm) that are not amenable to routine endotherapy - where pain is the predominant symptom. The aim is to break the calculi to fragments of ≤ 3 mm, so that they can be removed by subsequent endoscopic retrograde cholangiopancreatography (ERCP). Calculi in the head and body are targeted during ESWL.

ESWL is not indicated in patients with extensive calculi in the head, body and tail of the pancreas, or in patients with isolated calculi in the tail area because of increased chance of collateral damage to the spleen are high. Patients with multiple stricture, head mass, pancreatic ascites or pseudocysts are not treated by ESWL. Cholangitis or coagulopathy due to biliary stricture are treated before subjecting the patient to ESWL.

Procedure protocol

The protocol followed for ESWL of pancreatic calculi at our institute is depicted in Figure 2.

A third-generation electromagnetic lithotripter (Delta Compact; Dornier Med Tech, Weissling, Germany.) (Figure 3) is used to deliver a maximum of 5000 shocks are delivered per session. Repeat sessions are carried out on successive days until the stone fragments are < 3 mm in diameter. An intensity of 5-6 (15 000-16 000 kV) on a scale of 1-6 with a frequency of 90 shocks per minute is used for fragmentation^[7,8].

A few centers have advocated ESWL alone without any subsequent endotherapy for large PD calculi, stating that good fragmentation is followed by spontaneous expulsion of the fragments^[18,19]. At our center, ERCP, pancreatic sphincterotomy and pancreatic ductal clearance is always performed after ESWL because of the dense nature of the calculi present in patients with idiopathic chronic pancreatitis^[7]. Pancreatic sphincterotomy prior to

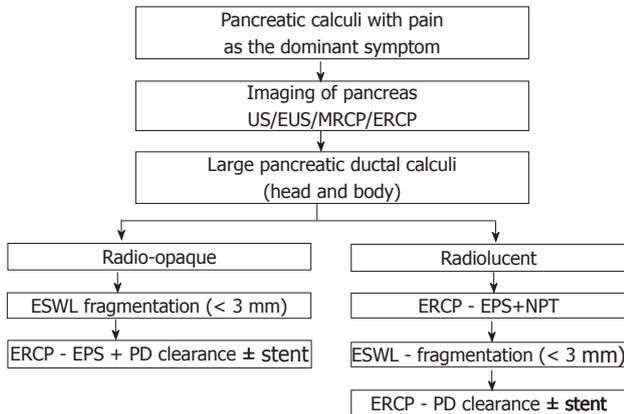


Figure 2 Protocol followed at Asian Institute of Gastroenterology, for extracorporeal shockwave lithotripsy of large pancreatic duct calculi^[7]. EPS: Endoscopic pancreatic sphincterotomy; US: Ultrasound; EUS: Endoscopic ultrasound; MRCP: Magnetic resonance cholangiopancreatography; ERCP: Endoscopic retrograde cholangiopancreatography; PD: Pancreatic duct; ESWL: Extracorporeal shock wave lithotripsy; NPT: Naso-pancreatic tube.

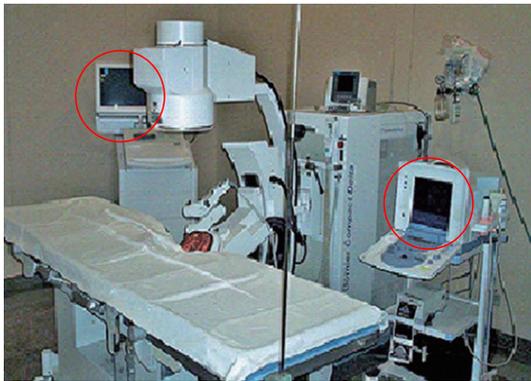


Figure 3 Third-generation lithotripter with fluoroscopic and ultrasound imaging facility.

ESWL is technically challenging because these dense calculi tend to obstruct the pancreatic duct completely and prevent deep cannulation. In our study, the majority of ESWL procedures were carried out under epidural anesthesia (EA)^[20]. However, general anesthesia or total intravenous analgesia has also been used for this procedure.

Efficacy and pain relief

Over 95% of our patients require three sessions or fewer of ESWL for adequate fragmentation. In 76% of patients, the PD cleared completely, in 17% partially, and there was clearance failure in the remaining 7%^[7] (Table 1 and Figures 4 and 5). Similar results for clearance of the PD, ranging from 37% to 100% have been reported earlier^[9]. Comparative efficacy of ESWL for pancreatic calculi is shown in Table 2. Short-term pain relief was seen in 84% of our patients. This experience is also similar to others who have reported short-term pain in 82%-94% of the patients^[9,10,21-23]. The results of a large meta-analysis have indicated that ESWL has a significant impact on improvement of pain. The mechanism of pain relief is due to decompression of the main PD following clearance of the

Table 1 Details of extracorporeal shockwave lithotripsy in 1006 patients treated at Asian Institute of Gastroenterology

No. of sessions	n (%)	Shock waves (n)	
		Mean	Range
1	292 (29)	4450	(4250-4900)
2	370 (37)	9270	(8800-9940)
3	300 (30)	13 250	(11 800-14 700)
4	32 (3)	18 900	(18 100-19 400)
≥ 5	12 (1)	23 550	(22 100-27 750)
Clearance			
Complete cleared	762 (76)		
Partially cleared	173 (17)		
Failed clearance	71 (7)		

Table 2 Efficacy of extracorporeal shockwave lithotripsy for pancreatic calculi

Author	No. of patients	Complete clearance (%)	Pain relief (%)	Follow-up (mo)
Delhaye <i>et al</i> ^[10]	123	59	85	14
Costamagna <i>et al</i> ^[12]	35	74	72	27
Kozarek <i>et al</i> ^[14]	40	-	80	30
Farnbacher <i>et al</i> ^[27]	125	64	48	29
Dumona ceau <i>et al</i> ^[20]	29	-	55	51
Adamek <i>et al</i> ^[16]	80	-	76	40
Tandan <i>et al</i> ^[7]	1006	76	84	6

Table 3 Pain relief and analgesic use, pre- and post-extracorporeal shockwave lithotripsy^[7]

	Post-ESWL (n = 846)		Pre-ESWL (n = 711)		
Pain relief			0	326	
VAS (Scale 0-10)	6/10	212	1/10	161	
	7/10	320	2/10	96	P < 0.001
	8/10	204	3/10	85	
	9/10	110	4/10	43	
Analgesic use	0	-	0	326	
Doses/mo	1-5	48	1-5	258	
	6-10	190	6-10	127	P < 0.001
	11-15	385			
	> 15	223			

VAS: Visual analog score; ESWL: Extracorporeal shockwave lithotripsy.

obstruction^[9]. Pain relief was reflected by decreased use of analgesics and of number of hospitalizations during the follow-up period^[7,14] (Table 3). Failure of pain relief, despite adequate clearance could be because of multiple mechanisms of pain in patients of chronic pancreatitis. These include pancreatic inflammation, ischemia, and associated lesions such as duodenal and biliary strictures. Surgery is often considered as the gold standard in the management of chronic pancreatitis. However, even after duodenum-preserving resection of the head of the pancreas, which is considered to be the best surgical approach for chronic pancreatitis, almost 25% of patients experience recurrence of pain^[21]. In addition, there is considerable procedure-related morbidity and mortality. ESWL followed by PD clearance on ERCP is therefore increasingly used in the management of CCP, with results that

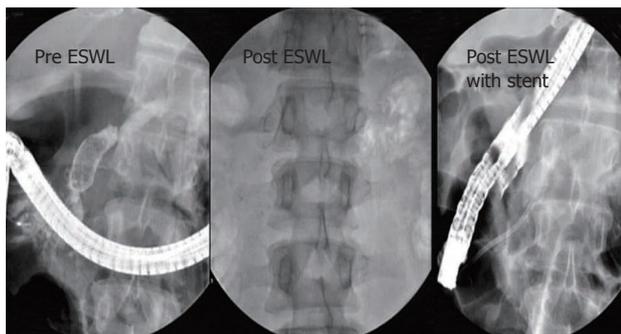


Figure 4 Large pancreatic calculi in head and genu, cleared by extracorporeal shockwave lithotripsy followed by pancreatic stenting. ESWL: Extracorporeal shockwave lithotripsy.

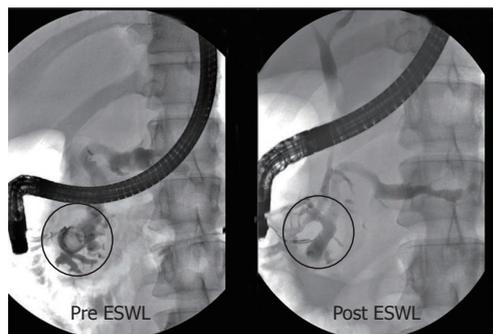


Figure 5 Large pancreatic calculi in head. Post extracorporeal shockwave lithotripsy (ESWL) reduction in diameter of main pancreatic duct.

are comparable to surgery^[10-12,19].

Limitations of ESWL in CCP

Long-term follow-up is necessary to evaluate the role of ESWL in PD clearance of patients with CCP. Data on this issue are conflicting. Although no benefit on glandular function and pain has been reported by some workers^[16,24], others have shown a definite improvement in exocrine and endocrine functions^[23]. We feel that more long-term follow-up studies are required to define the role of ESWL in pain and exocrine or endocrine dysfunction, as well as the possibility of carcinoma development, which are all potential sequelae of CCP. This is especially true in tropical regions and in patients with idiopathic chronic pancreatitis in whom the disease begins at a young age.

Although the numbers are small, failure of complete fragmentation has been reported at most centers. It would be ideal to identify this set of patients, so that they can be subjected to surgery directly. Yet another limitation is the failure to prevent recurrence following ESWL, which has been reported in 22%-35% of patients^[22,24].

In conclusion, ESWL is a good technique for extraction of large PD calculi in patients with CCP and offers good pain relief. It is conceivable that ESWL done at a young age, followed by intensive medical therapy could alter the course of the patients with CCP, besides obviating the need for surgery^[8].

ESWL FOR LARGE COMMON BILE DUCT STONES

Conventional therapy for common bile duct (CBD) stones involves endoscopic sphincterotomy and extraction by balloon catheter or Dormia basket. Between 80% and 90% of CBD stones can be extracted using these techniques^[4,5,25]. The rest are categorized as difficult CBD stones and include large stones (> 15 mm diameter), impacted stones in patients with narrow distal CBD and/or difficult anatomy. Large stones can either be fragmented or the CBD passage dilated to facilitate extraction. Fragmentation of large CBD stones can be carried out by me-

chanical lithotripsy, electro hydraulic lithotripsy (EHL)^[26], intraductal laser lithotripsy (ILL)^[27], spy scope and holmium laser^[28], and ESWL. The narrow distal CBD can be subjected to balloon dilatation to facilitate extraction of large calculi^[29-31]. Sauerbruch and colleagues first demonstrated the efficacy in achieving CBD stone disintegration successfully and with minimal side effects^[8].

Indications and contraindications

ESWL is indicated all patients with large CBD calculi that are not extractable by routine techniques of sphincterotomy followed by basket or balloon tawl. It is especially useful for patients with post-cholecystectomy retained stones, isolated or primary CBD stones, and in those who refuse or are unfit for surgery.

Acute cholangitis and coagulopathy are relative contraindications and ESWL can be performed once these conditions are treated.

Procedure protocol

The majority of CBD calculi are radiolucent. An initial ERCP is performed and a nasobiliary tube (NBT) is placed in the CBD. This is used to opacify the calculi for targeting and fragmentation. It is also used to bathe the stones in saline - a simple technique that aids fragmentation. ESWL is carried out at an intensity of 4 (in a scale of 1-6) corresponding to 11 000-16 000 kV) at a rate of 90 shocks/min. A maximum of 5000 shocks are given per session. The aim is to break the calculi into fragments < 5 mm in diameter for extraction by subsequent ERCP. Stenting is done if clearance is partial or an associated stricture is present. The protocol followed at our institute is shown in Figure 6.

Efficacy

In our experience, complete clearance was achieved in 84.4%, partial in 12.3%, and failure in 3.1% of patients^[3] (Figure 7). ESWL was useful in clearing intrahepatic calculi also. Similar successful clearance has been reported at other centers (Table 4). Over 75% of our patients needed ≤ 3 less sessions of ESWL. Clearance of the CBD in patients with post-cholecystectomy calculi or primary CBD calculi by ESWL and subsequent ERCP

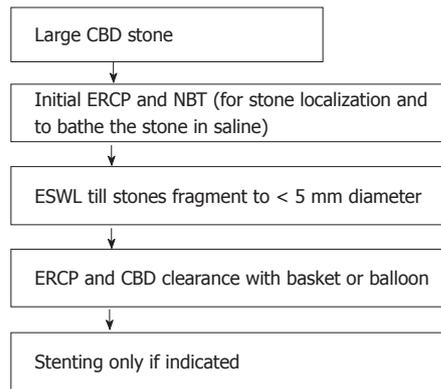


Figure 6 Protocol for extracorporeal shockwave lithotripsy of large common bile duct calculi. CBD: Common bile duct; ERCP: Endoscopic retrograde cholangiopancreatography; NBT: Nasobiliary tube; ESWL: Extracorporeal shockwave lithotripsy.

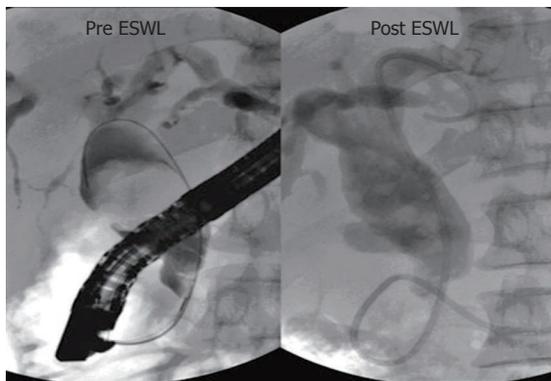


Figure 7 Large common bile duct calculi with narrow distal common bile duct. Good fragmentation achieved with extracorporeal shockwave lithotripsy (ESWL).

obviates the need for surgery^[3]. ESWL was successful in patients in whom mechanical lithotripsy or surgical extraction of CBD stones was unsuccessful^[5]. Non-operative options for removing large CBD calculi include laser lithotripsy, EHL or ESWL. The success rates are similar at between 80% and 95%^[6]. No significant differences in CBD clearance was seen in comparison of ESWL with EHL^[25]. Laparoscopic CBD exploration (LCBDE) is a well-established technique for management of large CBD stones. Despite several studies supporting LCBDE, current surgical practice suggests an overwhelming preference for preoperative ERCP^[33]. Potential explanations for this preference include the challenging nature of LCBDE, lack of necessary equipment, lack of formal training in LCBDE, and increased operative time^[34,35].

Factors that influence fragmentation of CBD stones

Factors that promote better fragmentation and patient compliance have been analyzed at our center^[3]: (1) Use of EA. EA provides good sensory block and reduces patient movement^[20]. This helps with better targeting and reduces the total number of shocks. The same catheter can be used for three sessions of ESWL, which is

Table 4 Efficacy of extracorporeal shockwave lithotripsy for large common bile duct calculi

Author	No. of patients	Complete clearance (%)
Adamek <i>et al</i> ^[27]	79	78.5
Neuhaus <i>et al</i> ^[29]	30	73
Binmoeller <i>et al</i> ^[28]	10	70
Ellis <i>et al</i> ^[5]	83	83
Tandan <i>et al</i> ^[3]	283	84
Kocdor <i>et al</i> ^[32]	20	85

often adequate to fragment calculi in the majority of patients; (2) Frequency of shocks at 90/min. At a higher frequency, the ongoing shock waves and those reflected from the surface of the calculus tend to cancel each other out^[17]; (3) The presence of fluid around the calculi aids better fragmentation. Saline irrigation *via* the NBT is carried out during ESWL; and (4) Radiolucent calculi fragment easier compared to radio-opaque ones.

At our center, patients with large CBD calculi undergo ESWL, under EA, at a shock wave frequency of 90/min, with saline irrigation *via* an NBT. Limitations include failure to prevent recurrence, which is reported around 14% at 1 year following ESWL^[36]. The other limitation is failure to identify the calculi that are not amenable to fragmentation, prior to ESWL.

COMPARISON WITH COMPETING STRATEGIES

Mechanical lithotripsy, ILL, EHL and Spy Glass are the other modalities of stone fragmentation for large CBD calculi. Neuhaus *et al*^[27] have reported better clearance with ILL as compared with ESWL, whereas Adamek and colleagues have found no difference between these modalities^[25]. The availability of instrumentation and expertise at a center often determines the choice of the procedure adopted at that center. Very few studies have compared different strategies for fragmentation of large CBD stones.

COMPLICATIONS OF ESWL

A number of rare and serious complications have been reported following ESWL^[37-40]. These occur infrequently and appear to be limited in number. The complications include perirenal hematoma, biliary obstruction, bowel perforation, splenic rupture, lung trauma, and necrotizing pancreatitis. In our experience, as well as in most of the other centers with high patient volume, complications are minimal and mild, and are managed conservatively without extension of hospital stay^[3,7,25,27]. Pain at the site of shock wave delivery, skin ecchymosis, abdominal pain, occasional fever, and hemobilia were observed in some of our patients. No blood transfusion or intervention was required in any of these cases. There is no increased incidence of pancreatitis following ESWL and ERCP. Accurate targeting achieved by the

third-generation lithotripter, as well as reduced patient movements with EA, are responsible for reducing collateral tissue damage and minimizing complications^[3,7].

AREAS OF FUTURE RESEARCH

Although fragmentation of pancreatic and biliary calculi by ESWL is satisfactory, there is ample scope for future research to improve results and minimize complications. The long-term results of ESWL in CCP, especially in young patients, are yet to be determined. Data on post-ESWL long-term follow-up are conflicting, with no benefit being shown in some studies^[16], whereas others have revealed good long-term results^[23]. Can intervention at an early age change the course of CCP and help avoid surgery and long-term sequelae^[7]? A long term prospective study in this regard would give us a clearer insight. Another focus of research would be to identify the small percentage of pancreatic and biliary calculi that do not respond to ESWL, so that they can be subjected to alternate modes of endoscopic therapy or surgery. An improvement in the focusing and intensity of shock waves would minimize the failure rate as well as help target pancreatic calculi in the tail region while avoiding splenic complications^[38,39]. Recurrence of pancreatic and biliary calculi after initial successful clearance is known to occur^[22,36]. Identification of this subset of patients and prevention of such recurrences using pharmacological agents would be ideal.

CONCLUSION

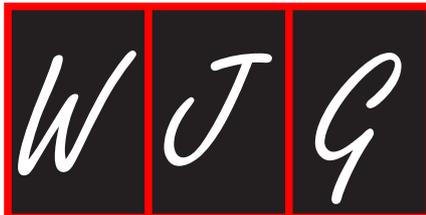
ESWL is an excellent therapeutic modality for large pancreatic and CBD calculi. The high efficacy, non-invasive nature of the procedure, along with the low complication rate make it a procedure of choice and can be offered as first-line therapy for selected patients with large pancreatic and CBD calculi.

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Current status of thiopurine analogues in the treatment in Crohn's disease

Peter Laszlo Lakatos, Lajos S Kiss

Peter Laszlo Lakatos, Lajos S Kiss, 1st Department of Medicine, Semmelweis University, H-1083 Budapest, Hungary
Author contributions: Lakatos PL and Kiss LS co-authored this paper.

Correspondence to: Dr. Peter Laszlo Lakatos, MD, PhD, 1st Department of Medicine, Semmelweis University, H-1083 Budapest, Koranyi S 2A, Hungary. kislakpet@bell.sote.hu

Telephone: +36-20-9117727 Fax: +36-1-3130250
Received: March 22, 2011 Revised: June 21, 2011

Accepted: June 28, 2011

Published online: October 21, 2011

Abstract

In the last decades, with the development of biological therapy, the treatment paradigms in patients with Crohn's disease have continuously evolved. Several studies focusing on the optimal use of both traditional immunosuppressants and biological therapy have been published, investigating conventional, accelerated step-up and top-down approaches. In addition, much emphasis has been placed in recent years on the determination of important predictive factors that could enable early patient stratification, which would lead to a tailored management strategy. In this review, the authors try to highlight new evidence on the optimal timing, benefits, and risks of immunosuppressants alone, or in combination, in patients with Crohn's disease.

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Key words: Crohn's disease; Immunosuppressives; Azathioprine; Thiopurine methyltransferase; Biologicals

Peer reviewer: Ali Keshavarzian, MD, Josephine M. Dyrenforth Professor of Medicine, Professor of Pharmacology and Molecular Biophysics and Physiology Director, Digestive Diseases and Nutrition Vice Chairman of Medicine for Academic and Research Affairs, Rush University Medical Center 1725 W Harrison, Suite 206, Chicago, IL 60612, United States

Lakatos PL, Kiss LS. Current status of thiopurine analogues in the treatment in Crohn's disease. *World J Gastroenterol* 2011; 17(39): 4372-4381 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4372.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4372>

INTRODUCTION

Inflammatory bowel disease (IBD) is a multifactorial disease with probable genetic heterogeneity^[1]. In addition, several environmental risk factors (e.g., diet, smoking, measles or appendectomy) may contribute to its pathogenesis. During the past several decades, the incidence pattern of both forms of disease, Crohn's disease (CD) and ulcerative colitis, has changed significantly^[2], showing some common characteristics yet also quite distinct features between the two disorders.

The phenotypic classification of CD plays an important role in determining the treatment, and may assist in predicting the likely clinical course of disease. In 2005, the Montreal revision of the Vienna classification system was introduced^[3]. Using the Vienna classification system, it has been shown in clinic-based cohorts that there can be a significant change in disease behavior over time, whereas disease location remains relatively constant^[4]. Population-based studies have demonstrated that up to one-third of the patients had evidence of a stricturing or penetrating intestinal complication at diagnosis, and half of all patients experienced an intestinal complication within 20 years after diagnosis^[4,5]. Similarly, these complications occurred in more than 50% of children, after a median follow-up of 84 mo^[6]. Half of the adult patients required surgery within 10 years after diagnosis, while in children, 34% of patients required surgery within 5 years of diagnosis. The risk of postoperative recurrence was approximately 44%-55% after 10 years. These data suggest that Crohn's disease is a chronic progressive disease,

where effective intervention prior to the onset of bowel damage (stricture, fistula, abscess) is required in order to improve the outcome. Of note, however, not all patients with CD will show disease progression. Thus, recognizing patients at the highest risk of developing a disabling disease or complications at an early stage is crucial. In CD cohorts from referral centers, an initial need for steroids, an age below 40 years, the presence of perianal or stricturing disease and a significant weight loss were associated with the development of disabling disease^[7,8].

A systematic review published in 2004, which analyzed population-based studies in CD with a complete follow-up, failed to demonstrate a significant improvement in disease outcome during the past four decades^[9]. Of note, disease activity, occurrence of complications, and need for surgery did not significantly change during that period. For example, time to intestinal surgery did not change despite the more frequent use of immunosuppressants in CD patients from the end of the 1990s^[10]. According to the authors' conclusion, the timing of immunosuppressants use might have been inappropriate. Nevertheless, data support that azathioprine (AZA) allows not only for the maintenance of remission and weaning off steroids in approximately two-thirds of patients with steroid-dependent CD, but may lead to complete or near-complete mucosal healing and histological remission in a significant proportion of CD patients^[11].

More recently, Peyrin-Biroulet *et al.*^[12] published a systematic review on the natural history of CD in population-based cohorts. The authors conclude that the impact of changing treatment paradigms with the increasing use of immunosuppressants and biological agents on the natural history of CD is poorly understood. To investigate this question, two approaches may be appropriate; (1) to conduct a disease-modification trial using the newly proposed definition of "early Crohn's disease"^[13]; (2) to investigate the evolution of the disease phenotype and complications in population-based cohorts with unified patient management. The limitation of the first approach is that only the relatively short-term outcomes (e.g., clinical remission, endoscopic healing, short-term risk of hospitalization and/or surgery) can be investigated with adequate statistical power. In contrast, in the second setting, the follow-up is complete in every patient; however, patient management is more individualized and variable.

Treatment paradigms have been evolving in the last two decades, with the inclusion of biological therapy. In the last several years, numerous studies focusing on the optimal use of traditional immunosuppressants and biological therapy have been published. In addition, much emphasis has been placed in recent years on the determination of important predictive factors to identify patients at risk for disease progression as soon as possible, in order to enable a tailored management strategy^[14]. In this review, the authors try to highlight some of the new, available evidence on the benefits, timing, and risks of immunosuppressants alone, or in combination, in pa-

tients with CD.

NEW DATA AND NEW STRATEGIES ON THE USE OF THIOPURINE ANALOGUES: ALONE OR IN COMBINATION?

Efficacy of conventional immunosuppressants

In CD, the efficacy of immunosuppressive therapy with purine analogues has been established in controlled trials, which assessed the role of AZA/6-mercaptopurine (6-MP), both as induction agents and as steroid-sparing agents in a withdrawal study^[15,16]. The study reported by Present *et al.*^[15] in 1980, was the first to demonstrate with certainty the efficacy of 6-MP in the induction of remission in CD. By using a dose of 1.5 mg/kg per day, 67% of patients responded to therapy as compared to only 8% of patients who received placebo. However, not all controlled trials report such a positive clinical response to thiopurines in the induction of disease remission in CD. The notion of the delayed onset of action of 6-MP also stems from the study by Present *et al.*^[15], which reported that the mean time to response was 3.1 mo, with 89% of responders doing so within 4 mo of initiating therapy. However, it should be noted that the first clinical evaluation in this study was not performed until the 12-wk mark. Thus, it is likely that a proportion of patients were already responding before the first assessment. It is important to note that if therapy is started relatively late in the disease course, when the anatomical damage is irreversible, these medications will not prevent the occurrence of complications. Until recently, immunosuppressants were introduced relatively late during the disease course, mainly in steroid-dependent/resistant or postoperative patients^[17]. Thiopurines were started in the majority of patients years after the diagnosis. Even so, in clinical cohorts, the efficacy of thiopurine therapy was defined as optimal in approximately 47% of the patients. Similar results were published by the Oxford clinic^[18], where the mean remission rate was 45% and the proportion of patients remaining in remission at one-, three-, and five-years was 95%, 69% and 55%, respectively. In general, it is recommended that thiopurines be added to the therapeutic regime in patients failing to wean off corticosteroids during their first attempt at tapering the dose or alternatively after a second attempt.

The most convincing data to support a benefit from early use of AZA, however, come from the pediatric literature^[19], where in a randomized controlled trial involving 55 children, the early use of 6-MP was associated with a significantly lower relapse rate (only 9%) compared with 47% in controls ($P = 0.007$). Moreover, the duration of steroid use was shorter ($P < 0.001$) and the cumulative steroid dose was lower at 6, 12 and 18 mo ($P < 0.01$). The benefit of an early aggressive treatment was also demonstrated in another pediatric study^[20], where 80.5% of children with newly diagnosed moderate-to-severe CD were treated with immunomodulators within

the first year. Early immunomodulator use was associated with reduced corticosteroid exposure and fewer hospitalizations per patient. Candy *et al*^[21] similarly showed that AZA offers a therapeutic advantage over placebo (47% *vs* 7% remission rate at 15 mo; $P < 0.001$) in the maintenance of remission in CD patients. Both studies showed no difference in the proportion of patients who had achieved remission at 12 wk, since corticosteroids served as the induction therapy for both groups. These results highlight the steroid-sparing benefits of thiopurines and suggest that the short-term use of corticosteroids for the induction of remission can serve as a bridge to the more long-term maintenance of a steroid-free remission with thiopurines.

The benefit of thiopurines was also demonstrated in cohort studies. In the pediatric setting, since the year 2000, the more systematic introduction of AZA at the time of diagnosis led to a 2-fold longer first remission period^[22]. Similarly, the long-term beneficial effect of early AZA treatment was demonstrated in an adult referral cohort study from Hungary, where early AZA treatment was independently associated with a decreased risk for disease behavior change and resective surgery. It also prevented the deleterious effects of smoking^[23,24]. Similarly, a lower risk of surgery (HR: 0.41; 95% CI: 0.21-0.81) in non-penetrating non-stricturing CD patients with an immunomodulator use lasting more than 6 mo was also reported from the United States^[25]. An important clinical question is of course, patient adherence to treatment. A wide range of non-adherence was reported in Germany, for patients taking AZA and in long-term remission, ranging from 7.1% to 74.3%^[26]. Limited data are available with regards to factors predicting effectiveness and failure of long-term thiopurine use in IBD patients. There is evidence to suggest that 6-methylmercaptopurine (6-MMP) concentration and the 6-MMP/6-thioguanine nucleotides (6-TGN) ratio may be associated with therapeutic failure^[27]. In patients with suboptimal response on AZA and high 6-MMP levels, the addition of allopurinol was effective and safe in optimizing 6-TGN production, leading to improved disease activity scores, reduced corticosteroid requirements, and normalization of liver enzymes, but careful monitoring for adverse effects and profiling of thiopurine metabolites is essential^[28].

The efficacy of thiopurine analogues for the induction of maintenance was also proven in recent reviews by the COCHRANE group^[29,30]. The odds ratio (OR) of a response to AZA or 6-MP therapy compared with placebo in active CD was 2.43 (95% CI: 1.62-3.64), 54% in AZA-treated patients and 34% in the placebo arms. This corresponded with a number needed to treat (NNT) equaling about five. When the two trials using 6-MP in active disease were excluded from the analysis, the OR was 2.06 (95% CI: 1.25-3.39). Treatment for longer than 17 wk resulted in an OR of 2.61 (95% CI: 1.69-4.03); however, a significant benefit was not observed for a shorter treatment period. A steroid-sparing effect was seen with an OR of 3.69 (95% CI: 2.12-6.42),

corresponding to a NNT of about three, in order to observe steroid-sparing in one patient. Similarly, AZA was effective in maintaining remission in the seven trials with AZA and one with 6-MP. AZA and 6-MP had a positive effect on maintaining remission (OR: 2.32; 95% CI: 1.55-3.49) with a NNT of six. The OR for the maintenance of remission with 6-MP was 3.32 (95% CI: 1.40-7.87) with a NNT of four. Higher doses of AZA improved response (AZA 1 mg/kg, OR: 1.20; 2 mg/kg, OR: 3.01; 2.5 mg/kg, OR: 4.13). A steroid-sparing effect with AZA was noted, with an OR of 5.22 (95% CI: 1.06-25.68) and a NNT of three. The Cochrane analysis reported a response rate of 55% with thiopurine therapy *vs* 29% for placebo, a pooled OR of 4.58 (95% CI: 0.49-42.82) also favored fistula healing. It should be noted that there was only a small number of patients evaluable for this analysis, and with the confidence interval crossing 1 this result is statistically insignificant.

In clinical practice, it is still uncertain if and when immunosuppressive therapy should be interrupted in patients in long-term (4-6 years) remission on thiopurines. In a recent withdrawal study by the GETAID group^[31], the authors have provided evidence for the benefit of long-term AZA therapy beyond 5 years in patients with prolonged clinical remission. The cumulative probabilities of relapse at 1, 3, and 5 years were 14.0%, 52.8%, and 62.7%, respectively. A C-reactive protein (CRP) concentration of 20 mg/L or greater (risk: 58.6), a hemoglobin level of less than 12 g/dL (risk: 4.8), and a neutrophil count 4×10^9 /L or greater (risk, 3.2) were independently associated with an increased risk of relapse. Among the 32 relapsing patients, 23 were retreated by AZA alone, with all but one leading to a successful remission.

Finally, in adults, a recently published clinical strategy trial from Belgium and the Netherlands^[32] randomized 133 patients with active CD, naïve to both steroids and AZA, to either a conventional step-up strategy [with full courses of steroids (prednisolone or budesonide) and introduction of AZA when the patients experienced a flare-up after tapering off or became dependent on steroids] or top-down (infliximab induction therapy and AZA at the first presentation). From week 6, AZA was continued as monotherapy, thus, a long-term combination was not administered. Up to the one-year mark after the initiation of therapy, steroid-free remission was more frequent in the early combined immunosuppressive group (61.5% *vs* 42.2%, difference: 19.4%, 95% CI: 2.4-36.3, $P < 0.05$). The median time to relapse was also longer in the early combined immunosuppressive - the "top-down" group [329.0 d, interquartile range (IQR) 91.0-∞ *vs* 174.5 d, IQR 78.5-274.0, $P < 0.03$]. In contrast, the difference was not significant after 52 wk. This open-label trial was liable to the intrinsic observer bias. Furthermore, patients in the conventional group had to fail two courses of steroids before the start of the immunosuppressant, which added a delay of appropriate treatment in at least one-third of the patients. None-

theless, a significant difference was found concerning complete ulcer healing during endoscopy, with 73.1% of evaluated patients (19/26) in the early combined immunosuppressants group *vs* 30.4% (7/23) in the controls, in a subgroup of patients who underwent ileocolonoscopy at week 104. In addition, the majority of patients (15/17) with mucosal healing in the early combined immunosuppressive group, after two years of therapy, remained in remission off steroids and did not need further infliximab (IFX) therapy in the subsequent two years of follow-up^[33]. The authors concluded that CD can be effectively treated without steroids, if patients are offered an early combined therapy of immunosuppressants. An interesting secondary result of the study was that approximately 10%-20% of patients required IFX after the induction period.

Current use of thiopurines and anti-TNF blockers: Alone or in combination?

There is no consensus on the appropriateness of concomitant immunomodulators with anti-tumor necrosis factor (anti-TNF) therapy for CD. Some patients benefit from concomitant immunomodulators, but there are increasing concerns related to infections and the risk of lymphoma. Until recently, anti-TNF antibodies have usually been initiated as second or third line immunosuppressants in patients failing or dependent on steroids and/or AZA. In 2003, immunosuppressants were shown to inhibit the development of neutralizing anti-infliximab antibodies, when this drug was used in an episodic, on-flare strategy^[34]. Moreover, IFX serum levels were also significantly higher in patients with concomitant immunosuppressive therapy^[35]. Therefore, theoretically, combined therapy may have synergistic immunosuppressive effects resulting in increased efficacy, but it may also increase the long-term toxicity. However, in randomized controlled trials (discussed previously in detail) in CD patients with long disease duration, often after multiple surgical interventions, a synergistic effect was not observed. Concomitant immunosuppressive and/or steroid therapy was not more efficacious compared to the anti-TNF agent alone in patients on scheduled maintenance therapy^[36]. Thirty to seventy percent of patients in these trials received either of the drugs. In PRECISE 1, for example, 23% of patients on certolizumab with concomitant immunosuppressants *vs* 23% without immunosuppressants, showed a drop of more than 100 points at weeks 6 and 26 in the Crohn's disease activity index (CDAI). The numbers were identical for patients with and without concomitant steroid therapy^[37]. Similarly, in PRECISE 2, at week 26, 61% of patients receiving concomitant immunosuppressive agent and 64% without demonstrated a clinical response. A similar tendency was also reported for adalimumab in CHARM. Clinical remission rates with or without concomitant immunosuppression were not significantly different either at week 26 or 52 [37% *vs* 33% for adalimumab every other week (EOW) and 39% *vs* 50% for adalimumab every

week (EW)]. In addition, similar to certolizumab, the clinical efficacy was significantly different based on the disease duration. This tendency was also similar for IFX in the ACCENT I and II trials, although the rate of infusion reactions was lower (12.5%) in patients receiving concomitant immunosuppression compared to those without (22.0%), and the rate of formation of antibodies was higher^[37]. In contrast, reported IFX concentrations were not different over time. Although significance was not reported, in ACCENT I, the clinical response and remission rate in the 5 mg/kg group was reported as 54% and 38%, respectively, in patients with an immunosuppressant, and was 34% and 26% without immunosuppressant therapy.

Additionally, a recent, prospective, open-label study demonstrated that withdrawing immunosuppressants in patients with CD on a combined maintenance schedule of IFX and immunosuppressive therapy for at least six months did not affect efficacy over two years of follow-up, but tended to decrease IFX trough levels and CRP elevation^[38]. This indicates that the impact of withdrawing antimetabolites in patients treated with biologicals has no, or only limited, risk of loss of efficacy, although the impact on IFX trough levels warrants further long-term follow-up. Noteworthy, however, is that most patients had been failing AZA therapy before having entered the trial. As a final point, in a recent large Belgian cohort study^[39], concomitant AZA or methotrexate (MTX) therapy did not influence the outcome of IFX treatment during a median follow-up of five years. Importantly, 49.7% of patients were on AZA and 9.4% on MTX at the time of anti-TNF induction therapy. 34.1% of those on AZA at baseline stopped its use after a median of 15 mo; however, in 41.3% of these patients MTX was started later during the follow-up, based on the clinical indication. Moreover, 26% of the patients needed one intervention (increasing the dose to 10 mg/kg or decreasing the interval) during IFX maintenance therapy, while 10% and 14% needed two or three modifications, respectively. Therefore, the results should be interpreted with caution, since an alternative conclusion might be that patients with more aggressive disease course were able to maintain similar clinical benefit with a combination therapy and/or modifications in the dose or interval of the biological therapy.

More recently, however, anti-TNF agents have been used earlier in the disease course, including in patients naïve to AZA. The first piece of evidence arises from the pediatric literature^[40]. In 112 children with moderate-to-severe disease, IFX induction and scheduled maintenance therapy, every 8 wk, in the REACH study was associated with 63.5% and 55.8% clinical response and clinical remission rates, respectively. All patients were required to have started concomitant immunomodulators (AZA, 6-MP or MTX) at least 8 wk prior to study entry and approximately one third of the patients were also simultaneously receiving steroids. The average disease duration was as low as two years. Although the defini-

tion of remission was different (instead of CDAI, the pediatric index, PCDAI, was used) and disease duration was short, these were among the highest remission rates reported for anti-TNF agents.

In adult CD patients with early disease (< 2 years) naïve to purine analogues and MTX, the outcome was similar to that found in the pediatric population. The large, blinded, double-dummy, controlled SONIC trial compared AZA monotherapy (2.5 mg/kg per day), IFX monotherapy, and combined IFX and AZA therapy^[41]. The average disease duration was 2.3 years (range 0-43 years). At 26 wk, the steroid-free remission rates in patients receiving combined immunosuppressive therapy with IFX and AZA were higher than with IFX monotherapy (56.8% *vs* 44.4%, $P < 0.05$). In turn, these were also higher than remission rates in patients receiving AZA monotherapy (30.0%, $P < 0.01$). A course of steroids was allowed in all patients until week 12, to compensate for the slow onset of the therapeutic effect of AZA. The proportion of patients receiving systemic steroids at baseline in combination with AZA, IFX or in the combination group was similar ($n = 40, 52$ and 47 patients, respectively); however, the dose used was below that recommended for induction therapy (mean dose of 24 mg/d). It is even more difficult to explain the large difference in clinical remission off steroids at week 26, since the number of patients receiving steroids at this time point ($n = 60, 60$ and 58 , respectively) and the mean dose were virtually identical (range: 9.4-11.6 mg/d) in all three groups. Therefore, the lower clinical efficacy is not reflected by differences in steroid use. Moreover, steroids should have been tapered off by week 12, where possible. As a consequence, the end result in at least one-third of the patients reflects an insufficient steroid induction therapy in combination with either AZA, IFX or the combination of the two drugs. Nevertheless, the total disappearance of mucosal ulcers was also higher in the combined IFX-AZA group (43.9% IFX and AZA *vs* 16.5% AZA, $P < 0.001$). Nonetheless, a significant bias cannot be excluded, since patients with lesions at baseline who did not undergo endoscopy at week 26, or who had results that could not be evaluated were assumed to have a lesion. These patients numbered 50 of 109 (45.9%) in the AZA group, 29 of 93 (31.2%) in the IFX group, and 31 of 107 (29.0%) in the combination-therapy group. In addition, it is difficult to interpret the data since a significant proportion of the patients had negative ileocolonoscopy at inclusion.

At week 50, assuming that patients not entering the study extension would not be in a steroid-free remission, the overall proportion of patients in steroid-free remission was 46.2% with the IFX-AZA combination, 34.9% under IFX monotherapy, and 24.1% with AZA monotherapy ($P < 0.03$). To select patients with objective signs of inflammation (an elevated C-reactive protein and/or active disease at endoscopy) seems to be important, since in a subgroup analysis, benefit from more aggressive combination therapy was restricted to patients with

objective signs of active inflammation.

Whether these results would affect the management of non-immunosuppressive-naïve patients remains debated. Of note, in a very recent cohort study by Sokol *et al*^[42] IBD flare-ups, perianal complications, and a switch to adalimumab were less frequently observed in patient-semester with combined immunosuppressant and biological use than in those without immunosuppressives (19.3% *vs* 32.0%, $P = 0.003$; 4.1% *vs* 11.8%, $P = 0.03$; 1.1% *vs* 5.3%, $P = 0.006$). Maximal C-reactive protein (CRP) level and IFX dose/kg observed during the semesters were lower in semesters with immunosuppressives. In a multivariate analysis, immunosuppressive co-treatment was associated with a decreased risk of disease flare-up (OR: 0.52; 95% CI: 0.35-0.79). Moreover, the effectiveness of co-treatment with immunosuppressants was time-independent.

POSTOPERATIVE MANAGEMENT: IMMUNOSUPPRESSANTS OR MORE?

Early postoperative use of AZA at a dose of 2-2.5 mg/kg per day) seemed to delay endoscopic postoperative recurrence in comparison to historical series or placebo groups in randomized controlled trials^[43]. Furthermore, in a recent controlled, randomized, prospective trial, AZA administered for 12 mo together with metronidazole for 3 mo was more effective in preventing endoscopic postoperative recurrence assessed at 12 mo, compared to metronidazole alone in patients previously only minimally exposed to AZA^[44]. In a meta-analysis, Peyrin-Biroulet *et al*^[45] have shown that purine analogues were more effective than control arms in preventing clinical recurrence at 1 year (mean difference: 8%, NNT = 13 and 2 years, respectively (mean difference: 13%, NNT = 8)). The efficacy of purine analogues was also superior to that of placebo for the prevention of clinical and endoscopic recurrence at 1- and 2-years (mean differences: 13%, NNT = 7, and 23%, NNT = 4), respectively. At 1-year, purine analogues were more effective than control arms in preventing severe (≥2-4) endoscopic recurrence (mean difference: 15%, NNT = 7); however, the rate of adverse events leading to drug withdrawal was higher in thiopurine-treated patients.

In a more recent study from Austria,^[46] the authors evaluated the impact of thiopurine treatment on surgical recurrence in patients after the first intestinal resection for CD. In a Cox regression analysis, treatment with thiopurines for no more than 36 mo (HR: 0.41; 95% CI: 0.23-0.76, $P = 0.004$) and smoking (HR: 1.6; 95% CI: 1.14-2.4, $P = 0.008$) were identified as independent predictors for surgical recurrence. In addition, a multicenter study led by Reinisch *et al*^[47] investigated the efficacy of AZA therapy for the prevention of clinical relapse in patients with endoscopic recurrence (≥2-4, but CDAI < 200). Treatment failure-defined as a CDAI score > 200 and an increase of > 60 points from baseline, or study drug discontinuation due to lack of efficacy or intolerable adverse drug reaction-occurred in 22.0% (9/41) of

AZA-treated patients and 10.8% (4/37) of mesalazine-treated patients. The difference was mainly due to the discontinuation of AZA and the adverse drug reactions that only occurred in AZA-treated patients [9/41 (22.0%) *vs* 0%, $P = 0.002$]. In contrast, clinical recurrence was significantly less frequent in patient treated with AZA *versus* mesalazine [0/41 (0%) *vs* 4/37 (10.8%), $P = 0.031$]. Hence, the efficacy of AZA, while clearly established, must be balanced against its side-effect profile, resulting in a high rate of discontinuation. Finally, preliminary data support biological therapy as a possible therapeutic option, at least in selected patient populations^[48].

ADVERSE EVENTS

6-Mercaptopurine (predominantly used as a chemotherapeutic agent) and its pro-drug, AZA (an immune modifier agent), are purine analogues that competitively interfere with nucleic acid metabolism by acting as substrate competitive antagonists for the hypoxanthine-guanine phosphoribosyl transferase enzyme (anti-metabolite activity)^[49]. Consequently, both drugs reduce cell proliferation and have immune-modifier properties. Adverse events are frequent and lead to cessation of therapy in 9% to 25% of patients^[50]. Adverse events associated with AZA and 6-MP include nausea, allergic reactions, flu-like illness, malaise, fevers, rash, abdominal pain, pancreatitis, hepatotoxicity, myelosuppression, and an increased risk of lymphoma^[51]. Classically, AZA-related adverse events have been categorized into two types: allergic, idiosyncratic or non-dose-dependent and dose-dependent.

Advances in the understanding of AZA and 6-MP drug metabolism have led to genetic and metabolite tests that help clinicians optimize the use of these drugs. A deficiency of the thiopurine methyltransferase (TPMT) enzyme appears to account for some dose- and metabolism-dependent toxicities, such as leukopenia (and possible subsequent infection), thrombocytopenia, and malignancy. TPMT exerts these side effects by limiting the production of 6-TGNs by converting 6-MP to 6-thiouric acid and 6-MMP^[52], and major 6TGN accumulation may lead to profound, potentially life-threatening myelotoxicity. Population studies have shown that the distribution of TPMT activity is trimodal: 0.3%-0.5% of the population have low to absent activity (TPMTL/TPMTL), around 10% have intermediate activity (TPMTL/TPMTH), and approximately 90% inherit normal to high enzyme activity (TPMTH/TPMTH)^[52]. In this regard, a correlation between the TPMT genotype and enzyme activity has been proven. Approximately 5% of the white population carries one or more variant TPMT alleles, with more than ten variant alleles reported^[53]. The functional consequences of alleles *2, *2A, *3B and *3C, accounting together for more than 90% of mutant alleles, have been extensively characterized.

Nevertheless, it is clear that there are many other causes of myelotoxicity. This was accurately demon-

strated by Colombel *et al.*^[54], who found that only 27% of CD patients with myelosuppression had a documented low TPMT activity. Other confounding genetic and environmental factors include, for instance, the patient's age, renal function, AZA formulation, co-administration of mesalazine (a reversible TPMT inhibitor) and allopurinol (XO inhibitor). Thus, the determination of TPMT activity is not an exclusive test to rely on when predicting the risk of myelotoxicity. It may only be helpful in identifying a certain group of high-risk patients but as the negative predictive value is rather low, it is not beneficial in ruling out possible side effects. Also, as the prevalence of double carriage of variant TPMT alleles is as low as 1/300, continuous monitoring of red blood cell counts remains mandatory in clinical practice.

Other toxicities such as rash-fever-arthralgias (2.3%), pancreatitis (1.4%), hepatitis, nausea (1.4%), non-pancreatic abdominal pain, and diarrhea appear to be hypersensitivity reactions^[55]. Mercaptopurine may be tolerated in up to 60% of AZA-intolerant patients, and treatment should be considered, particularly if intolerance was due to hepatotoxicity, arthralgia, nausea, vomiting, flu-like illness or rash^[56,57]. A less well-known, and relatively rare, side-effect of AZA is nodular regenerative hyperplasia (NRH). In a recent French study^[58], the cumulative risk calculated was 0.5% at 5-years and 1.25% at 10-years in patients on a median AZA dose of 2 mg/kg per day.

According to a recent review by the Cochrane group, adverse events requiring withdrawal from an induction trial, principally allergy, leukopenia, pancreatitis, and nausea, were increased with active therapy with an odds ratio of 3.44 (95% CI: 1.52-7.77), and were observed in 9.3% of treated patients and in 2.3% of patients in the placebo arms^[50]. The NNT to observe one adverse event on AZA or 6-MP was 14.

In 2005, Kandiel *et al.*^[59] performed a meta-analysis utilizing data from six of these cohort studies. The authors were able to pool calculated standardized incidence ratios (SIR) from all studies. When data were pooled across all studies, there were 11 observed lymphomas compared to the expected 2.63 cases, resulting in an SIR of 4.18 (95% CI: 2.07-7.51). Due to significant variability in SIR estimates amongst the studies, sensitivity analyses were performed, where each study was excluded from the group and SIR was recalculated (SIR range: 3.49-5.21). The authors concluded that IBD patients on thiopurines seemed to have a 4-fold increased risk of lymphoma, but whether this risk was due to the medications themselves or the underlying disease severity has not yet been elucidated. Nevertheless, there may be a small but real risk of lymphoma. Interestingly, treatment with AZA or 6-MP appeared to be associated with a small increased risk of Epstein-Barr virus (EBV)-positive lymphoma^[60]. Of note, EBV is a hallmark of lymphomas and lymphoproliferative disorders that arise in patients on immunosuppressive agents, which are used to limit rejection of bone marrow or solid organ transplants [post-transplant lymphoproliferative dis-

Table 1 Current status of thiopurine analogues in the treatment in Crohn's disease: Take home messages

<p>In Crohn's disease treatment paradigms have been evolving in the last decades, with biological therapy becoming available</p> <p>The efficacy of immunosuppressive therapy with purine analogues is well established in controlled trials (induction-maintenance, steroid-sparing agents, postoperative setting)</p> <p>New data indicate that earlier use of immunosuppressants alone may be more effective in maintaining remission, reducing further corticosteroid exposure, and decreasing the risk of hospitalization and surgery</p> <p>Adverse events during thiopurine therapy are frequent and lead to cessation of therapy in 9%-25% of patients</p> <p>Despite intensive research, there is still controversy in the literature regarding the clinical relevance of thiopurine S-methyltransferase (TPMT) testing. Based on recent data, the determination of TPMT activity may be helpful in identifying high-risk patients for developing major complications, especially myelosuppression. In contrast, the negative predictive value is rather low, and it is not beneficial in ruling out the possibility of a side effect. Similarly, there is no established rationale to use TPMT activity for adjusting the dose of azathioprine to enhance therapeutic efficacy. For general practice, regular, frequent monitoring of clinical symptoms and laboratory check-ups continue to be recommended</p> <p>Combination therapy with infliximab-azathioprine may have an added benefit in inducing steroid-free remission and mucosal healing compared to either infliximab or azathioprine alone, in azathioprine-naïve patients with early onset of disease</p> <p>At present, the risks and benefits of combination therapy should be assessed on a per-case basis and should be discussed with the patient in the everyday clinical practice</p>

order (PTLD)]. More recently, the CESAME group^[61] confirmed the above findings, through a study involving 19 486 IBD patients. The incidence rate of lymphoproliferative disorders were 0.90 per 1000 (95% CI: 0.50-1.49) patient-years in those receiving thiopurines, 0.20/1000 (95% CI: 0.02-0.72) patient-years in those who had discontinued therapy, and 0.26/1000 (95% CI: 0.10-0.57) patient-years in those who had never received thiopurines ($P = 0.0054$). The multivariate-adjusted hazard ratio of lymphoproliferative disorder between patients receiving thiopurines and those who had never received the drugs was 5.28 (95% CI: 2.01-13.9, $P = 0.0007$). Most cases associated with thiopurine exposure matched the pathological range of post-transplant disease. Importantly, there was a significant imbalance amongst the forms of disease, since 76% of patients on thiopurine therapy were CD patients *versus* only 48% of patients who never received immunosuppression. Moreover, anti-TNF therapy was also not included in the multivariate analysis, which introduced a significant bias, since there was a 7795 patient-year exposure to anti-TNF therapy and the SIR was significantly increased in patients who had received but discontinued anti-TNF therapy (SIR: 6.92) and exponentially increased in patients on combination therapy (SIR: 10.2).

Whether combined AZA/6-MP and anti-TNF therapy increases toxicity in the long-term, is still debated, but recent studies of 17 hepatosplenic T-cell lymphomas in young male patients with combined therapy have raised considerable concerns^[62]. Unfortunately, most cases were fatal. More data are needed but, in selected patients, particularly those previously exposed to purine analogues or AZA, and scheduled, long-term, maintenance monotherapy with anti-TNF antibodies is certainly a valid option. In contrast, the risk of infections was not higher during combined AZA/6-MP and anti-TNF therapy (OR: 1.6; 95% CI: 0.1-19)^[63]. In a case-control study from the Mayo Clinic, the use of steroids, AZA/6-MP was associated with a 2.2-3.4-fold elevated risk, but the risk was infinite if all three drugs were used.

CONCLUSION

In patients with Crohn's disease, treatment paradigms have been evolving in the last decades, with biological therapy becoming available. In Crohn's disease, the efficacy of immunosuppressive therapy with purine analogues is well established in controlled trials, both as induction agents and as steroid-sparing agents, showing efficacy also in the postoperative setting. In the past several years, numerous studies focusing on the optimal use of both, traditional immunosuppressants and biological therapy, investigating the conventional, accelerated step-up, and top-down approaches, have been published. Emerging new data indicate that earlier use of immunosuppressants is more effective in maintaining remission, reducing further corticosteroid exposure, and decreasing the risk of hospitalization and surgery. However, adverse events are frequent and lead to cessation of therapy in 9% to 25% of patients. Consequently, the benefit of azathioprine, while clearly established, must be balanced against its side-effect profile resulting in a high rate of discontinuation (Table 1).

Additionally, combination therapy with infliximab-azathioprine may have an added benefit in inducing steroid-free remission and mucosal healing compared to either infliximab or azathioprine alone, in azathioprine-naïve patients with early onset of disease. The added benefit of a biological-thiopurine combination is less well-established in non-azathioprine-naïve patients. Long-term combination, however, may potentially be associated with an increased risk for infection and malignancy. In recent years, several important studies on the safety of immunosuppressants, including anti-tumor necrosis factor agents, have been published and the cumulative body of evidence suggests that combined immunosuppressive therapy in patients with inflammatory bowel disease increases toxicity. At present, the risks and benefits of combination therapy should be assessed on a per-case basis and should be discussed with the patient in the everyday clinical practice. Moreover, much emphasis should be placed on defining the important predic-

tive factors in order to enable early patient stratification, thus leading to a tailored management strategy. Certainly, more research is needed in the area, since the impact of changing treatment paradigms with the increasing use of immunosuppressants and biological agents on the natural history is poorly understood. In the future, choosing among treatment paradigms, whether traditional immunosuppressants, biological or a combination in inflammatory bowel diseases may become highly dependent on the individual patient risk profile, the drugs already tried, and disease severity.

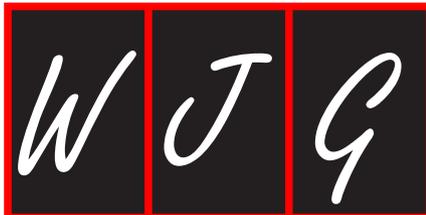
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S- Editor Sun H L- Editor Webster JR E- Editor Zhang DN



Natural orifice transluminal endoscopic surgery: New minimally invasive surgery come of age

Chen Huang, Ren-Xiang Huang, Zheng-Jun Qiu

Chen Huang, Zheng-Jun Qiu, Department of General Surgery, Affiliated First People's Hospital, Shanghai Jiao Tong University, Shanghai 200080, China

Ren-Xiang Huang, Digestive Endoscopy Center, Affiliated Huadong Hospital, Fudan University, Shanghai 200040, China

Author contributions: Huang C and Huang RX equally contributed to this paper; Qiu ZJ designed this review; Huang C and Huang RX drafted the manuscript; all authors have read and approved the final manuscript.

Supported by Fund for scientific research of Shanghai Shenkang Hospital Development Center, No. SHDC12006102

Correspondence to: Zheng-Jun Qiu, MD, PhD, Department of General Surgery, Affiliated First People's Hospital, Shanghai Jiao Tong University, 100 Haining Road, Shanghai 200080, China. qiuwryb@online.sh.cn

Telephone: +86-21-63240090 Fax: +86-21-63240825

Received: March 23, 2011 Revised: June 21, 2011

Accepted: June 28, 2011

Published online: October 21, 2011

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Key words: Laparoscopic surgery; Natural orifice transluminal endoscopic surgery; Endoscopy

Peer reviewer: Pietro Valdastrì, MScEE, PhD, Assistant Professor of Industrial BioEngineering, The BioRobotics Institute, Scuola Superiore Sant'Anna, Polo Sant'Anna Valdera, Viale Rinaldo Piaggio, 34, 56025 Pontedera, Italy

Huang C, Huang RX, Qiu ZJ. Natural orifice transluminal endoscopic surgery: New minimally invasive surgery come of age. *World J Gastroenterol* 2011; 17(39): 4382-4388 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4382.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4382>

Abstract

Although in the past two decades, laparoscopic surgery, considered as a great revolution in the minimally invasive surgery field, has undergone major development worldwide, another dramatic surgical revolution has quietly appeared in recent years. Ever since Kalloo's first report on transgastric peritoneoscopy in a porcine model in 2004, interest in a new surgical procedure named natural orifice transluminal endoscopic surgery (NOTES) has blossomed worldwide. Considering that a NOTES procedure could theoretically avoid any abdominal incision, operation-related pain and scarring, many surgeons and endoscopists have been enthusiastic in their study of this new technique. In recent years, several NOTES studies have been carried out on porcine models and even on humans, including transvaginal cholecystectomy, transgastric appendectomy, transvaginal appendectomy, and transvesical peritoneoscopy. So what is the current situation of NOTES and how many challenges do we still face? This review discusses the current research progress in NOTES.

INTRODUCTION

Since the first laparoscopic cholecystectomy was conducted by Mouret in 1987^[1], conventional surgery using laparotomy has been largely replaced in the two ensuing decades by laparoscopic surgery, due to its lower level of trauma and faster recovery. Currently, laparoscopic cholecystectomy has become a classical approach and laparoscopic surgery has become the standard treatment for many gastrointestinal conditions. Based on the minimally invasive surgery, a novel approach to the endoluminal endoscopic surgery named the natural orifice transluminal endoscopic surgery (NOTES) is currently emerging because it has the advantage of avoiding surface incision. This advantage could help reduce surgical pain, decrease anesthesia and analgesia, shorten recovery time, avoid hernia formation and adhesions, and eliminate any surgical site infection and visible scarring. Ever since Kalloo's first report on transgastric peritoneoscopy in a porcine model in 2004^[2], this dramatic surgical revolution has triggered many surgeons and endoscopists to study this new technique. This complex technique involves breaching the wall of the stomach, colon, vagina or bladder by endoscopic means to gain access into the peritoneum to perform the novel endoscopic therapy. In recent

years, several NOTES experiments have been carried out on porcine models and even on humans, including transvaginal cholecystectomy, transgastric appendectomy, transvaginal appendectomy and transvesical peritoneoscopy. In response to the clinical potential of NOTES, in 2005, a working group, named the Natural Orifice Surgery Consortium for Assessment and Research, composed of Society of American Gastrointestinal and Endoscopic Surgeons and American Society for Gastrointestinal Endoscopy was established. This working group generated a white paper that encouraged future NOTES research and outlined key research areas that needed to be addressed^[3].

NOTES PROCEDURES

NOTES procedures are frequently performed with existing endoscopic techniques and a number of accessories, such as snares, endoscopic biopsy forceps, endoscopic grasping forceps, endoloops and endoscopic clips^[4]. An endoscope may be introduced through a natural external orifice such as the mouth, anus, vagina or urethra to visualize various cavities, or through incisions and sutures to create internal orifices for entry into the free peritoneal cavity and access different viscera^[5].

Transesophageal route

This is probably used for transesophageal intracardiac and mediastinal procedures, including biopsies. Considering the high difficulty for thoracic NOTES technique nowadays, few surgeons and endoscopists have such experiences, with the notable exceptions of Fritscher-Ravens *et al*^[6], who accessed the heart under endoscopic ultrasound (EUS) guidance through the transesophageal route, and von Delius *et al*^[7], who reported transesophageal NOTES mediastinoscopy in eight porcine models.

Transgastric route

To date, most of the published clinical cases report experience with the transgastric approach. The anterior wall of the stomach is usually the ideal incision site for access to the peritoneal cavity^[8], while the posterior wall may be selected to explore the retroperitoneum. After sterilization, a double-channel endoscope enters the stomach through a sterile overtube, and then an endoscopic needle knife is used to create a 2-4 mm incision with electrocautery. A dilation balloon is advanced over a catheter, and the incision is radially dilated to ensure free access of the endoscope to the peritoneal cavity^[4]. Previously, a wide range of NOTES procedures of various complexity were carried out on experimental porcine models, such as peritoneoscopy^[9,10], lymphadenectomy^[11], tubal ligation^[12], oophorectomy^[13], cholecystectomy^[14], cholecystogastrostomy^[15], gastrojejunostomy^[16], distal pancreatectomy^[17], and splenectomy^[18]. Lee *et al*^[19] have successfully performed transgastric endoscopic cecectomy with laparoscopic assistance on three canine models. The first transgastric appendectomies in humans were performed

by Rao and Reddy in India in 2004 (unpublished results). In 2008, Marescaux *et al*^[20] reported the first human case of NOTES cholecystectomy. Also in 2008, Rao *et al*^[21] reported transgastric appendectomy, tubal ligation and liver biopsy in patients. Besides, Horgan *et al*^[22] have successfully carried out transgastric appendectomy in a 42-year-old man and Wang *et al*^[23] have reported transgastric liver cyst fenestration.

Transgastric bariatric NOTES is another promising application for the treatment of obesity. To date, several successful experimental endoscopic interventions for obesity have been reported, such as endoscopically delivering duodenal-jejunal bypass sleeves^[24,25], using the TOGA System, a set of transoral endoscopically guided staplers that are being used to create a stapled restrictive pouch along the lesser curve of the stomach^[26,27], and endoscopically injecting botulinum toxin-A^[28]. However, rigorous testing of the standard transgastric bariatric NOTES techniques is still lacking.

Transcolonic route

Although transcolonic NOTES has been a rarely explored approach to the peritoneal cavity because of concerns related to fecal contamination and intra-abdominal infectious complications^[29], a few surgeons and endoscopists still perform transcolonic cholecystectomy and transcolonic appendectomy^[30,31]. Bazzi *et al*^[32] have successfully performed hybrid transrectal NOTES nephrectomy in three porcine models.

Transvaginal route

Currently, transvaginal access is the preferred approach in humans because this route obviates the risk of intestinal content leakage *via* an imperfectly closed access site^[29]. The first transvaginal cholecystectomy in humans was carried out by Marescaux *et al*^[20] in 2007. In 2009, Horgan *et al*^[22] reported a series of successful transvaginal cholecystectomies in nine patients and one transvaginal appendectomy in a 24-year-old woman. Tarantino *et al*^[33] have reported a study which aimed to evaluate the feasibility and safety of transvaginal rigid-hybrid NOTES anterior resection in 40 patients with symptomatic diverticular disease, and the results were satisfactory. Suzuki *et al*^[34] have found that transvaginal cholecystectomy resulted in cardiopulmonary stability and well-preserved immune function similar to those in laparoscopic cholecystectomy in an experiment that involved 10 porcine models. Haber *et al*^[35] have reported that hybrid robotic transvaginal NOTES pyeloplasty, partial nephrectomy and radical nephrectomy were feasible and safe in a porcine model.

Transvesical route

Considered as another novel surgical route, transvesical peritoneoscopy was performed by Lima *et al*^[36] on a porcine model in 2006. In 2007, Gettman *et al*^[37] have reported transvesical peritoneoscopy in a 56-year-old man.

All of these routes have their own advantages in NOTES procedures. However, no one is perfect to date. Although

the transgastric route is regarded as the easiest way to get into the abdominal cavity, and initially, many NOTES experiments were done *via* the transgastric route, a drawback limiting the use of the transgastric route is the lack of a secure and reliable way to close the gastrotomy, which is an essential step in the procedure. The transcolonic route is similar to the transgastric route except that the former has an increased risk of contamination due to the fecal bacterial load^[38]. The benefits of transcolonic access include in-line endoscopic visualization and the ability to create and close the colotomy with existing transanal endoscopic microsurgery equipment^[29]. The transvesical route allows straight access to the upper abdominal organs such as the gallbladder, which is mechanically more advantageous than the transgastric approach. By far, the most clinical experience has been obtained with transvaginal access used as an accessory entry point to the peritoneal cavity during cholecystectomy. Data from several NOTES registries show that this access is associated with a low complication rate (3%-8%) and has a low technical threshold^[39,40]. Closure of the colpotomy can also be performed under direct vision using standard surgical techniques. However, this route is only suitable for female patients^[29,38]. The urinary tract is normally sterile, therefore, using the transvesical route can reduce infection risk. Anatomical relationships of the lower urinary tract to the peritoneum and retroperitoneum appear to be in the direct line of sight. Thus, all abdominal structures can theoretically be accessed. Closure of bladder access is simplified because catheterization alone promotes healing with considerably less risk of fistula formation and no risk of bowel leak^[37]. However, as the urethra is quite narrow and short, it is controversial to date whether it is possible to experience NOTES through the transvesical route, other than peritoneoscopy, and how surgical specimens can be taken out of the body through the narrow urethra.

CURRENT CHALLENGES OF NOTES

Although the potential benefits of NOTES such as no scarring, no pain and shorter hospitalization represent a new frontier of surgery, many technological challenges still exist. NOTES will not receive widespread adoption for clinical application until these problems are solved.

Surgical platform

The endoscopes that we use nowadays only offer small instrument and suction channels, which makes retraction and dissection of tissues difficult. Therefore, to develop a new platform that is larger, stronger and eventually articulated, instruments that can pass through large working channels are necessary. There are various operative platforms under investigation currently. Basically, all systems for performing NOTES-related procedures that are currently available can be classified into three different types^[41]: (1) mechanical platforms such as EndoSamurai (Olympus, Tokyo, Japan); Anubis (Karl Storz, Tuttlingen,

Germany); Direct Drive System (Boston Scientific, Natick, MA, United States) and Endosurgical Operating System (EOS, USGI Medical, San Clemente, CA, United States), which allows passage of additional larger-caliber endoscopic instruments, without possibility of triangulation^[42]; (2) computer-assisted platforms such as the master and slave transluminal endoscopic robot (University of Singapore) or da Vinci system (Intuitive Surgical, Sunnyvale, CA, United States); and (3) non-tethered systems such as mechanical or magnetic capsules. Cho *et al.*^[43] have successfully performed transgastric NOTES sigmoidectomy on a survival canine model with a custom-paired magnetic intraluminal device. Scott *et al.*^[44] have carried out complete transvaginal cholecystectomy using Magnetic Anchoring and Guidance System (MAGS) instruments on porcine models.

Pneumoperitoneum

Endoscopic insufflation may be used to maintain pneumoperitoneum, but this approach is more difficult to manage and measure than a standard laparoscopic port approach, which is specifically designed for intra-abdominal insufflation. A wider variation in pressure is observed than with laparoscopic insufflation^[45]. On the other hand, a laparoscopic port and insufflation system ensures that any excess insufflation is noted and quickly addressed. The port also allows passage of a single laparoscopic instrument into the abdomen. Horgan *et al.*^[22] have suggested that, until better instruments are developed, having one port available for use with well-developed minimally invasive instruments is important for safe natural orifice surgery at this stage. However, in 2010, von Delius *et al.*^[46] performed pressure-controlled endoscopic insufflation and found that CO₂ insufflation for NOTES showed minor advantages compared with insufflation with room air, regarding intra-abdominal visualization, but resulted in an increase in cardiac afterload.

Spatial orientation

Orientation can be a challenge for NOTES in the peritoneum, because the triangulation used by surgeons during laparoscopy is impossible. Some organs appear relatively easy to find, such as the uterus and ovaries, while others are somewhat surprisingly difficult to localize (gallbladder and spleen)^[3]. Some workers consider that the aid of EUS and miniprobes (MPs) can resolve this problem. Fritscher-Ravens *et al.*^[6] successfully accessed the heart under the guidance of EUS through transesophageal route in 2007. Varas Lorenzo *et al.*^[47] consider that EUS-guided pancreatic pseudocyst or abscess drainage represents a notable advance for NOTES, and in the future, distal pancreatectomy will probably require EUS support along the greater curvature of stomach to locate an entry point for distal pancreatic resection. MPs may also help in selecting an entry point. Fowler *et al.*^[48] have reported that their Queen's NOTES group has devised a novel method of orientation by using a magnetic device that passes within an endoscope channel allowing for 3D imaging of

the shape and orientation of the endoscope. Best *et al*^[49] have found that MAGS instrumentation for NOTES procedures did not cause tissue damage or adverse clinical outcomes in porcine abdominal walls. Fernández-Esparrach *et al*^[50] considered that it was helpful to use a CT-based image-registered navigation system to identify safe gastrointestinal access sites for NOTES and intra-peritoneal structures.

Triangulation of instruments

To date, in NOTES experience, when the target tissue is reached, retraction and dissection are virtually impossible due to the lack of the triangulation of endoscopic instruments, which can provide efficient grabbing and dissection capabilities. Dallemagne *et al*^[29] have reported some novel instruments that are currently under investigation. One prototype of operating endoscope is known as Anubis (Karl Storz). This unique four-way articulating flexible endoscope, with a built-in light and video source, has a 16-mm diameter insertion shaft with an 18-mm diameter distal articulating vertebrae section and distal head. The distal head incorporates two opposing, movable arms with 4.2-mm working channels. Another instrument named as Direct Drive System (Boston Scientific) is an ergonomic, table-mounted, operative platform providing five degrees of freedom to the tip of the instruments^[51]. MAGS, which provides a longer access port (50 cm) that provides easier deployment of instruments and suitable reach, more robust cauterizer with a longer, more rigid, pneumatically deployed tip with better reach and sufficient torque to allow blunt dissection, and a more versatile tissue retractor with bidirectional dual flexible graspers, which provides excellent cephalad fundus retraction and inferolateral infundibulum retraction^[44]. EndoSamurai (Olympus) also aims at providing triangulation of the instruments, using a different operating mode^[29]. NOTES instruments are still developing and few researchers have compared these various instruments.

Closure technique

Among the challenges of the NOTES technique, closure and suture techniques are thought to be critical in view of perforation and infection risks, especially for the transgastric and transcolonic routes. To date, several animal cases of microabscesses, peritonitis and death have been related to unsatisfactory closure of the transluminal access sites^[52]. Currently, clips are frequently used to close the defects, but these have proven to be inadequate. Ryou *et al*^[53] have compared several gastric closure methods including endoclips, surgical suturing and a suction-based suturing device. The investigators were disappointed that mucosal closure with endoclips resulted in significant air and fluid leakage *via* the line of the endoclips. Shabbir *et al*^[54] have compared gastrotomy closure with either hand-sewn, endoloop or endoclip techniques in 24 *ex vivo* porcine stomachs, and found that endoclips seem to be better for gastrotomy closure than endoloops because of their potential to endure relatively

higher pressure without any prolongation of application time. However, two leaks were still noted at the clip bite site. Meanwhile, other scholars have reported some positive experimental outcomes. McGee *et al*^[55] have demonstrated that the NDO Plicator, which is an endoscopic device designed to treat gastroesophageal reflux disease by reducing the inner diameter of the gastroesophageal junction, resulted in leak-proof gastric closure in a porcine model. Meireles *et al*^[56] have used an automated stapler (Surg ASSIST) for reliable closure of the gastrotomy incision in a live porcine model. Schoenberg *et al*^[57] have reported transgastric uterine horn ligations of porcine models with the specific absorbable NOTES loops, which are recommended for use during NOTES appendectomy. However, to date, a commercially available, simple, safe and effective endoscopic instrument for closing these puncture sites has not been created.

Perioperative complications

To date, the perioperative outcomes have been favorable in most reported studies. However, as closure technique is not mature enough, intraperitoneal infection remains a primary concern for NOTES. Yang *et al*^[58] have reported that 45 porcine models underwent transgastric or transvaginal NOTES peritoneoscopy and transumbilical laparoscopic cholecystectomy under NOTES view and found that, after antiseptic preparation such as gastric or vaginal lavage and antibiotic peritoneal irrigation, the bacterial load significantly decreased in the transgastric group, which seems as safe as the sterile transvaginal approach. von Delius *et al*^[7] consider that transesophageal NOTES mediastinoscopy carries a substantial risk of inadvertent development of pneumothorax after animal experiments. Biliary leaks have been reported in a NOTES study involving transvaginal cholecystectomy from Pugliese *et al*^[59], which were treated successfully by endoscopic drainage and stenting. The risk of infertility after transvaginal NOTES procedures is unknown, but Horgan *et al*^[22] have suggested that avoidance of bleeding and inflammation of the pelvis should minimize this potential risk.

NOTES AND LAPARO-ENDOSCOPIC SINGLE-SITE SURGERY

Closely related to NOTES, laparo-endoscopic single-site surgery (LESS) describes minimally access surgical procedures that are performed through a single incision/location^[60]. Rane *et al*^[61] published the first true LESS experience in abstract form in 2007, performing a transumbilical laparoscopic nephrectomy. As a result of the lack of ideal novel endoscopic instruments, NOTES experiences have been much more limited than LESS, and clinical experience with LESS has been more extensively reported^[62]. However, Raman *et al*^[63] have reported a retrospective case-controlled study comparing the outcomes of 11 LESS nephrectomies to 22 matched, conventional laparoscopic nephrectomies, which failed to demonstrate any significant improvement in analgesic use or conva-

lescence. To date, although few studies have focused on the comparison between NOTES and LESS, we still believe that NOTES, considered as the developing terminal minimally invasive surgery, must have a promising future.

CONCLUSION

After the familiar laparoscopic surgical techniques, NOTES has become the next worldwide focus of minimally invasive therapy. The novel surgical procedures, with fast recovery and without general anesthesia, visible scarring, postoperative hernia formation and adhesions, are attractive for most surgeons and endoscopists. Although the novel procedure is far away from being mature and many technical problems have to be overcome before its widespread application in clinical cases, NOTES is undoubtedly a promising procedure for the future. More clinical studies and creation of new NOTES-specific instruments will make NOTES a reality.

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S- Editor Wu X L- Editor Kerr C E- Editor Xiong L

Paris chinensis dioscin induces G2/M cell cycle arrest and apoptosis in human gastric cancer SGC-7901 cells

Lin-Lin Gao, Fu-Rong Li, Peng Jiao, Ming-Feng Yang, Xiao-Jun Zhou, Yan-Hong Si, Wen-Jian Jiang, Ting-Ting Zheng

Lin-Lin Gao, Yan-Hong Si, Department of Pathology and Pathophysiology, Taishan Medical University, Taian 271000, Shandong Province, China

Fu-Rong Li, School of Pharmaceutical Science, Taishan Medical University, Taian 271000, Shandong Province, China

Peng Jiao, Ming-Feng Yang, Institute of Basic Medical Sciences, Taishan Medical University, Taian 271000, Shandong Province, China

Xiao-Jun Zhou, Wen-Jian Jiang, Ting-Ting Zheng, Department of Clinical Medicine, Taishan Medical University, Taian 271000, Shandong Province, China

Author contributions: Gao LL and Jiao P performed the majority of experiments; Gao LL and Li FR designed the research; Zhou XJ, Si YH, Zheng TT and Jiang WJ participated in cell culture and part of laser scanning confocal microscopic detection; Yang MF analyzed the data of the detection results; Li FR and Jiao P provided the vital reagents and analytical tools and were involved in editing the manuscript; Gao LL and Li FR edited the manuscript; Li FR and Jiao P analyzed the data.

Supported by The grant from the Department of Education of Shandong Province, China, No. J10LF18

Correspondence to: Lin-Lin Gao, MD, Department of Pathology and Pathophysiology, Taishan Medical University, No. 2, Yingshengdong Road, Taian 271000, Shandong Province, China. ytgd98@yahoo.cn

Telephone: +86-538-6225010 Fax: +86-538-6222036

Received: February 14, 2011 Revised: May 6, 2011

Accepted: May 13, 2011

Published online: October 21, 2011

Abstract

AIM: To investigate the anti-tumor effects of *Paris chinensis* dioscin (PCD) and mechanisms regarding cell cycle regulation and apoptosis in human gastric cancer SGC-7901 cells.

METHODS: Cell viability was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. Cell apoptosis was evaluated by flow cytometry and laser scanning confocal microscope (LSCM) using Annexin-V/propidium iodide (PI) staining, and the cell cycle was evaluated using PI staining with flow cytometry.

Intracellular calcium ions were detected under fluorescence microscope. The expression of cell cycle and apoptosis-related proteins cyclin B1, CDK1, cytochrome C and caspase-3 was measured by immunohistochemical staining.

RESULTS: PCD had an anti-proliferation effect on human gastric cancer SGC-7901 cells in a dose- and time-dependent manner. After treatment of SGC-7901 cells with PCD, apoptosis appeared in SGC-7901 cells. Morphological changes typical of apoptosis were also observed with LSCM by Annexin V/PI staining, and the cell number of the G0/G1 phase was decreased, while the number of cells in the G2/M phase was increased. Cell cycle-related proteins, such as cyclin B1 and CDK1, were all down-regulated, but caspase-3 and cytochrome C were up-regulated. Moreover, intracellular calcium accumulation occurred in PCD-treated cells.

CONCLUSION: G2/M phase arrest and apoptosis induced by PCD are associated with the inhibition of CDK-activating kinase activity and the activation of Ca²⁺-related mitochondrion pathway in SGC-7901 cells.

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Key words: CyclinB1/CDK1; Cell cycle arrest; Caspase-3, Ca²⁺; Cytochrome C

Peer reviewers: Dr. Lucia Ricci Vitiani, Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena, 299, Rome 00161, Italy; Dr. Jianyuan Chai, Assistant Professor, Research (09-151), VA Long Beach Healthcare System, 5901 E. 7th St, Long Beach, CA 90822, United States

Gao LL, Li FR, Jiao P, Yang MF, Zhou XJ, Si YH, Jiang WJ, Zheng TT. *Paris chinensis* dioscin induces G2/M cell cycle arrest and apoptosis in human gastric cancer SGC-7901 cells. *World J Gastroenterol* 2011; 17(39): 4389-4395 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4389.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4389>

INTRODUCTION

Gastric cancer is the most common cause of death from cancer in China^[1,2]. Recent evidence suggests that cell apoptosis is closely related to the occurrence, progress and metastasis of tumors^[3-5]. The mechanisms of apoptosis in tumor cells is an important field of study for tumor treatment and molecular cancer biology^[6].

The progression of cells through the cell cycle is tightly controlled by the sequential activation and inactivation of a family of serine-threonine kinases known as the cyclin-dependent kinases (CDKs). In particular, CDK1 controls progression from the S phase through G2 and into the M phase. Similarly, progression from the G1 to S phase is controlled sequentially by CDK4/6 and CDK2. CDK activity is regulated by binding to cyclin partners and the action of endogenous inhibitory peptides^[7,8].

Loss of cell cycle control, leading to uncontrolled proliferation, is common in cancer. Therefore, the identification of potent and selective cyclin dependent kinase inhibitors is a priority for anti-cancer drug discovery.

Paris chinensis (Liliaceae) is distributed in many regions of the world, such as India, China, Vietnam, and Germany. As a traditional Chinese medicine, it grows wildly throughout South China and has been used mainly as a folk remedy for treatment of abscesses, throat swelling and pain, thanatophidia bites, contused wounds and convulsions^[9] for centuries. It is also the major component of the famous Chinese patent medicine *Yunnan Baiyao Powder* and snake-bite therapeutics. It also has been used to treat liver cancer in China for many decades^[10-12]. The active components of *Paris chinensis* are the saponin steroids polyphyllin D, dioscin, and balanitin 7. Among its three chemical constituents, polyphyllin D has been previously reported^[13-15] to circumvent drug resistance and elicit apoptosis in HepG2 and R-HepG2 cells *via* mitochondrial damage. However, as there has been no documentation of the use of the other important steroid saponin dioscin in the treatment of cancer, its mechanisms in human gastric cancer cells remain unknown.

Therefore, the aim of the present study was to evaluate the effects of *Paris chinensis* dioscin (PCD) on human gastric cancer SGC-7901 cells and the signaling pathways involved in PCD-induced apoptosis.

MATERIALS AND METHODS

Chemicals and reagents

PCD with a purity of 99% was purchased from Yuancheng Science and Technology Corporation (Wuhan, China). RPMI-1640 medium, 4-hydroxyethyl piperazine ethanesulfonic acid (HEPES), fetal calf serum and trypsin were purchased from Gibco BRL Life Technologies Inc. (Grand Island, New York, United States). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin, streptomycin and trypsin were purchased from Amresco Chemical Co. Ltd. (United States). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were purchased from Sigma (St. Louis, United States). The fluorescent probe Fluo-3/AM is a prod-

uct of Molecular Probes Incorporated (United States). The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from BD Biosciences (United States). The primary antibodies for cyclinB1, CDK1, caspase-3, cytochrome C and β -actin and the secondary antibody were acquired from Santa Cruz Biotechnology. Fetal bovine serum (FBS) was purchased from Hyclone (United States), and all chemicals were of analytical grade and were obtained from Tianjin Chemical Reagents Co. Ltd. (Tianjin, China).

Cell culture

SGC-7901 cells were obtained from the Chinese Type Culture Collection (Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, China). SGC-7901 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 μ g/mL) at 37°C in a humidified atmosphere of 95% air and 5% CO₂; the medium was changed every other day. When the cultures were 80%-90% confluent, the SGC-7901 cells were washed with phosphate-buffered saline (PBS), detached with 0.25% trypsin, centrifuged and re-plated onto 96- or 24-well plates at an appropriate density according to each experimental scale.

Cell viability and cytotoxicity

The cultured cells at the exponential growth phase were harvested from the culture flasks by trypsin and then resuspended in fresh medium. The cell suspensions were dispensed into a 96-well microplate at 100 μ L/well and incubated in an incubator with 5% CO₂ at 37°C. After 24 h, 200 μ L of various concentrations (0-500 μ g/mL) of PCD were added and incubated for 12, 24, 36, 48, 60 and 72 h to evaluate their anti-proliferation effects on SGC-7901. The cell proliferation in the microplate was determined using the MTT assay^[16] after incubation. Twenty microliters of PBS solution containing 5 mg/mL MTT was added to each well. After incubation for 4 h, the cells from each well were solubilized with 100 μ L DMSO for optical density determination at 570 nm. Cell proliferation activity was expressed as the percentage of MTT counts of treated cells relative to those of the control (% of control). The IC₅₀ was taken as the concentration that caused 50% inhibition of cell viabilities and was calculated by the Logit method.

Observation of morphological changes

The SGC-7901 cells were seeded into six-well plates (2.0 \times 10⁵ cells/well) and incubated in RPMI-1640 at 37°C in an atmosphere of 5% CO₂ for 24 h. The cells were treated with several concentrations of PCD. After incubation for 24 h, cellular morphology was observed under a phase contrast microscope (Nikon, Japan). The photographs were taken at a magnification \times 40.

Cell cycle analysis

SGC-7901 cells (2 \times 10⁶ cells/mL) in 100-mm culture dishes were incubated with PCD for 24 h, then harvested by trypsinization and fixed with 90% ice-cold ethanol.

The fixed cells were incubated with a staining solution containing 0.2% NP-40, RNase A (30 µg/mL), and propidium iodide (PI) (50 µg/mL) in a phosphate-citrate buffer (pH 7.2). Cellular DNA content was analyzed by flow cytometry (BD FACS Calibur, United States). At least 10 000 cells were used for each analysis, and the results were displayed as histograms.

Flow cytometry and LSCM analysis of cell apoptosis

SGC-7901 cells were cultured in RPMI-1640 with 10% fetal bovine serum. Before the cell density was modulated to 1×10^5 cells, cell synchronization was conducted to force the cells to the G0 phase *via* a serum-free culture for 12 h, and the cells were washed twice with PBS before being suspended in a binding buffer (10 mmol/L HEPES pH 7.4, 140 mmol/L NaOH, and 2.5 mmol/L CaCl₂). Five microliters of fluorescein isothiocyanate (FITC)-labeled Annexin V was mixed with 100 µL cell suspensions containing 1×10^5 cells, and the cells were incubated at room temperature for 5 min. Thereafter, 50 µL PI solution (10 µg/mL) was added to the cells, followed by an additional 5-min incubation. The scatter parameters of the cells (20 000 cells per experiment) were analyzed using a FACS flow cytometer and Cell Quest analysis software (Becton-Dickinson, CA). Four cell populations were identified according to the following interpretations: viable population in the lower-left quadrant (low-PI and FITC signals), early apoptotic population in the lower-right quadrant (low-PI and high-FITC signals), necrotic population in the upper-left quadrant (high-PI and low-FITC signals), and late apoptotic or necrotic population in the upper-right quadrant (high-PI and high-FITC signals).

At this point, cells treated in the manner described above were examined on a glass slide using a laser-scanning confocal microscope (LSCM) (Bio-Rad Radiance2100, United States) with 488-nm excitation and 525-nm emission wavelengths. Bright green fluorescence was manifested in membranes of the cells undergoing prophase apoptosis because of Annexin V-FITC staining, while nuclear cardinal red fluorescence was associated with advanced stage apoptosis because of PI staining.

Measurement of intracellular calcium

The intracellular calcium ion ($[Ca^{2+}]_i$) was measured as previously described^[17]. After confluence, SGC-7901 cells on a coverslip were loaded by the $[Ca^{2+}]_i$ indicator Fluo-3/AM in HEPES solution at 37°C in the dark for 30 min. HEPES solution contains (concentration in mmol/L): NaCl 118, KCl 4.8, CaCl₂ 2.5, KH₂PO₄ 1.2, HEPES 5, and glucose 10. The pH was brought to 7.4 with NaOH. The final concentration of Fluo-3/AM was 5 µmol/L. After loading with Fluo-3/AM, a fluorescence image of $[Ca^{2+}]_i$ was taken using a laser-scanning confocal microscope (Bio-Rad Radiance2100, United States) at 600×, and qualitative changes of $[Ca^{2+}]_i$ were inferred from the fluorescence intensity using SimplePCI imaging systems (Simple PCI, Compix Inc., United States).

Western blotting analysis

Twenty µg of protein in each 20-µL sample was electrophoresed through 10% SDS-PAGE gels as previously described^[18]. Separated proteins were incubated with primary antibodies overnight at 4 °C, transferred to nitrocellulose membranes, and blocked with a 5% skim milk solution. They were incubated with secondary antibodies for 1 h at 37 °C. Each antigen-antibody complex was visualized by enhanced chemiluminescence Western blotting detection kits (Amersham Pharmacia Biotech, Piscataway, NJ), and band densities were determined using Chemi Doc Software (BioRad); β-Actin was used as a loading for normalization.

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are given as the mean ± SE. Statistical analysis was performed using the statistical software package SPSS 13.0 (SPSS). A *P* value of 0.05 (two-sided) was considered statistically significant.

RESULTS

Cytotoxic activity of PCD on SGC-7901 cells

As shown in Figure 1A, vehicle-treated SGC-7901 cells (control) grew well with clear skeletons, whereas cells treated with PCD exhibited cytoplasmic shrinkage and either detached from each other, floated in the medium, or became distorted and blurry under a phase contrast microscope. The number of sloughed cells increased with increasing drug concentrations. The MTT assay showed that PCD significantly inhibited the viability of SGC-7901 cells (Figure 1B). The cells were incubated in the absence or presence of various concentrations of PCD for specified time periods, and the IC₅₀ values were 13.77 ± 0.18 , 8.73 ± 0.41 , and 3.62 ± 0.29 mg/mL for 24, 48 and 72 h, respectively. The MTT assay showed that PCD decreased the viability of SGC-7901 cells in a concentration- and time-dependent manner (*P* < 0.05 and *P* < 0.01, respectively).

Effect of PCD on cell cycle phase distribution of SGC-7901 cells

To investigate whether PCD affects the cell cycle of SGC-7901 cells, the cell cycle distribution of synchronized cells treated with or without PCD were analyzed by measuring the DNA content with PI after exposure to PCD for 24 h. As shown in Figure 2B, compared to vehicle treatment ($59.26\% \pm 5.12\%$), PCD treatment reduced the percentage of the cells in the G1 phase to $43.58\% \pm 1.79\%$, $49.58\% \pm 1.79\%$ and $45.58\% \pm 1.79\%$, respectively (*P* < 0.05). The percentage of G2/M cells was $12.48\% \pm 1.71\%$ in control cells and increased to $24.48\% \pm 1.62\%$, $33.00\% \pm 3.16\%$ and $38.32\% \pm 3.90\%$ in the cells treated with 10, 50 and 250 µg/mL of PCD for 24 h, respectively. These results showed that PCD exerted its effect of G2/M phase cell cycle arrest rather than S phase arrest induction in SGC-7901 cells, which contributed to the effects of PCD on decreasing viability

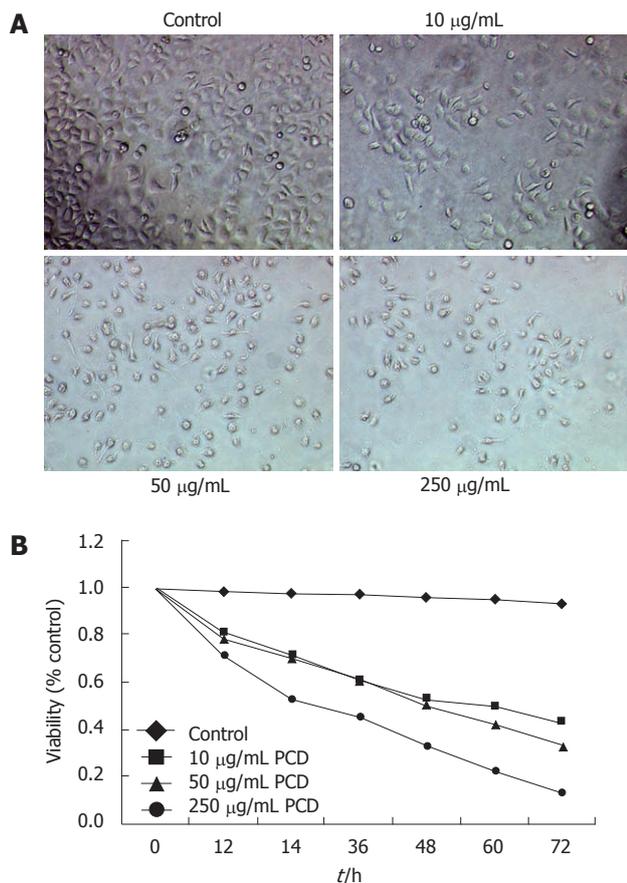


Figure 1 *Paris chinensis* dioscin inhibits the viability of SGC-7901 cells. A: Morphological changes of SGC-7901 cells exposed to *Paris chinensis* dioscin (PCD) for 24 h imaged under a phase contrast microscope at 40 ×; B: Effect of PCD on SGC-7901 viability. SGC-7901 cells were treated with PCD at the indicated concentrations for 0-72 h. Cell viability was then determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and expressed as the mean ± SD, n = 3. The optical density value at 570 nm is proportional to the number of cells with PCD.

against SGC-7901 cells.

To gain insight into the mechanism of G2/M phase cell cycle arrest induced by PCD, we examined the expression of cyclins B and CDK1, which are closely related to G2/M cell cycle progression, using the Western blot assay. As shown in Figure 2C, the expression of cyclin B1 and CDK1 was decreased after PCD treatment for 24 h.

Effect of the PCD on apoptosis in SGC-7901 cells

To identify whether PCD induces apoptosis, the treated cells were also stained with Annexin V-FITC/PI, and the population of apoptotic cells was analyzed by flow cytometry. As seen in Figure 3A, the drug treatment significantly increased the proportion of apoptotic cells. In the vehicle treated cells, 12.04% ± 1.62% were positive for Annexin V-FITC staining, while PCD treatment resulted in increases of 17.18% ± 2.58%, 24.75% ± 2.72% and 54.91% ± 3.35% in apoptosis when cells were treated with PCD (P < 0.05 and P < 0.01, respectively). These results demonstrate the ability of PCD to induce apoptosis in SGC-7901 cells. The morphologic changes of cells treated in the manner described above were also observed under LSCM by Annexin V/PI staining. As shown in Figure 3B, typical

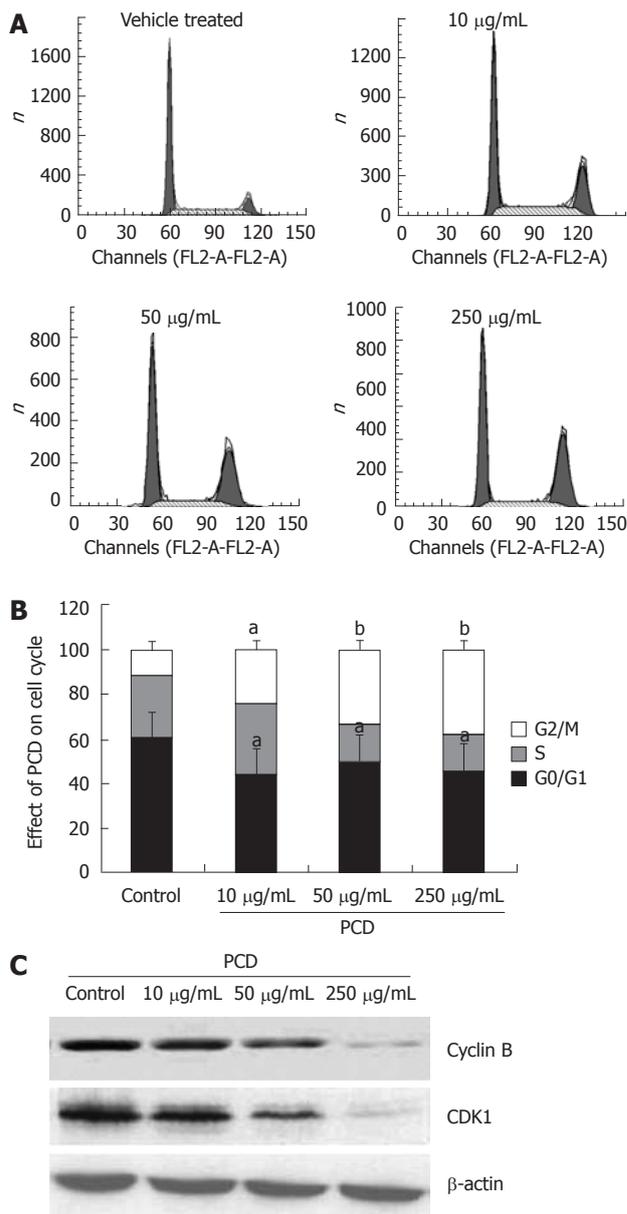


Figure 2 *Paris chinensis* dioscin induces G2/M cell cycle arrest in SGC-7901 cells. A: Cell cycle distribution was monitored by flow cytometry using a propidium iodide staining assay; B: Histogram of cell cycle distribution after treatment with *Paris chinensis* dioscin (PCD) for 24 h. Cell cycle distribution was monitored by flow cytometry using a propidium iodide staining assay. Each histogram represents three parallel experiments, and each bar represents the mean ± SE (One-way ANOVA). ^aP < 0.05, ^bP < 0.01 vs vehicle treated (control); C: Western blotting analysis of the expression of cyclin B and CDK1 with or without PCD treatment of SGC-7901 cells.

morphological changes, such as the formation of apoptotic bodies, appeared after the cells were treated for 24 h with 250 µg/mL PCD, whereas the vehicle-treated cells did not show evident apoptotic morphological changes.

To determine whether apoptosis induced by PCD was due to a mitochondrial-dependent caspase pathway, we further tested whether cytochrome C could be released from the mitochondria into the cytoplasm. We next investigated the levels of cytochrome C and caspase-3, which was the core protein in the caspase cascade in the soluble cytosolic fractions of SGC-7901 cells, after PCD treatment for 24 h. Figure 3C shows that PCD increased the

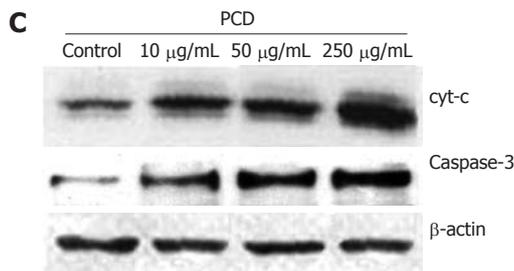
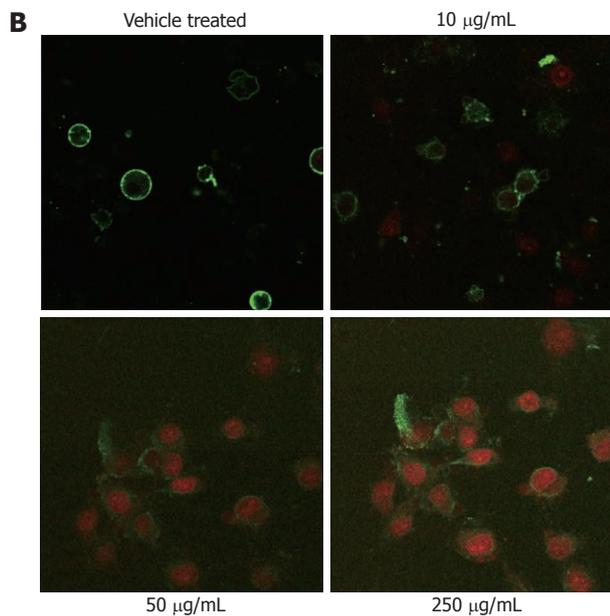
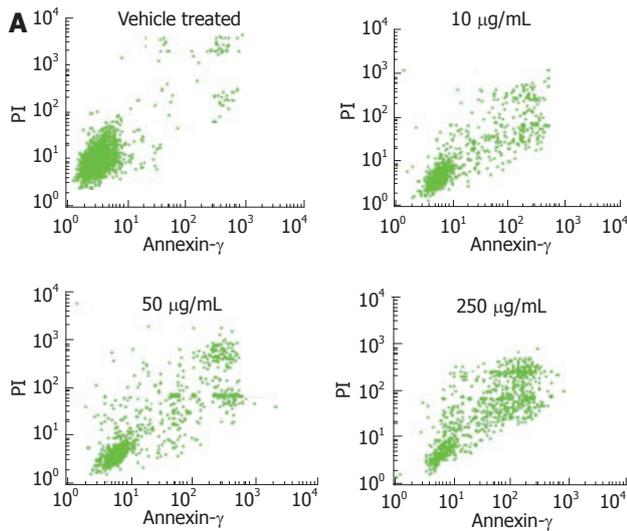


Figure 3 *Paris chinensis* dioscin induces apoptosis in SGC-7901 cells. A: Apoptotic cells determined by flow cytometry assay; B: Morphological changes of SGC-7901 cells as determined with a laser scanning confocal microscope at 600 × treated with *Paris chinensis* dioscin; C: Western blotting analysis of the expressions of caspase-3, cytochrome C and β-actin (internal control) in control and PCD-treated SGC-7901 cells. PCD: *Paris chinensis* dioscin. PI: Propidium iodide.

level of cytochrome C released into the cytosol, and the expression of caspase-3 was increased after PCD treatment for 24 h compared with the vehicle-treated cells ($P < 0.05$), which indicated that PCD increased the caspase-3 level in SGC-7901 cells. Moreover, cells treated with PCD

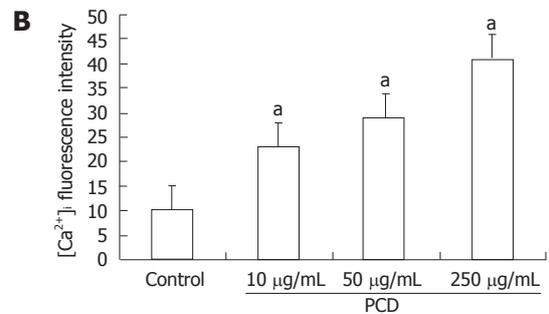
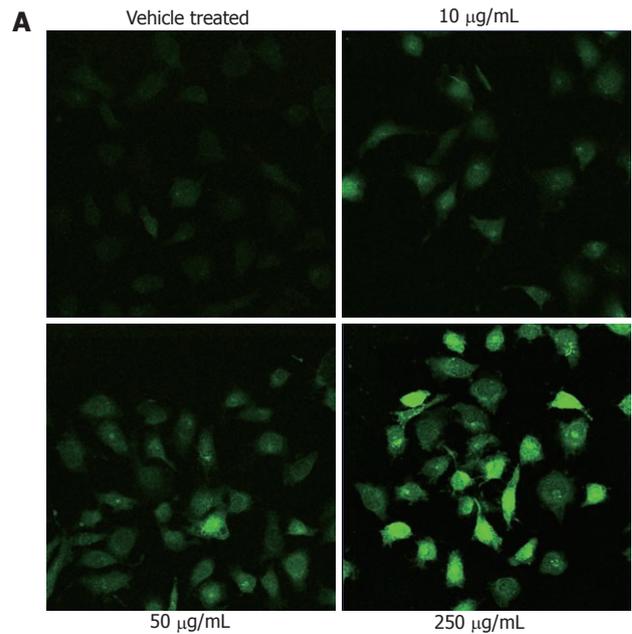


Figure 4 Effects of *Paris chinensis* dioscin on intracellular $[Ca^{2+}]_i$ expression in human gastric cancer SGC-7901 cells. A: Fluorescence image of $[Ca^{2+}]_i$ under laser scanning confocal microscope at 600 ×; B: Qualitative changes of $[Ca^{2+}]_i$ were inferred from the fluorescence intensity after *Paris chinensis* dioscin (PCD) treatment for 24 h, using SimplePCI imaging systems. Data are presented as mean ± SD (error bar). * $P < 0.01$ vs control.

exhibited a dose-dependent increase at this level ($P < 0.05$).

Effect of PCD on $[Ca^{2+}]_i$ in SGC-7901 cells

To explore whether PCD-induced apoptosis involved $[Ca^{2+}]_i$, we used the $[Ca^{2+}]_i$ indicator Fluo-3/AM to detect $[Ca^{2+}]_i$ changes after PCD treatment with various densities. As shown in Figure 4, $[Ca^{2+}]_i$ fluorescence intensity in the group treated with 250 μg/mL PCD was higher than in the vehicle-treated and lower concentration groups ($P < 0.01$), and PCD treatment with 10, 50 and 250 μg/mL induced an increase by 41% ± 4.72%, 66% ± 5.61%, and 86% ± 7.25% *vs* the vehicle-treated cells (25.33% ± 2.17%) ($P < 0.01$, $n = 6$) in Fluo-3/AM fluorescence intensity after 24 h treatment, respectively. These results suggest that the PCD can induce a dose-dependent $[Ca^{2+}]_i$ influx and might induce apoptosis or necrosis that follows *via* calcium ion overload.

DISCUSSION

Natural products with anticancer properties could be

valuable substances in cancer treatment, and this study examined the effect of PCD and its underlying mechanisms on the inhibition of tumor cell proliferation. In this study, we assessed the inhibitory effects and molecular mechanisms of PCD using human gastric cancer SGC-7901 cells. MTT showed that PCD inhibited the growth of SGC-7901 cells in both time-dependent and concentration-dependent manners (Figure 1B). To determine whether the cytotoxic activity of PCD was due to apoptosis, SGC-7901 human stomach carcinoma cells were treated for 24 h with various concentrations of PCD. Not only were morphological changes such as cytoplasmic shrinkage, detachment from each other, floating in the medium, distortion and some blurring under a fluorescence microscope observed (Figure 1A), but marked chromatin condensation and apoptotic body formation in PCD-treated cells were also observed in cells stained with Annexin V-FITC/PI using an LSCM (Figure 3B). Flow cytometry with Annexin V-FITC/PI staining showed that the drug treatment significantly increased the proportion of apoptotic cells, confirming that PCD induced apoptosis in SGC-7901 cells. Dysregulation of the cell cycle mechanism has also been shown to play an important role in the growth of various types of cancer cells, and the induction of cancer cell apoptosis is recognized as an important target in cancer therapy. In this study, PCD-inhibited SGC-7901 cell proliferation resulted partly from an accumulation of cells in the G2/M phase of the cell cycle. The G2/M phase is associated with DNA synthesis and the mitotic preparation period, which plays a crucial role in cell cycle progression. The complex formation of cyclins with CDKs results in an active agent that phosphorylates substrates involved in cell cycle progression^[19]. The mitosis-promoting factor, which comprises a complex of CDK1 and cyclin B, is thought to be the key controller of the progression from G2 to mitosis^[20-22]. In this study, PCD induced G2/M phase cell cycle arrest (Figure 2A and B), the cells of G2/M phase were present at 3.2 folds of the typical concentration after 24 h treatment, and Cyclins B1 and CDK1 were downregulated (Figure 2C), indicating that cell cycle-related proteins were involved in the PCD-induced cell cycle arrest in SGC-7901 cells.

The accumulated data suggest that the mitochondria-initiated death pathway plays an important role in triggering apoptosis in response to those stimuli. In the mitochondria-initiated death pathway, mitochondria undergoing permeability transition release apoptogenic proteins such as cytochrome C or apoptosis-inducing factor from the mitochondrial inter-membrane space into the cytosol. Released cytochrome C can activate caspase-9, and activated caspase-9 in turn cleaves and activates executioner caspase-3.

The apoptotic process is preceded by the collapse of the mitochondrial potential, the opening of a multi-protein structure named the permeability transition pore, which could be triggered by multiple stimuli such as changes in Ca^{2+} , oxygen radicals, pH, swelling of the matrix and rupture of the outer membrane with ensuing changes in the permeability of the outer mitochondrial membrane, and release of apoptogenic factors including cytochrome C from mitochondria^[23-26]. Changes in cell cycle arrest and apoptosis are listed below.

In this study, Western blot showed that cytochrome C increased in cytoplasm accompanying caspase-3 upregulation after PCD treatment (Figure 3C), indicating that the mitochondrial apoptotic pathway played a pivotal role in PCD-induced apoptosis of SGC-7901 cells.

Aside from the mechanisms described above, Ca^{2+} plays a critical role in this process, and intracellular Ca^{2+} overload appears to mediate the lethal effects of receptor overactivation^[27].

Ca^{2+} overload has even been suggested to be the final common pathway for all types of cell death. Over the last few years, several studies have shown that increases of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_e$) occur, both at early and late stages of the apoptotic pathway^[28-33].

More specifically, it has been suggested that both Ca^{2+} release from the endoplasmic reticulum (ER) and capacitive Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels are apoptogenic^[34-36]. There are also data suggesting that very high intracellular Ca^{2+} levels can promote cell death through necrosis, whereas lower intracellular Ca^{2+} increases induced by milder insults promote cell death through apoptosis^[37,38]. In this study, the $[Ca^{2+}]_e$ fluorescence intensity of cells loaded with Fluo-3/AM under a fluorescence microscope in the group treated with 250 $\mu\text{g}/\text{mL}$ PCD was obviously higher than in the control and lower concentration groups (Figure 4).

Corbiere *et al.*^[39] reported that diosgenin-induced apoptosis in different human cancer cells is caspase-3-dependent and is concomitant with a fall in the mitochondrial membrane potential. We characterized the mechanisms by which PCD exerts its inhibitory effects on SGC-7901 cells by inducing G2/M cell cycle arrest and Ca^{2+} - cytochrome C- apoptosis.

Therefore, our results suggest that PCD may be a potential candidate as a novel therapeutic agent originating from a natural source, and the induction of apoptosis by PCD in other cancer cell lines is the subject of on-going investigations.

COMMENTS

Background

Gastric cancer is the most leading cause of death from cancer in China and majority in the world. Currently, no effective treatment is available. Therefore, there is a critical need to develop effective chemotherapeutic strategies for gastric cancer.

Research frontiers

There has been no documentation of the use of the other important steroid saponin dioscin in the treatment of cancer, its mechanisms in human gastric cancer cells remain unknown.

Innovations and breakthroughs

This is the first report on the anti-proliferation, induction of apoptosis by *Paris chinensis* dioscin (PCD) on human gastric cancer SGC-7901 cells. The authors characterized the mechanisms by which PCD exerts its inhibitory effects on SGC-7901 cells by inducing G2/M cell cycle arrest and Ca^{2+} - cytochrome C- apoptosis.

Applications

PCD might be useful as an adjuvant drug in human gastric cancer treatment.

Terminology

Paris chinensis (Liliaceae) is a Traditional Chinese Medicine and has been used mainly as a folk remedy for treatment of thanatophidia bites and convulsions for centuries. The active components of *Paris chinensis* are the saponin steroids polyphyllin D, dioscin, and balanitin 7.

Peer review

This is an interesting and good paper mainly due to its potential clinical application.

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Physical activity and nutrition attitudes in obese Hispanic children with non-alcoholic steatohepatitis

Lana N Hattar, Theresa A Wilson, Leanel A Tabotabo, E O'Brian Smith, Stephanie H Abrams

Lana N Hattar, Leanel A Tabotabo, Stephanie H Abrams, Division of Pediatric Gastroenterology, Hepatology, and Nutrition, Baylor College of Medicine, Houston, TX 77030, United States
Lana N Hattar, Theresa A Wilson, Leanel A Tabotabo, E O'Brian Smith, Stephanie H Abrams, Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, United States
Author contributions: Hattar LN and Abrams SH designed the research; Hattar LN, Tabotabo LA and Abrams SH performed the research; Wilson TA contributed with the School Physical Activity and Nutrition Survey questionnaire experience-analytic tools; Hattar LN, Smith EO and Abrams SH analyzed the data; and Hattar LN and Abrams SH wrote the paper with other authors as members of the writing committee.

Supported by National Institute of Health [NIH K12 RR 17665 (Morey Haymond, MD (PI); C Wayne Smith, MD (Mentor)]

Correspondence to: Lana N Hattar, MD, Division of Pediatric Gastroenterology, Hepatology, and Nutrition, Baylor College of Medicine, Houston, TX 77030, United States. lhattar@yahoo.com
Telephone: +1-316-2688040 Fax: +1-316-2914880

Received: February 13, 2011 Revised: April 21, 2011

Accepted: April 28, 2011

Published online: October 21, 2011

Abstract

AIM: To assess nutrition, physical activity and healthful knowledge in obese children with biopsy-proven non-alcoholic steatohepatitis (NASH or NA) compared to children without liver disease.

METHODS: Children with biopsy-proven NASH comprised the NASH group. Age, sex and ethnicity matched control groups consisted of obese (OB) and lean (CO) children with no liver disease. Subjects were administered the School Physical Activity and Nutrition Survey and one blood draw was obtained.

RESULTS: Fifty-seven patients were enrolled with a mean age of 12.1 ± 2.1 years, and all were Hispanic. Even though the OB and NA had a similar increased body mass index (%), 35% of the NA group always read nutrition labels compared to none in the OB (P

< 0.05), and more NA children felt their diet is "less healthy". NA consumed the least amount of fruits with only 25% having ≥ 1 fruit/d *vs* 45% in OB and 64.7% in CO ($P < 0.05$ NA *vs* CO). Only 15% of NA subjects performed light exercise *vs* 35% and 59% of OB and CO groups, respectively ($P = 0.02$). The mean physical activity score was lowest in the NA group ($P < 0.05$). Amongst the subjects with NASH, we found that 100% of patients with grade 2 or 3 fibrosis had a sedentary score > 2 compared to only 63.6% of those with grade 1 or no fibrosis ($P < 0.05$).

CONCLUSION: Children with NASH had increased sedentary behavior, decreased activity, and fruit intake. Larger studies may determine the benefit of changing these behaviors as treatment for NASH.

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Key words: Non-alcoholic steatohepatitis; Hispanic; Pediatric; Nutrition; Physical activity; School physical activity; Nutrition survey

Peer reviewer: Dr. Seyed Mohsen Dehghani, MD, Associate Professor, Department of Pediatrics, Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, Iran

Hattar LN, Wilson TA, Tabotabo LA, Smith EO, Abrams SH. Physical activity and nutrition attitudes in obese Hispanic children with non-alcoholic steatohepatitis. *World J Gastroenterol* 2011; 17(39): 4396-4403 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4396.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4396>

INTRODUCTION

The prevalence of obesity is increasing in epidemic proportions in both adults and children. This has tripled from 1980 for the age group 6 to 19 years, with national health and nutrition examination survey (NHANES) reporting

17.4%-18.8% of children being obese^[1]. The evidence is clear; obese children become obese adults. In fact, obese 10 to 14 years old adolescents are more than 20 times more likely to become obese adults^[2]. This is unfortunate, since obesity is associated with metabolic syndrome, which has been reported in up to 50% of severely obese children^[3], and other obesity-associated diseases, such as fatty liver disease.

As the epidemic of childhood obesity progresses, it is predicted that the prevalence of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steato-hepatitis (NASH or NA) will also increase^[4]. NAFLD is an umbrella term that includes simple steatosis and simple steatosis with the presence of lobular inflammation. These two forms of NAFLD are generally considered to be non-progressive. Once a patient has portal inflammation, ballooning degeneration of hepatocytes or fibrosis the patient has the type of NAFLD that has been shown to be progressive, or NASH. A diagnosis of NAFLD may be made when the patient has elevated liver enzymes in combination with radiography demonstrating fatty infiltration in the liver. NAFLD is now considered to be the most common cause of chronic liver disease in the pediatric population^[5]. Studies using non-invasive techniques have been performed worldwide and have shown that NAFLD may be present in up to 77% of obese children^[6]. Schwimmer *et al.*^[7] recently reported a population-based study where 13% of all children had biopsy-proven NAFLD and 3% had NASH.

The pathogenesis of the development of NAFLD is not completely understood. In 1999, Day and James^[8] published the current theory “the two hit hypothesis”. The first “hit” is believed to be the development of steatosis, while the second “hit” leads to the progression to inflammation, necrosis and fibrosis. Oxidized by-products are harmful adducts that can cause liver injury, resulting in subsequent fibrosis^[9]. Day^[10] has subsequently pointed out that genetic factors influence the host response to endotoxin, oxidative stress, and severity of steatosis and that environmental factors, such as a diet high in saturated fat or low in antioxidant vitamins, influence the host’s susceptibility to fatty liver disease.

There are still no approved pharmacological therapies for NAFLD/NASH. Therefore, current management is based upon the presence of associated risk factors and aims to improve an individual’s quality of life, thus reducing NAFLD-associated morbidity and mortality. Today, lifestyle intervention (diet and exercise) is the treatment of choice for NAFLD/NASH. Significantly high levels of serum triglycerides, glucose, insulin, alanine aminotransferase (ALT), increased body mass index (BMI) and waist circumference (central adiposity) are all possible clinical features of pediatric NAFLD, which suggests that interventions on these variables can help to treat fatty liver, as well as to prevent progression to NASH^[11]. On the other hand, resolution of histological abnormalities revealed by liver biopsy is, at this time, the main target of NASH treatment^[12]. Studies on pediatric subjects have

shown that moderate weight loss can improve BMI and serum levels of ALT, and reduce fatty liver infiltration and necro-inflammation, although no change has been demonstrated in degree of fibrosis^[13].

One way to assess a child’s diet and physical activity is the School Physical Activity and Nutrition (SPAN) questionnaire^[14], which was developed as a surveillance instrument to measure physical activity, nutrition attitudes, and dietary and physical activity behaviors in children and adolescents. This tool can assess many dietary and physical behaviors over the 24 h prior to questionnaire administration. The food behaviors measured by this instrument include recall of certain foods from the previous day. This type of short-term recall is better for children because their cognitive skill has not developed sufficiently to estimate averaging and frequency as found in a traditional food frequency questionnaire^[15]. It has been validated and found to be reproducible^[16,17].

We hypothesize that there are differences in the dietary behaviors, physical activity and healthful knowledge between obese children who develop NASH and obese and lean children with no evidence of liver disease. These differences would be of greater clinical relevance when examined in subjects who share a similar genetic background and who are affected most frequently by NASH, i.e., being of Hispanic origin. Therefore, identifying those potential environmental differences may have important implications which may guide us towards treatment and possibly even prevention of this condition in the future.

MATERIALS AND METHODS

To test the hypothesis that there are differences in the dietary and physical activity habits between obese children with NASH and obese and lean children with no evidence of liver disease, we enrolled 57 children between the ages of 8 and 16 years into this pilot study. We categorized them into three different groups: the “NA” or NASH group which included 20 obese children with biopsy-proven NASH, the or obese control group (OB) which included 20 obese children with no evidence of liver disease, and the or lean control group (CO) which included 17 lean children with no evidence of liver disease.

Inclusion and exclusion criteria

Each subject in the NA group had a history of chronic serum ALT elevation defined by an ALT > 40 U/L on two separate occasions separated by at least 3 mo (90 d apart). Subjects also underwent a work-up for other causes of chronic hepatitis of unknown etiology including serologic evaluation for alpha-1-antitrypsin with phenotype, hepatitis B surface antigen, hepatitis C antibody, copper, ceruloplasmin, anti-nuclear antibody, anti-smooth muscle antibody (F-actin), anti-liver kidney microsomal antibody, and total immunoglobulin G. Subsequently, the NA subjects had a standard of care liver biopsy to identify the etiology and severity of chronic transaminitis. To be included in the study, the biopsy demonstrated a minimum of 5% of

hepatocytes with macrovesicular fat, without other etiologies for the presence of fat being identified and a pattern of injury consistent with NASH, as determined by an anatomic pathologist. All biopsies were also scored according to the NAFLD Activity Score (NAS), which is the sum of grades for steatosis (0-3), ballooning degeneration (0-2) and lobular inflammation (0-3)^[18]. Each biopsy also received a fibrosis score. All NA subjects had their liver biopsy confirming the diagnosis of NASH within 60 d of study enrollment.

In the OB group, each subject had a BMI percentile > 95% for age/sex as determined on growth chart by the Center for Disease Control (CDC), and no history of abnormal liver enzymes or chronic liver disease. If serum ALT levels were not drawn within the last 6 mo, subjects underwent screening ALT as a requirement for eligibility. If the subject had an ALT > 40 U/L on the day of the study visit, the subject was excluded ($n = 2$).

For inclusion in the CO group, each subject had a normal weight as defined by having a BMI percentile > 5% but less than 85% for age/sex as determined on a growth chart by the CDC. This was followed by Dual X-ray Absorptiometry (DXA) demonstrating less than 30% fat mass for females and less than 25% for males. Two male subjects were excluded for DXA % fat mass > 30% on the day of the study visit. CO subjects also had no history of abnormal liver enzymes or chronic liver disease.

We excluded any subject who had a disease considered by the study physician to be significant, including history of cancer or immunosuppressed state. We also excluded anyone with history of significant alcohol intake, use of medication known to cause NAFLD, or history of total parenteral nutrition. This protocol was reviewed and approved by the institutional review board of Baylor College of Medicine.

SPAN

All 57 subjects underwent the same evaluation; one study visit which included obtaining one blood draw and the administration of the SPAN^[14] questionnaire. This questionnaire was developed as a surveillance instrument to measure physical activity, nutrition attitudes, and food behaviors in children and adolescents. It has two versions, one for elementary students and the other for middle/high school students. We used the elementary school version, which is 10 pages long, with 54 questions. This version was found to have a reading level appropriate for a 9-year-old child and contains pictures to help the child understand the questions. Also, this questionnaire has been validated and has shown good to excellent reproducibility^[16,17].

Each question is generally directed towards one of three parameters of interest: food intake, physical activity and healthful knowledge. Most of the questions that address the child's food intake or physical activity are formatted to ask the child how much he/she has eaten of that food item on the day prior to the administration

of the questionnaire (i.e., "Yesterday, did you eat fruit?"). This is intended to minimize any recall bias. However, the questions that assess their healthful knowledge are formatted in a more general way (i.e., "Do you ever read the nutrition labels on food packages?").

For the purpose of better comparisons between the three groups, we created a scoring system for some of the questions of interest. For example, we created the physical activity score based on the sum of the answers of four different questions in the SPAN questionnaire that addressed physical activity. The higher the score, the more physical activity the child reported. We also added up the answers of two other questions and created the sedentary score (i.e., Sedentary score = the answer to question 35 "Yesterday, how many hours did you watch TV or video movies?" + the answer to question 40 "How many hours per day do you usually spend on the computer or playing video games, like Nintendo®, Sega®, or arcade games?"). Again the higher the score the more sedentary behavior the subject reported.

For other questions, we divided the children's answers into "No" or "Yes". "No" if they had no intake of that food item or "Yes" if they had at least one portion of that food item on the prior day but could be two, three or more portions. For example the answers to "Fruit intake \geq once per day" are either "No", which meant they had no fruit intake the day prior to the administration of the questionnaire or "Yes" which meant that they had at least one fruit the day before but could have had more.

Serological analysis

One blood draw was performed at the time of the visit after an overnight fast of at least 8 h. Serological analysis was performed for insulin, glucose, lipid profile, and hepatic profile in Texas Children's Hospital's clinical laboratory. Heights and weights were measured for all children and BMI, BMI percentile for age/sex, and BMI Z-score were calculated. To assess insulin sensitivity, we determined the Quantitative Insulin Sensitivity Check Index (QUICKI) using the calculation: $1/[\log(\text{fasting insulin in } \mu\text{U/mL}) + \log(\text{fasting glucose in mg/dL})]$ ^[19]. As a measure of insulin resistance, the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated using the equation: $(\text{fasting insulin in } \mu\text{U/mL} \times \text{fasting glucose in mmol/L})/22.5$ ^[19]. A QUICKI value of < 0.339 represents impaired insulin sensitivity in children, while a HOMA-IR value of > 2 represents insulin resistance.

Statistical analysis

The data collected (serological levels and questionnaire answers) were analyzed using the Statistical Package for the Social Sciences (SPSS, Version 18.0). Descriptive statistics (means, standard deviations, and percentages) were calculated to describe sample characteristics. Analysis of Variance with Tukey's multiple comparison analysis (Post Hoc Test) was used to compare the three experimental groups. Significance was defined as $P < 0.05$.

Table 1 Patients' demographics

	CO	OB	NA	P value ^c
Male: Female (male %)	12:5 (71)	15:5 (75)	15:5 (75)	1
Age, yr (mean ± SD)	12.4 ± 2.1	11.9 ± 2	12.2 ± 2	0.9
BMI (%)	48.95 ± 21.85 (12.2-83.3)	98.31 ± 1.42 ^a (95.3-99.9)	98.05 ± 1.73 ^a (93.4-99.5)	1
Z-Score	-0.04	2.27 ^a	2.17 ^a	0.8
BMI % males	49.9	98.4 ^a	97.8 ^a	1
BMI % females	50.4	97.9 ^a	98.7 ^a	1
Fasting insulin (μU/mL)	2.7 ± 2.5 (1-11)	12.1 ± 8.6 ^a (1-31)	19.4 ± 10.4 ^{a,c} (5-41)	0.02
Fasting glucose (mg/dL)	78.7 ± 7.6 (62-91)	84.1 ± 5.9 (75-95)	85.9 ± 8.5 ^a (68-111)	0.7
HOMA-IR	0.5 ± 0.5 (0.1-2.0)	2.5 ± 1.9 ^a (0.2-7.0)	4.1 ± 2.3 ^{a,c} (1-9.1)	0.02
QUICKI	0.467 ± 0.069 (0.344-0.558)	0.364 ± 0.073 ^a (0.289-0.527)	0.319 ± 0.027 ^a (0.280-0.383)	0.05
Cholesterol (mg/dL)	158.9 ± 25.2 (130-223)	165.2 ± 30.4 (116-243)	179.4 ± 37.8 (95-245)	0.3
TG (mg/dL)	62.5 ± 28 (25-123)	114.8 ± 38 ^a (63-208)	140.3 ± 81.4 ^a (38-424)	0.3
HDL (mg/dL)	56.7 ± 12.2 (37-87)	43.4 ± 7.9 ^a (32-62)	43.9 ± 9.7 ^a (22-68)	1

^a*P* < 0.05, *vs* lean controls (CO) group; ^b*P* < 0.05, *vs* obese controls (OB) group. Values represent the mean ± SD (range). BMI: Body mass index; HOMA-IR: Homeostatic model assessment for insulin resistance; QUICKI: Quantitative insulin sensitivity check index; TG: Triglyceride; HDL: High-density lipoprotein; NA: Non-alcoholic steatohepatitis subjects.

RESULTS

Patients' demographics

The study population (Table 1) included a total of 57 children; 20 in the NA group, 20 in the OB group and 17 in the CO group. All three groups were age, sex and ethnicity matched. The mean age was 12.1 ± 2.1 years. All subjects were Hispanic, and 74 % of the cohort was males (42 males and 15 females). The mean BMI % was 98.1% for the NA group (Z-score 2.2), 98.3% for the OB group (Z-score 2.3), and 48.9% for the CO group (Z-score -0.04). The mean fasting insulin levels were 19.4, 12.1 and 2.7 for the NA, OB and CO groups respectively (*P* < 0.05 NA *vs* OB). Increased insulin resistance and decreased insulin sensitivity were reflected in the HOMA-IR and the QUICKI (Table 1).

Body image and dieting behavior

While BMI % and BMI Z-score did not differ between NA and OB, differences in body image and dieting behavior between all 3 groups were elucidated (Table 2). We found that 55% of the NA group felt that they weighed too much compared to other students in their grade who were as tall as they were, while only 45% of the OB group and 5.9% of the CO group felt that way (*P* < 0.01, NA *vs* CO; *P* < 0.01, OB *vs* CO). Upon questioning the subjects if they have ever tried to lose weight, 85% of the NA group and 85% of the OB group answered yes compared to only 11.8% of the CO group. (*P* < 0.001, NA *vs* CO; *P*

Table 2 Body image and dieting behavior (%)

Question	CO	OB	NA
Compared to other students in your grade, who are as tall as you, do you think you weigh:			
The right amount	52.9	50	35
Too much	5.9	45 ^a	55 ^a
Too little (or not enough)	41.2	5 ^a	10 ^a
Are you trying to lose weight?			
No	94.1	20 ^a	5 ^a
Yes	5.1	80 ^a	95 ^a
Have you ever tried to lose weight?			
No	88.2	15 ^a	15 ^a
Yes	11.8	85 ^a	85 ^a
Do you ever read the nutrition labels on food packages?			
Almost never or never	41.2	20	10 ^a
Sometimes	52.9	80	55
Almost always or always	5.9	0	35 ^{a,c}
The foods that I eat and drink now are healthy:			
No	0	0	10
Yes, sometimes	94.4	65	80
Yes, all the time	5.9	35 ^a	10

Values represent percentage of children with each answer, ^a*P* < 0.05, *vs* lean controls group; ^c*P* < 0.05, *vs* obese controls (OB) group. CO: Lean controls; NA: Non-alcoholic steatohepatitis subjects.

< 0.001, OB *vs* CO). In response to the question "Are you trying to lose weight now?" 95% of the NA group, 80% of the OB group and 5.9% in the CO group stated "Yes" (*P* < 0.001, NA *vs* CO; *P* < 0.001, OB *vs* CO).

Looking into their healthful dietary knowledge, we found that 35% of the NA group answered that they always or almost always read the nutrition labels on food packages compared to only 5.9% of the CO group and none in the OB group (*P* < 0.05, NA *vs* OB; *P* < 0.05, NA *vs* CO). Interestingly, only 10% of the NA group and 5.9% of the CO group felt that they always eat and drink healthy foods, compared to 35% of the OB subjects (*P* < 0.05, OB *vs* CO).

Food consumption

Children reported food intake of milk, fruits, and fries (Figure 1). The NA group consumed the least amount of fruits when compared to the other groups, with only 25% of NA subjects reporting that they consumed ≥1 fruit per day compared to 45% and 64.7% of the OB and CO groups respectively (*P* = 0.05, between the 3 groups). This difference was even more significant between the NA and CO groups (*P* < 0.05).

The NA group reported the least amount of dairy consumption, with only 55% having ≥ 1 cup of milk per day, compared to 60% of the OB group and 64.7% of the CO group; however this was not statistically significant. There were no statistically significant differences between the 3 groups regarding reported consumption of cereal, grains, vegetables, or sugar sweetened beverages. Also, there were no differences between the NA and OB groups in regards to having breakfast the day prior to the questionnaire or taking a vitamin pill.

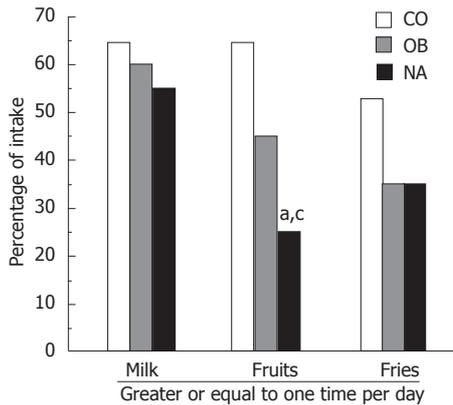


Figure 1 Intake of milk, fruits and fries. This figure plots the percentage of subjects responding to school physical activity and nutrition questions regarding food consumption. Non-alcoholic steatohepatitis subjects reported consuming significantly less fruits than obese and lean control subjects. CO: Lean controls; OB: Obese controls; NA: Non-alcoholic steatohepatitis subjects. ^a*P* < 0.05, vs CO group; ^c*P* < 0.05, vs OB group.

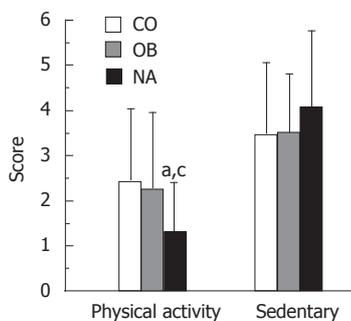


Figure 2 Physical activity and sedentary behavior. This figure demonstrates the physical activity scores and sedentary scores of obese children with non-alcoholic steatohepatitis (NASH) compared with obese controls and lean controls. NASH subjects reported significantly less physical activity than obese and lean controls. CO: Lean controls; OB: Obese controls; NA: Non-alcoholic steatohepatitis subjects. ^a*P* < 0.05, vs CO group; ^c*P* < 0.05, vs OB group.

Physical activity and sedentary behaviors

Differences in reported light to vigorous exercise and sedentary behaviors were evaluated in this cohort. We found that 45% of the NA group performed vigorous exercise (e.g., basketball, running or jogging, fast dancing, swimming laps, tennis, fast bicycling or similar aerobic exercises) for at least 20 min the day prior to the administration of the questionnaire, compared to 64.7% in the CO group and 25% in the OB (*P* = 0.05, between the 3 groups; *P* < 0.05, OB *vs* CO). However, only 15% of the NA subjects had performed light exercise (defined as fast walking, slow bicycling, skating, pushing a lawn mower, or mopping the floors) for at least 30 min the day before, compared to 35% and 59% of the OB and CO groups, respectively (*P* < 0.01, NA *vs* CO, *P* < 0.05 OB *vs* CO). The mean physical activity score was lowest in the NA group (NA = 1.3 *vs* OB = 2.3 *vs* CO = 2.4; *P* < 0.05), but the mean sedentary score was not significantly different between the groups (NA = 4.1 *vs* OB = 3.5 *vs* CO = 3.5; *P* = NS) (Figure 2).

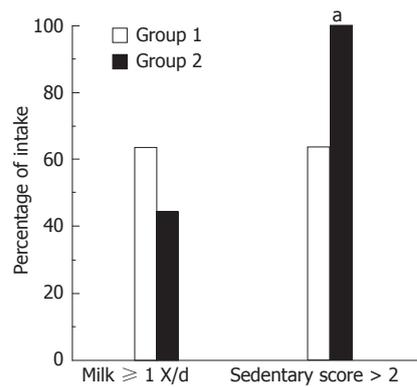


Figure 3 Milk intake and sedentary score by severity of fibrosis in non-alcoholic steatohepatitis subjects. This figure demonstrates milk consumption and sedentary scores amongst subjects with non-alcoholic steatohepatitis. All subjects with advanced fibrosis (grades 2 or 3), had a sedentary score > 2 compared to only 63.6% of those with grade 1 or no fibrosis (^a*P* < 0.05, vs group 1).

Behaviors in subjects with NASH

In an attempt to clarify possible differences in diet and activity amongst obese children with differing degrees of liver injury, a sub-group analysis of the NA group was performed (Figure 3). Amongst the subjects with NASH, we found that 100% of patients with grade 2 or 3 fibrosis had a sedentary score > 2 compared to only 63.6% of those with grade 1 or no fibrosis (*P* < 0.05). We also noted that 44.4% of patients with grade 2 or 3 fibrosis had ≥ 1 cup of milk per day, compared to 63.6% of patients with no fibrosis or with grade 1 fibrosis; however, this difference was not statistically significant. Also, 16.7% of patients with grade 3 steatosis reported having pasta on at least 2 occasions the day prior to the questionnaire while none of the subjects with grade 1 or 2 steatosis had that amount (*P* < 0.05). Comparing the patients’ NAFLD Activity Score (NAS), we found that there were no differences between subjects with NAS score ≥ 4 and those with NAS score < 4 in regards to their dietary habits, physical activity and healthful knowledge.

Subjects with NASH vs non-NASH

Upon comparing subjects with NASH to those with no evidence of liver disease (“non-NASH” = CO + OB groups), we found that 75% of the NA group reported no fruit intake at all compared to only 45.9% in the non-NASH group (*P* < 0.05). Additionally, 35% of the NA group “always” or “almost always” read nutrition labels, compared to only 2.7% of the non-NASH group (*P* < 0.01). In evaluating light exercise, we found that 85% of the NA group reported not performing any light exercise on the prior day, compared to 54.1% in the non-NASH group (*P* < 0.05). And finally, 95% of the NA group stated that they were currently trying to lose weight compared to only 45.9% of the non-NASH group (*P* < 0.001).

DISCUSSION

Our pilot study demonstrates clear differences in dietary

behaviors, physical activity and healthful knowledge between lean healthy children, obese children with no evidence of liver disease and obese children with NASH. One potential caveat to our study is that we didn't obtain a liver biopsy to confirm the absence of NAFLD in the CO and OB groups. However, given the invasive nature of liver biopsy and normal transaminases in these subjects, it would be unethical to perform this procedure on children with no other clinical indication. Hence, in order to minimize this possibility, we excluded any child with a history of liver disease of any sort, history of elevated ALT or ALT > 40 U/L on the day of the study visit.

We performed this study in a cohort of Hispanic children; this must be taken into account when considering generalizing this to the population as a whole. Ogden *et al.*^[19] recently reported that the highest rate of obesity in children of all age groups was among teenage African American girls followed by Hispanic boys and girls of all ages with Hispanic males having the highest percentage of obesity compared to white and African American males^[20]. Additionally, the role of gender, race and ethnicity has been described in a population based study on NAFLD by Schwimmer *et al.*^[21], where they noted that obese adolescent boys were six times more likely to have fatty liver compared to obese adolescent girls. This study also supported the observation that NAFLD is more common in Hispanic adolescents compared to other ethnicities, with Hispanic ethnicity making a child five times more likely to have NAFLD than an African American child^[7]. Given the prevalence and severity of obesity and NAFLD in Hispanic children, our study population provides a unique insight into this population.

In our cohort, all subjects were Hispanic, and 74% of them were males. This male:female ratio is similar to Schwimmer's report of 82% of children with NASH being male^[7]. There were no differences between the 3 groups in regards to age and sex; however, there were clear differences between the groups in regards to their fasting glucose and insulin levels - findings that are consistent with previous studies (e.g., Louthan *et al.*^[22] and Vos *et al.*^[23] studies). Evidence supports the concept that a trio of obesity, dyslipidemia, and insulin resistance play an important role in the pathogenesis and severity of NASH in children, and we found that children with NASH had an increase in HOMA-IR and a decrease in QUICKI when compared to the obese and lean control groups^[19,22,23]. The NA and OB groups had a significantly higher level of triglyceride (TG) and lower level of high-density lipoprotein (HDL) when compared to the lean control subjects. Those values were comparable to the values from other studies such as Viva La Familia study by Butte *et al.*^[24] and Quirós-Tejeira *et al.*^[25], as the mean HDL in our NA subjects was 43.85 ± 9.7 (in Viva La Familia; HDL: 46.6 ± 0.5 mg/dL) and mean TG was 140.3 ± 81.4 (in Quirós-Tejeira *et al.*^[25]; TG for boys: 157.2 ± 86.9 mg/dL and for girls: 145.0 ± 71.7 mg/dL).

Recent studies have shown that the majority of Hispanic mothers of overweight children may not perceive

their children as being overweight^[26-28]. In another study by Mikhail *et al.*^[29] both children and parents underestimated the child's body fat with parents' estimates being slightly, but not significantly, better (unpublished data). Similarly, we found that only half of the NA children found themselves heavier than their classmates; however, the majority of them had tried and/or are currently trying to lose weight.

Interestingly, even though the NA and the OB groups had the same mean BMI % (98%), similar proportions reported feeling heavier than their classmates, and a similar percentage had tried or are currently trying to lose weight, the NA and OB groups had notable differences in their healthful knowledge. For example about one third of the NA children read food labels on a regular basis compared to none in the OB group, and more NA than OB children recognized that their diets are "less healthy". This finding may be attributed to the fact that the NA children had multiple physician's visits including seeing a specialist (i.e., a hepatologist), and had undergone multiple blood draws and a liver biopsy for obesity-related health issues, while the OB group were recruited generally during their well-child visit, through Baylor Pediatric Residents Primary Care clinics at Texas Children's Hospital. Therefore, the NA subjects may have a heightened sense that their diet is not as healthy as it should be and may truly be reading food labels on a more regular basis.

A growing body of evidence supports that oxidative stress may play a vital role in the development of NASH^[30,31]. Intake of antioxidants may help prevent the occurrence of this disease and antioxidant vitamin supplementation studies have been performed^[13,32,33]. In our study, there were no differences between the three groups in regards to their daily intake of a vitamin pill to explain a difference in their antioxidant intake; however, we noted that the NA group had the lowest intake of fruits per day while the CO group had the highest intake. Fruits are rich in antioxidants^[34-36], and this difference in fruit intake between the groups may give insight into why some obese Hispanic children develop NASH while others do not.

In an analysis of NHANES looking at whole grain consumption and weight status, it was found that women consuming at least one serving of whole grain had a significantly lower mean BMI and waist circumference than women with no whole grain consumption^[37]. In another study done in the United Kingdom, it was found that a higher intake of whole grains (about three servings per day) was associated with lower BMI and central adiposity^[38]. Nicklas *et al.*^[39] have found that consuming ready-to-eat cereal at breakfast was associated with improved weight and nutrient adequacy in African American children. Surprisingly, in our study there were no differences between the 3 groups in regards to having breakfast or cereal intake. This, however, may be due to the small sample size in our pilot study.

While differences between the 3 groups were noted in respect to vigorous activity, it was even more notable in regards to light exercise, with only 15% of the NA sub-

jects performing light exercise for at least 30 min on the prior day, compared to one third and almost two thirds of the OB and CO groups, respectively. While we have not evaluated for patatin-like phospholipase domain-containing protein 3 (PNPLA 3) variants in our study subjects, it has been recognized that PNPLA 3 variants have been associated with NASH, especially in Hispanics. Johansson, *et al*^[40] reported that PNPLA 3 mutations were associated with decreased light physical activity. This highlights the need for studies to evaluate PNPLA 3 mutations in children with NASH.

Amongst the subjects with NASH, we found that all patients with advanced fibrosis had a sedentary score > 2 compared to less than two thirds of those with grade 1 or no fibrosis. Realizing that this was a one-time study visit, we can not be certain as to where our subjects are in regards to the natural history of disease progression. However, in a study out of Italy by Nobili *et al*^[41], children with NASH who improved their BMI, partially by increased exercise, decreased the degree of liver injury. This could explain the above finding in our study.

In conclusion, both genetic and environmental factors play major roles in the development of NASH and determine why only a minority of obese, insulin resistant individuals progress from simple steatosis to inflammation and fibrosis^[10]. This pilot study aimed to use a validated tool, the SPAN questionnaire, to assess the role of specific environmental factors such as diet and physical activity in pediatric NAFLD. Our results suggest that pediatric hepatologists should consider counseling children with NASH regarding the potential benefits of decreasing sedentary behavior, increasing light activity level, and increasing fruit intake. We conclude that future studies identifying environmental factors involved in pediatric NASH may help target therapeutic measures and preventative measures.

COMMENTS

Background

As the epidemic of obesity in childhood is increasing, it is predicted that non-alcoholic fatty liver disease and non-alcoholic steato-hepatitis (NASH or NA) prevalence will increase as well. Currently, there is no approved therapy for this condition, so life style changes, e.g., diet and exercise have become the mainstays of therapy.

Research frontiers

The authors hypothesize that there are differences in the dietary behaviors, physical activity and healthful knowledge between obese children who develop NASH and obese and lean children with no evidence of liver disease. Therefore, identifying those potential environmental differences may have important implications in regards to treatment and possibly even prevention of this condition in the future.

Innovations and breakthroughs

The authors demonstrated clear differences between obese children with NASH and obese and lean children with no evidence of liver disease. Children with NASH had increased sedentary behavior, decreased activity level, and decreased fruit intake.

Applications

This pilot study highlights the potential benefits of decreasing sedentary behavior, increasing light activity level, and increasing fruit intake in obese children as this may help prevent the development of NASH in this population.

Peer review

In this manuscript the authors evaluated nutrition, physical activity and healthful knowledge in obese children with NASH compared to children without liver disease and concluded that children with NASH had increased sedentary behavior and decreased activity. **This study is interesting.**

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S- Editor Tian L L- Editor O'Neill M E- Editor Xiong L

Prevalence of restless legs syndrome in patients with irritable bowel syndrome

P Patrick Basu, N James Shah, Nithya Krishnaswamy, Tommy Pacana

P Patrick Basu, Gastroenterology, Hepatology, and Liver Transplant, Columbia University College of Physicians and Surgeons, New York, NY 10032, United States

P Patrick Basu, Gastroenterology, Hepatology, and Liver Transplant, New York Hospital Queens, Flushing, NY 11355, United States

P Patrick Basu, Internal Medicine, Forest Hills Hospital, Forest Hills, NY 11375, United States

N James Shah, Nithya Krishnaswamy, Tommy Pacana, Internal Medicine, Forest Hills Hospital, Forest Hills, NY 11375, United States

Author contributions: This study was designed, organized, and executed by Basu PP; Manuscript development, writing and review was performed by Basu PP, Shah NJ, Krishnaswamy N and Pacana T.

Correspondence to: P Patrick Basu, MD, Internal Medicine, Forest Hills Hospital, 5 Station Square, Forest Hills Gardens, NY 11375, United States. patbasumd@aol.com

Telephone: +1-718-8970584 Fax: +1-718-8965571

Received: November 9, 2010 Revised: February 15, 2011

Accepted: February 22, 2011

Published online: October 21, 2011

Abstract

AIM: To determine the prevalence of restless legs syndrome (RLS) in patients with irritable bowel syndrome (IBS).

METHODS: Patients with diarrhea-predominant IBS ($n = 30$), constipation-predominant IBS ($n = 30$), or mixed-symptom IBS ($n = 30$) were recruited from the community between March 2008 and February 2009. Rifaximin 200 mg three times daily was administered empirically to alleviate small intestinal bowel overgrowth in all patients. The presence of RLS was assessed *via* an RLS questionnaire and polysomnography.

RESULTS: Twenty-six patients with IBS (29%) were diagnosed with RLS using the RLS questionnaire. Twenty-four of the 26 patients (92%) underwent polysomnography,

and all had confirmation of RLS. A greater percentage of patients with RLS had diarrhea-predominant IBS (62%) compared with patients with constipation-predominant IBS (4%) or mixed-symptom IBS (33%).

CONCLUSION: Restless legs syndrome is prevalent in patients with IBS, especially those with diarrheal symptoms. Assessment of concomitant disorders may improve diagnosis and expand relevant treatment options for patients.

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Key words: Restless legs syndrome; Irritable bowel syndrome; Small intestinal bacterial overgrowth; Prevalence

Peer reviewer: Dr. Julian RF Walters, PhD, MD, BSc, MBBS, Department of Gastroenterology, Imperial College London, Hammersmith Hospital, Du Cane Road, London, W12 0HS, United Kingdom

Basu PP, Shah NJ, Krishnaswamy N, Pacana T. Prevalence of restless legs syndrome in patients with irritable bowel syndrome. *World J Gastroenterol* 2011; 17(39): 4404-4407 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4404.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4404>

INTRODUCTION

Irritable bowel syndrome (IBS) is a common gastrointestinal (GI) disorder that affects between 3% and 25% of the population in Western countries^[1]. It is characterized by chronically recurring symptoms associated with alterations in bowel habits, which may be classified as constipation predominant (IBS-C), diarrhea predominant (IBS-D), or mixed symptom (IBS-M)^[2,3]. Other symptoms include abdominal pain or discomfort, bloating, flatulence, straining, sensations of urgency, and incom-

plete evacuation^[3]. Symptoms may fluctuate in severity and frequency, with some periods of remission^[1]. The etiology of IBS is unknown, but multiple, possibly inter-related, pathophysiologic mechanisms may be involved, including psychological disturbances^[1,2,4], genetic predisposition^[1], alterations in inflammatory responses^[2,4], visceral hypersensitivity^[4], abnormal intestinal motility^[3], alterations in neurotransmitter levels^[2-4], and small intestinal bacterial overgrowth (SIBO)^[2]. Small intestinal bacterial overgrowth in particular may be an important factor because of its impact on mucosal inflammation. Inflammation may lead to increased cytokine activity, increased motility, and the release of neurotransmitters such as 5-hydroxytryptamine (5-HT), thereby disrupting central and enteric nervous system crosstalk^[4]. This may explain why IBS, which is associated with SIBO^[5-8], is often comorbid with other sensitivity disorders, including fibromyalgia and interstitial cystitis^[9-11].

Restless legs syndrome (RLS) is a common sensorimotor disorder^[12] affecting 1% to 10% of the population^[13]. It is clinically characterized as a compelling urge to move the legs that is often associated with discomfort, is worse during rest or inactivity, is relieved by movement, and is worse or only occurs in the evening or at night^[12]. The etiology of RLS remains incompletely characterized^[13,14]; however, dopaminergic dysfunction and altered control of iron homeostasis may contribute to the pathophysiology of RLS^[13,14]. Studies have also linked RLS with GI disorders, including Crohn's disease^[15], celiac disease^[16], and SIBO^[17,18]. Further, a small observational study of patients with IBS and RLS ($n = 13$) demonstrated that RLS symptoms improved following antibiotic therapy for SIBO^[18]. This study expounds on these observations by determining the prevalence of RLS in a large number of patients ($n = 90$) with IBS.

MATERIALS AND METHODS

This observational clinical study was conducted in patients ≥ 18 years of age diagnosed with IBS according to Rome III criteria. Participants were recruited between March 2008 and February 2009 from a community-based gastroenterology center and administered rifaximin 200 mg three times daily for 14 d as empiric therapy for SIBO before diagnosis of RLS. Patients with scleroderma, diabetes mellitus, Parkinson's disease, chronic kidney disease, hepatic encephalopathy, peripheral neuropathy, dysmotility disorder, high ferritin levels, or iron deficiency were excluded. Patients who were pregnant; who were taking dopamine antagonists, antidepressants, caffeine, alcohol, cocaine, or amphetamines; or who had used antibiotics 6 wk before enrollment were also excluded from the study. The study protocol, all protocol amendments, and the informed consent form were approved by the Central Institutional Review Board. All individuals provided written informed consent, and the study was conducted in accordance with ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975.

Table 1 Patient demographics and baseline disease characteristics n (%)

Characteristic	Patients ($n = 90$)
Mean age (range), yr	33 (20-55)
Male:Female	30:60
Race	
Hispanic	38 (42)
White	26 (29)
Asian	24 (27)
Black	2 (2)
Mean BMI, kg/m ²	28
Mean duration of IBS, yr	6
IBS subtype	
IBS-D	30 (33)
IBS-C	30 (33)
IBS-M	30 (33)

BMI: Body mass index; IBS: Irritable bowel syndrome; IBS-C: Constipation-predominant irritable bowel syndrome; IBS-D: Diarrhea-predominant irritable bowel syndrome; IBS-M: Mixed-symptom irritable bowel syndrome.

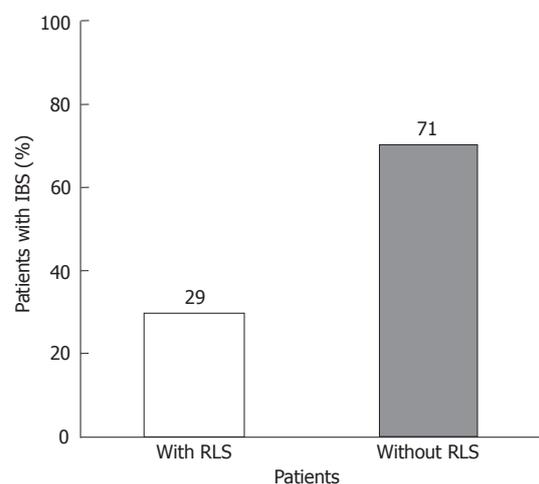


Figure 1 Prevalence of restless legs syndrome in patients with irritable bowel syndrome. Twenty-nine percent of patients with irritable bowel syndrome (IBS) were diagnosed with restless legs syndrome (RLS).

Initial diagnosis of RLS was made *via* the use of a standard RLS questionnaire formulated by the International Restless Legs Syndrome Study Group^[12]. The questionnaire was administered by medical students who were unaware of the study objective, and responses were interpreted by a gastroenterologist (*i.e.*, Basu). Diagnosis of RLS was confirmed *via* polysomnography conducted by a sleep specialist at a single center using criteria established by the Association of Sleep Disorders Centers^[19].

RESULTS

Ninety patients with IBS who met inclusion criteria and did not meet exclusion criteria were included in the study (Table 1). Of these patients, 30 (33%) displayed IBS-D, 30 (33%) exhibited IBS-C, and 30 (33%) presented with IBS-M. Initial diagnosis of RLS was established in 26 patients (29%) using the RLS questionnaire (Figure 1). Of

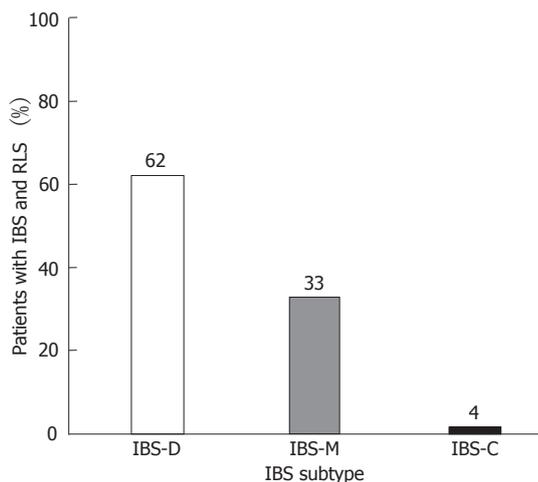


Figure 2 Distribution of irritable bowel syndrome subtypes in patients with restless legs syndrome, which was assessed in each patient using a questionnaire and sleep study analysis. IBS: Irritable bowel syndrome; IBS-C: Constipation-predominant irritable bowel syndrome; IBS-D: Diarrhea-predominant irritable bowel syndrome; IBS-M: Mixed-symptom irritable bowel syndrome; RLS: Restless legs syndrome.

these 26 patients, 24 (92%) underwent sleep study analysis to confirm RLS; two patients chose not to participate in this analysis and were excluded from the study.

Polysomnography confirmed RLS during REM and non-REM phases in all patients tested (100%). Salient sleep disturbances other than RLS included wakefulness > 30% of sleep time (63%), sleep maintenance < 30% of sleep time (100%), and disruption of sleep onset (100%). Moderate (myoclonus time > 30-40 s) and mild (myoclonus time > 10-30 s) involuntary jerks of limbs not related to RLS were also noted in 50% and 25% of patients, respectively. Patients with RLS were more likely to have IBS-D than IBS-C or IBS-M (Figure 2).

DISCUSSION

Irritable bowel syndrome is a common GI disorder with a sex-adjusted incidence rate of 196 cases per 100 000 people^[1], which is associated with approximately 8 billion dollars per year in medical costs^[4]. Individuals diagnosed with IBS take more time off work^[20], spend more days in bed^[20], and have an overall reduction in health-related quality of life compared with those without IBS^[21]. Although the etiology of IBS is unknown, it has been associated with psychological factors such as anxiety^[22] and verbal or emotional abuse^[23], as well as with sleep disturbances^[24]. Furthermore, alterations in GI motility (e.g., the migrating motor complex)^[25,26] and GI flora (e.g., SIBO)^[5,6,8] have been described in patients with IBS. Taken together, these data suggest disruption of communication between the central and enteric nervous system in IBS, which may explain the comorbidity of IBS with other sensory disorders^[9-11,18].

This study demonstrated that RLS was a comorbid condition with IBS in 26 of 90 patients (29%). This finding adds support to previous studies examining the prev-

alence of RLS in patients with intestinal disorders. In a study of 161 patients with an abnormal lactulose breath test indicative of SIBO, 19 patients (12%) perceived themselves to have RLS^[18]. Of these, 11 patients (58%) were confirmed to have RLS by the Johns Hopkins validated interview process. The prevalence of RLS in patients with celiac disease or Crohn's disease has also been examined, with 35% (30 of 85) and 43% (93 of 218) of patients reporting comorbid RLS, respectively^[15,16].

The mechanism underlying these comorbidities remains unknown, but the disruption of enteric and central nervous system communication because of alterations in inflammatory mediators and neurotransmitters triggered by SIBO has been implicated^[4]. It has been suggested that an excess of sulfate-reducing bacteria may lead to increases in hydrogen sulfide, which may act as a gaseous neurotransmitter^[11]. In addition, inflammation may lead to increased cytokine activity and altered release of 5-HT^[4]. This may explain why both the 5-HT antagonist alosetron and the minimally absorbed antibiotic rifaximin are effective in relieving IBS symptoms. Interestingly, rifaximin in combination with tegaserod (a prokinetic agent) and zinc reduced symptoms of RLS in patients with SIBO^[18], and treatment of 2 patients with RLS with tandospirone (a 5-HT_{1A}-receptor agonist) relieved RLS symptoms^[27]. The results presented herein suggest that factors in addition to SIBO may play a role in RLS because SIBO was eliminated in patients by prophylactic treatment with rifaximin before RLS diagnosis. Together, these studies suggest involvement of SIBO, altered neurotransmission, and other factors in the pathophysiology of RLS.

In conclusion, this study contributes to the available literature supporting an association between IBS and RLS. Screening of patients with IBS for RLS, or vice versa, may permit more accurate diagnosis based on revelation of potentially undisclosed symptoms. Concomitant diagnosis of these disorders may enhance treatment options for patients, given that some medications may provide relief for both conditions. Further investigations to determine the underlying mechanisms common in both disorders are needed to address the causality of this connection.

ACKNOWLEDGMENTS

Editorial assistance was provided under the direction of the authors by MedThink Communications with support from Salix Pharmaceuticals, Inc.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder characterized by abdominal pain or discomfort, bloating, flatulence, straining, urgency and incomplete evacuation. The pathophysiologic mechanisms underlying this disease are incompletely understood, but alterations in gastrointestinal and systemic inflammatory responses have been implicated. Disruption of inflammatory processes has also been associated with restless legs syndrome (RLS), a sensorimotor disorder associated with a compelling urge to move the legs that is relieved with movement and may result in sleep disruption. This prevalent

disease has been associated with a variety of inflammation-related gastrointestinal disorders including Crohn's disease, celiac disease, and small intestinal bacterial overgrowth, which have also been linked with IBS.

Research frontiers

Although common, RLS may be underdiagnosed because primary care physicians may be unaware of the manifestations of RLS and the impact these symptoms may have on patient quality of life. Identification of certain patient populations at risk for RLS may aid primary care physicians in recognizing and treating patients with RLS symptoms. In this study, the prevalence of RLS was evaluated in patients with IBS.

Innovations and breakthroughs

The results suggest that RLS is a common comorbid condition in patients with IBS, particularly in patients with diarrhea-predominant IBS. In addition, patients with RLS and IBS had sleep disturbances unrelated to RLS, suggesting that IBS may be a contributing factor in sleep disorders in general.

Applications

Based on these results, primary care physicians treating patients with IBS should be cognizant of patient reports of trouble sleeping and conditions that may be associated with poor sleep, such as difficulty concentrating. Furthermore, given the association of RLS with inflammatory gastrointestinal disorders including IBS, treatment and resolution of such disorders may be beneficial for patients with RLS.

Peer review

This is a straight-forward and well-written paper describing the association of RLS with IBS, in particular diarrhea-predominant IBS.

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S- Editor Tian L L- Editor O'Neill M E- Editor Li JY

Narrow-band imaging without magnification for detecting early esophageal squamous cell carcinoma

Edson Ide, Fauze Maluf-Filho, Dalton Marques Chaves, Sergio Eiji Matuguma, Paulo Sakai

Edson Ide, Fauze Maluf-Filho, Dalton Marques Chaves, Sergio Eiji Matuguma, Paulo Sakai, University of São Paulo, Gastrointestinal Endoscopy Unit, São Paulo 05403-900, Brazil
Author contributions: Ide E and Maluf-Filho F contributed equally to this work; Ide E, Maluf-Filho F and Chaves DM designed the research; Ide E, Maluf-Filho F and Matuguma SE performed the research and Ide E, Maluf-Filho F and Sakai P wrote the paper.

Correspondence to: **Edson Ide, MD**, University of São Paulo, Gastrointestinal Endoscopy Unit, São Paulo 05403-900, Brazil. contato@edsonide.med.br

Telephone: +55-11-82038285 Fax: +55-11-30696221

Received: January 13, 2011 Revised: March 14, 2011

Accepted: March 21, 2011

Published online: October 21, 2011

Abstract

AIM: To compare narrow-band imaging (NBI) without image magnification, and chromoendoscopy with Lugol's solution for detecting high-grade dysplasia and intramucosal esophageal squamous cell carcinoma (SCC) in patients with head and neck cancer.

METHODS: This was a prospective observational study of 129 patients with primary head and neck tumors consecutively referred to the Gastrointestinal Endoscopy Unit of Hospital das Clínicas, São Paulo University Medical School, Brazil, between August 2006 and February 2007. Conventional examinations with NBI and Lugol chromoendoscopy were consecutively performed, and the discovered lesions were mapped, recorded and sent for biopsy. The results of the three methods were compared regarding sensitivity, specificity, accuracy, positive predictive value, negative predictive value, positive likelihood value and negative likelihood value.

RESULTS: Of the 129 patients, nine (7%) were diagnosed with SCC, 5 of which were *in situ* and 4 which were intramucosal. All carcinomas were detected through NBI and Lugol chromoendoscopy. Only 4 le-

sions were diagnosed through conventional examination, all of which were larger than 10 mm.

CONCLUSION: NBI technology with optical filters has high sensitivity and high negative predictive value for detecting superficial esophageal SCC, and produces results comparable to those obtained with 2.5% Lugol chromoendoscopy.

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Key words: Gastrointestinal endoscopy; Squamous cell carcinoma; Esophageal neoplasms; Diagnosis; Lugol's solution

Peer reviewers: Dr. Hsu-Heng Yen, MD, Department of Gastroenterology, Changhua Christian Hospital, 135 Nanhsiao Street, Changhua 500, Taiwan, China; Dr. Shinji Tanaka, Director, Department of Endoscopy, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

Ide E, Maluf-Filho F, Chaves DM, Matuguma SE, Sakai P. Narrow-band imaging without magnification for detecting early esophageal squamous cell carcinoma. *World J Gastroenterol* 2011; 17(39): 4408-4413 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4408.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4408>

INTRODUCTION

In recent studies, narrow-band imaging (NBI) technology was found to be useful for detecting squamous cell carcinomas (SCC) of the pharynx and esophagus. Morphologic patterns in the changes of intrapapillary capillary loops were found by Muto *et al*^[1], Arima *et al*^[2] and Yoshida *et al*^[3], and can be useful for diagnosing SCC and even predicting the extent of this type of lesion. Accordingly, various reports on early-stage pharyngeal and esophageal SCC diagnosed through the use of NBI technology can be found in the literature. A small, su-

perforated SCC of the pharynx with a small, well-defined brown area was diagnosed by Muto *et al.*⁴¹, without the use of magnification. Using NBI technology without image magnification, Watanabe *et al.*⁴¹, in a prospective study, found six pharyngeal squamous cell carcinomas and Goda *et al.*⁵¹ found an esophageal SCC not identified by conventional endoscopy (obscure lesion).

Despite encouraging results with this new technology, esophageal Lugol staining remains the gold standard for detecting mucosal superficial neoplasias formed by glycogen-poor cells, including epidermoid carcinoma of the esophagus^{6,7}. Although Lugol staining is a simple and low-cost method, instillation of its solution may lead to complications, namely hypersensitivity to iodine, laryngitis, pneumonitis, as well as frequent painful sensations and nausea⁸⁻¹¹. A significant reduction in retrosternal discomfort was demonstrated by Kondo *et al.*¹¹ with the use of sodium thiosulphate costing \$0.15. However, Lugol's solution is not used in the pharynx or larynx. Therefore, the evaluation of alternative methods for "optical staining" such as NBI is desirable because these methods are potentially simpler, with no complications. The groups most likely to benefit from these methods are those at high risk of developing esophageal SCC, namely patients with malignant squamous cell neoplasias of the head and neck^{17,12-14}, because these patients routinely undergo endoscopic surveillance for this type of cancer.

The aim of this study was to compare NBI technology with Lugol staining during endoscopic examination of the esophagus for the detection of high-grade intraepithelial neoplasia and superficial SCC in this organ in patients with head and neck cancer.

MATERIALS AND METHODS

Patients and design

From August 2006 to February 2007, 136 consecutive patients with head and neck tumors were referred to the Gastrointestinal Endoscopy Unit of Hospital das Clínicas, São Paulo University Medical School, Brazil, for detection of esophageal SCC. Patients with head and neck SCC undergo annual upper gastrointestinal (GI) endoscopy and associated chromoendoscopy of the esophageal mucosa with Lugol's solution.

The inclusion criteria were indication of upper GI endoscopy examination for patients with head and neck SCC under surveillance for the detection of synchronous or metachronous lesions.

Exclusion criteria for patients were as follows: (1) clinical conditions precluding upper GI endoscopy examination and 2.0% Lugol staining; (2) **history of allergic reaction to iodine**; and (3) **diagnosis of esophageal neoplasia of advanced endoscopic appearance** defined as an ulcerated, infiltrative or stenotic lesion easily detected on conventional examination

All participants provided written informed consent. This study was approved by the Ethics Committee of the Gastroenterology Department of São Paulo University

Medical School, under research protocol No. 1083/06.

Endoscopy system

An Exera II Evis 180 GIF180 (Olympus, Tokyo, Japan) videoendoscope with high resolution (1080 dpi), 1.5-fold magnification and NBI technology was used. Examinations were performed conventionally under conscious sedation with midazolam and fentanyl chlorhydrate. A 2.0% Lugol's solution was used for staining. NBI and Lugol staining procedures were followed up by a single physician, and both methods were performed in one single procedure. First the organ was conventionally assessed with white light, and adhered residues or exudates were removed through potable water instillation. Assessment with NBI and 2.0% Lugol staining was subsequently performed. Upon mucosal analysis and biopsy, 0.5% sodium thiosulfate solution was instilled to remove the Lugol's solution from the mucosa to reduce spasm and pain. Therefore, the examination was divided into three phases. The first phase was the white light analysis, the second phase was the analysis of the mucosa with NBI, and the final phase was the assessment following Lugol staining. At the end of each phase, changes were documented in dynamic and static images and mapped using the anterior, posterior, and right and left lateral walls of the organ and the distance of the lesion from the anterior incisors as references. A biopsy was always performed after staining was completed.

For patients with malignant or actinic stenosis of the pharyngeal-esophageal tract, a smaller-diameter endoscope, GIF 180N model (Olympus, Tokyo, Japan), with a 4.9-mm caliber and 2.1-mm biopsy channel was used. Some of the possible complications resulting from Lugol's solution, such as laryngitis, chemical pneumonitis, hypersensitivity, and anaphylactic shock were registered.

When NBI was used, brown-stained areas of the mucosa were considered lesions suspected to be neoplasia (compared to "normal" mucosa, which is green independently of changes in surface or vascular texture). As for the Lugol dye solution, areas clearly not stained were suspected to be neoplasia, which is characterized by a white color in contrast with brown or brownish "normal" areas. The size and macroscopic shape were evaluated according to the Paris Classification¹⁵ for superficial esophageal lesions and their topography (cervical up to 5 cm of cricopharyngeal, thoracic and abdominal esophagus).

Histology

Histopathology was performed by a senior pathologist from the Department of Pathology of Hospital das Clínicas, São Paulo. The pathologist was aware of the endoscopic suspicion of esophageal SCC. Biopsy specimens were immersed in formaldehyde for fixation and stained using hematoxylin and eosin. The lesions were classified according to the Revised Vienna Classification. In the absence of lamina propria invasion, noninvasive neoplastic lesions were divided into two groups based on the degree of intraepithelial neoplasia: low grade and high grade.

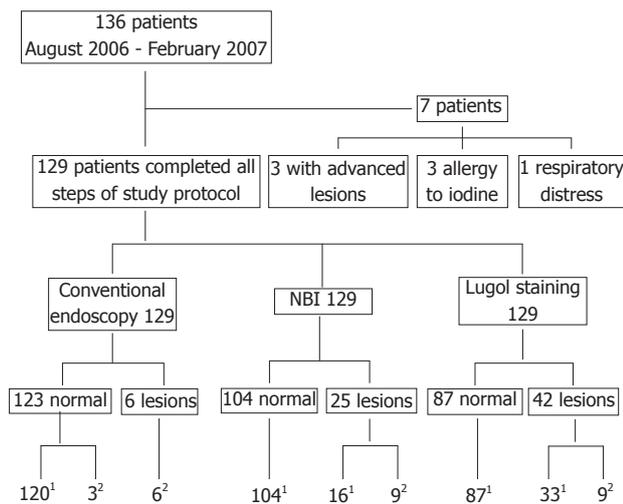


Figure 1 Flow chart of the study protocol. ¹Total patients without squamous cell carcinoma (SCC) ($n = 120$), ²Total of patients with SCC ($n = 9$). NBI: Narrow band imaging.

High-grade dysplasia, intraepithelial carcinoma and carcinoma *in situ* were considered equivalent entities^[15]. Whenever the lamina propria of the mucosa was invaded, the lesion was referred to as a microinvasive or intramucosal carcinoma.

In this study, only the findings of high-grade intraepithelial neoplasia (carcinoma *in situ*) and intramucosal carcinoma of squamous cells were considered true-positives for esophageal epidermoid carcinoma^[15].

Statistical analysis

Values were calculated for sensitivity, specificity, positive predictive value, negative predictive value, accuracy, positive and negative likelihood ratio, and their respective 95% confidence intervals (CIs).

RESULTS

Of the 136 patients, three were excluded as they had malignant esophageal lesions that were easily detected by conventional endoscopy, three as they had a prior history of allergy to iodine, and one had respiratory distress that prevented Lugol staining. Of the remaining 129 patients, there were 103 males and 26 females, aged 33 to 89 years (mean, 59 years).

One hundred and twenty-nine patients underwent all stages of the protocol of investigation for this study (Figure 1). For 42 patients in whom lesions were detected by NBI or Lugol dye, 9 (24.5%) proved to have esophageal neoplasias (four intramucosal neoplasias and five carcinomas *in situ*), and the remaining 33 patients exhibiting unstained lesions with Lugol’s solution had inflammatory disease only (Table 1).

Macroscopic classification, size, location, and histopathologic findings of the samples of the endoscopic or surgical resection are presented in Table 2. All carcinomas were found in the thoracic esophagus. The incidence was

Table 1 Histopathologic diagnosis by hematoxylin and eosin staining

Method	No. of endoscopic findings	SCC		Esophagitis
		<i>In situ</i>	Intramucosal	
Conventional	6	3	3	0
NBI	25	5	4	16
Lugol	42	5	4	33

NBI: Narrow-band imaging; SCC: Squamous cell carcinoma.

Table 2 Cases of squamous cell carcinoma (macroscopic and histopathologic findings)

Case	Macroscopic classification by conventional endoscopy	Size (mm)	Location at esophagus	Histopathologic examination
161	n/i	10	Thoracic	Intramucosal
175	0-II c	50	Thoracic	<i>In situ</i>
72	0-II c	30	Thoracic	Intramucosal
15	0-II b	20	Thoracic	<i>In situ</i>
94	0-II c	50	Thoracic	Intramucosal
64	0-II b	20	Thoracic	<i>In situ</i>
115	n/i	20	Thoracic	<i>In situ</i>
119	n/i	10	Thoracic	<i>In situ</i>
74	0-II b	25	Thoracic	Intramucosal

n/i: Not indentified.

Table 3 Comparison of performance across methods (95% confidence interval) (%)

	Conventional endoscopic examination	NBI	Lugol’s solution
Sensitivity	66.7 (35.9-97.5)	100 (100-100)	100 (100-100)
Specificity	100 (100-100)	86.7 (80.6-92.7)	72.5 (64.5-80.5)
PPV	100 (100-100)	36 (17.2-54.8)	21.4 (9.0-33.8)
NPV	97.6 (98.4-100)	100 (100-100)	100 (100-100)
Accuracy	97.7 (95.1-100)	87.6 (81.9-93.3)	74.4 (66.9-81.9)
PLR	n/c	7.5	3.6
NLR	0.33	0	0

NBI: Narrow-band imaging; PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; n/c: Not calculable.

4% for females and 8% for males.

Conventional endoscopy was able to identify most of the 10 mm lesions, and the sensitivity was 85.7% (95% CI, 59.8%-100%). However, this method failed in the diagnosis of smaller lesions (< 10 mm), where the sensitivity was 0%.

The performance of NBI was similar to that obtained by Lugol staining. Sensitivity and negative predictive value were 100% for both methods, and the specificity was 86.7% for NBI (95% CI, 80.6%-92.7%) and 72.5% (95% CI, 64.5%-80.5%) for Lugol’s solution.

Diagnostic performances for conventional endoscopic examinations, NBI and Lugol staining are presented in Table 3, and the performance results by lesion size are presented in Table 4.

Table 4 Comparison of diagnostic performance of the three procedures for lesions < 10 mm and > 10 mm (%)

	Conventional examination		NBI		Lugol's solution	
	< 10 mm	> 10 mm	< 10 mm	> 10 mm	< 10 mm	> 10 mm
Sensitivity (95% CI)	0 (0-0)	85.7 (59.8-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
Specificity (95% CI)	100 (100-100)	100 (100-100)	90 (84.6-95.4)	96.7 (93.5-100)	75.8 (68.2-83.5)	96.7 (93.5-100)
PPV (95% CI)	n/c	100 (100-100)	14.3 (4.0-32.6)	63.6 (35.2-92.1)	6.5 (2.2-15.1)	63.6 (35.2-92.1)
NPV (95% CI)	98.4 (96.1-100)	99.20 (97.6-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
Accuracy (95% CI)	98.4 (96.1-100)	99.2 (97.6-100)	90.2 (84.9-95.4)	96.9 (93.8-100)	76.2 (68.7-83.8)	96.9 (93.8-99.9)
PLR	n/c	n/c	10	30	4.1	30
NLR	1	0.1	0	0	0	0

n/c: Not calculable; NBI: Narrow-band imaging; PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio; NLR: Negative likelihood ratio.



Figure 2 Sequential endoscopic images of the esophagus. A: Lesion type 0 IIc; B: Narrow-band imaging image; C: After Lugol staining.

In the Lugol's solution group, there were 3 cases of chemical laryngitis and one of hypersensitivity to iodine (3% complication rate). No complications were reported with the conventional or NBI procedures.

DISCUSSION

Upper GI endoscopy with Lugol's solution staining is still considered the best method for tracking, diagnosing and delimiting superficial neoplasias of the esophagus^[16-18]. From 1965 to 1984, no intraepithelial and/or intramucosal lesion was diagnosed in the University Hospital of Kyushu, Japan^[19]. These lesions have only been observed since 1985, when Lugol's solution was initially used routinely for endoscopic evaluation of the mucosal surface. From 1985 to 1988, Sugimachi *et al*^[19] reported an increase in the number of patients with early stages of the disease receiving surgical treatment, ranging from 7% to 23% of patients undergoing surgical treatment. The results for the surgical treatment of esophageal-thoracic SCC in two major referral centers in Japan and China were compared by Fang *et al*^[20], and demonstrated a 2-year survival rate of 70.9% in Japan *vs* 56.2% in China. According to these authors, the use of Lugol chromoendoscopy to detect early lesions could explain the better results obtained at the Japanese center.

Lugol chromoendoscopy also proved useful for the detection of early esophageal SCC in patients with head

and neck cancer, a recognized high-risk group for the disease. In more than half of the head and neck cancer patients exhibiting the minimal mucosal changes associated with a lack of iodine impregnation (negative Lugol staining area), biopsies confirmed the presence of malignant neoplasia (sensitivity of 81.96% *vs* 59.1% when only the negative Lugol staining area was evaluated separately). Furthermore, no cases of neoplasia in normally-stained areas were found by Hashimoto *et al*^[7].

However, Lugol's solution irritates the mucosa and may lead to retrosternal chest pain and discomfort because of its alcoholic nature. Its use is limited by other factors, namely hypersensitivity to iodine and the risks of chemical esophagitis, laryngitis and bronchopneumonia. Several authors have reported necrosis and injury to esophageal and gastric mucosa caused by hypersensitivity to Lugol's solution^[8-10,12]. Furthermore, Lugol chromoendoscopy significantly increases the examination period^[11].

Slightly over 50% of early lesions are detected on conventional endoscopy, and the use of Lugol staining is still restricted to patients considered at high risk of developing this neoplasia. Furthermore, because of the previously mentioned risks and difficulties, a large portion of the population does not have access to efficient and safe examinations for the detection of early lesions, including those that are locally resectable. NBI does not have the limitations of Lugol chromoendoscopy and should therefore be considered as a replacement if it is equally

efficient in detecting esophageal SCC.

A few studies have evaluated the capacity of NBI without the use of image magnification for detecting esophageal SCC. A 2-fold capacity for detecting pharyngeal SCC compared with conventional white-light evaluation was found by Watanabe *et al*^[4]. In a multicenter study comparing the evaluation by narrow-band technology *vs* conventional white-light evaluation, the accuracies were 90.2% and 55.3%, respectively, ($P < 0.0001$)^[22]. Comparing Lugol chromoendoscopy to NBI technology with image magnification, equal results were found in the sensitivity of the two methods (92.3% *vs* 92.3%), but NBI had a better specificity (91.7% *vs* 72.2%)^[24].

The present study compared use of NBI technology with Lugol chromoendoscopy (a method considered the gold standard) for the detection of esophageal epidermoid carcinoma. This study was conducted in patients with head and neck cancer and without the use of image magnification. Many medical services do not have the resources for magnification; therefore, the aim of this study was to analyze whether NBI alone would suffice to detect small, superficial neoplasias of the esophagus. Of nine esophageal neoplasias, conventional white light could not detect three neoplasias, whereas both NBI and Lugol chromoendoscopy detected all neoplasias. The elevated likelihood ratio certifies the equivalence and high sensitivity of both methods. On the other hand, NBI esophagoscopy without image magnification, similar to Lugol chromoendoscopy, has a lower specificity for detecting early squamous cell neoplasias in the esophagus. Although Ponchon *et al*^[25] reported a 75% specificity for NBI, this may have been related to false detection of nonspecific inflammation. In two recent studies by Takenaka *et al*^[26] and Lee *et al*^[27], similar results were found: the sensitivity of NBI endoscopy for detecting esophageal SCC and high-grade intraepithelial neoplasia was 90.9% (95% CI, 58.7%-99.8%), specificity was 95.4% (95% CI, 90.3%-98.3%), and accuracy was 95.1% (95% CI, 90.1%-98.0%). Recently, transnasal endoscopy with NBI and Lugol staining were employed to screen patients with head and neck cancer whose condition prevented oral intubation with a standard endoscope and, as in our study, NBI and Lugol staining had the same sensitivity (88.9%) for the detection of high-grade mucosal lesions and performed far better than standard endoscopy (27.3% sensitivity)^[26,27]. The equivalent performance of NBI esophagoscopy and Lugol chromoendoscopy indicate that NBI esophagoscopy, without image magnification, is a potential surveillance method for patients at risk of esophageal squamous cell neoplasia. Therefore, this technology should be tested in other groups that are at risk of SCC (e.g., chronic corrosive esophagitis patients, tobacco smokers and alcohol users), and should be compared to Lugol chromoendoscopy.

The current study has some limitations. First, a sequential approach was adopted in which, in order, standard endoscopy, NBI and Lugol staining were employed by the same operator. This approach has the potential

bias of the same operator entering an examination phase having already detected any lesion in the prior phase. However, the sequential approach seems to be the best strategy for daily practice. Furthermore, the same methodology was used in similar studies^[22-25].

In conclusion, narrow-band technology with optical filters has a high sensitivity and high negative predictive value for detecting superficial squamous cell carcinomas of the esophagus (intramucosal carcinoma and carcinoma *in situ*). These results are comparable to those obtained with 2.5% Lugol chromoendoscopy but without the risks and technical difficulties related to this method. NBI could replace Lugol chromoendoscopy as a screening tool for detecting esophageal squamous cell carcinoma in patients with head and neck cancer.

COMMENTS

Background

Squamous cell carcinoma (SCC) of the esophagus is aggressive with high mortality. Early diagnosis has a major impact on survival and treatment costs. Narrow-band imaging (NBI) technology without magnification is simple to use and increases the rate of diagnosis of early lesions.

Research frontiers

In this study, the authors showed that NBI without magnification can replace Lugol staining in early diagnosis of lesions of the esophagus SCC.

Innovations and breakthroughs

The device used in Japanese studies are different from those used in Western countries (Lucera system), and led to the first use of NBI technology without magnification in detection of SCC of the esophagus.

Applications

NBI technology is easy to learn and use, increasing the diagnosis rate of early lesions in patients considered at risk of SCC.

Peer review

This is an interesting study comparing NBI to Lugo's staining and white light endoscopy for detection of early esophageal cancer in a Brazil medical center over 6 mo involving 129 patients. This study evaluated the usefulness of NBI in daily practice for screening of esophageal cancer. The study shows NBI is as effective as Lugol's chromoendoscopy for detecting early esophageal cancer.

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S- Editor Tian L L- Editor Cant MR E- Editor Xiong L

L-carnitine supplementation improves hematological pattern in patients affected by HCV treated with Peg interferon- α 2b plus ribavirin

Michele Malaguarnera, Marco Vacante, Maria Giordano, Massimo Motta, Gaetano Bertino, Manuela Pennisi, Sergio Neri, Mariano Malaguarnera, Giovanni Li Volti, Fabio Galvano

Michele Malaguarnera, Giovanni Li Volti, Fabio Galvano, Department of Biological Chemistry, Medical Chemistry and Molecular Biology, University of Catania, 95100 Catania, Italy
Marco Vacante, Maria Giordano, Gaetano Bertino, Sergio Neri, Mariano Malaguarnera, Department of Internal Medicine and Systemic Disease, University of Catania, 95125 Catania, Italy
Massimo Motta, Manuela Pennisi, Research Center "The Great Senescence", University of Catania, 95125 Catania, Italy
Author contributions: Malaguarnera M, Vacante M and Galvano F contributed to the study design, data analysis, and the drafting of the manuscript; Motta M, Bertino G and Neri S contributed to enrollment of patients and data interpretation; Giordano M helped with statistical analysis; Malaguarnera M, Pennisi M and Volti GL helped with data interpretation and data analysis. Supported by Ministero dell'Università e Ricerca Scientifica e Tecnologica

Correspondence to: Mariano Malaguarnera, AP, Department of Internal Medicine and Systemic Disease, Ospedale Cannizzaro, Viale Messina, 829-95125 Catania, Italy. malaguar@unict.it
Telephone: +39-95-7262008 Fax: +39-95-7262011

Received: January 10, 2011 Revised: February 19, 2011

Accepted: February 26, 2011

Published online: October 21, 2011

Abstract

AIM: To evaluate the efficacy of L-carnitine on alleviating anemia, thrombocytopenia and leukopenia, and minimizing dose reductions in patients with chronic hepatitis C virus (HCV) in treatment with Interferon α (IFN- α) plus ribavirin.

METHODS: Sixty-nine patients with chronic hepatitis C were enrolled in the study and divided into two groups. group A ($n = 35$) received Peg-IFN- α 2b plus ribavirin plus L-carnitine, and group B ($n = 34$) received Peg-IFN- α and ribavirin for 12 mo. All patients underwent laboratory investigations including: red cell count, hemoglobin, white cell count, platelets, bilirubin, alanine

aminotransferase (ALT), aspartate aminotransferase (AST), and viremia.

RESULTS: After 12 mo in group A compared to group B we observed significant differences in AST 108.8 vs 76.8 (IU/L; $P < 0.001$), ALT 137.9 vs 112.3 (IU/L; $P < 0.001$), viremia 4.04 vs 2.36 ($\times 10^6$ copies/mL; $P < 0.001$), Hb 1 vs 3.5 (g/dL; $P < 0.05$), red blood cells 0.3 vs 1.1 ($\times 10^{12}$ /L; $P < 0.001$), white blood cells 1.5 vs 3 ($\times 10^9$ /L; $P < 0.001$) and platelets 86 vs 85 ($\times 10^9$ /L; $P < 0.001$). The end treatment responders were 18 vs 12 (60% vs 44%) and the non responders were 12 vs 15 (40% vs 50%) [odds ratio (OR) 1.65, 95% CI = 0.65-5.37, $P < 0.05$]. In group A compared to group B there was a significant improvement of sustained virological response in 15 vs 7 patients (50% vs 25%), while the relapsers were 3 vs 5 (10% vs 18%) (OR 3.57, 95% CI = 0.65-19.3, $P < 0.001$).

CONCLUSION: L-carnitine supplementations modulate erythropoiesis, leucopoiesis and thrombocytopoiesis, and may be useful in patients treated for HCV. L-carnitine treatment offers the possibility of achieving a sustained virological response while preventing overtreatment.

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Key words: L-carnitine; Chronic hepatitis C; Anemia; Interferon

Peer reviewers: Natalia A Osna, MD, PhD, Liver Study Unit, Research Service (151), VA Medical Center, 4101 Woolworth Avenue, Omaha NE 68105, United States; Sabine Mihm, Professor, Department of Gastroenterology, Georg-August-University, Robert-Koch-Str.40, Göttingen D-37099, Germany

Malaguarnera M, Vacante M, Giordano M, Motta M, Bertino G, Pennisi M, Neri S, Malaguarnera M, Volti GL, Galvano F.

L-carnitine supplementation improves hematological pattern in patients affected by HCV treated with Peg interferon- α 2b plus ribavirin. *World J Gastroenterol* 2011; 17(39): 4414-4420 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4414.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4414>

INTRODUCTION

Interferon α (IFN- α), in combination with ribavirin (RBV) treatment, represents the most efficacious therapeutic tool in the management of chronic hepatitis C^[1,2]. The current standard of care results in successful outcomes in 70% of patients with hepatitis C virus (HCV) genotype 2 or 3 and in 48% of patients with HCV genotype 1. Systemic side effects such as fatigue, fever, chills, myalgia and nausea occur in most patients and normally disappear after a few weeks of treatment. Other side effects include hematologic, autoimmune, neurological and psychiatric disorders^[3-7]. The frequency of hematologic adverse events including thrombocytopenia, anemia and neutropenia is higher. A recent study^[5] reported anemia in 16%, thrombocytopenia in 20%, and leukopenia in 10% of Peg-IFN- α 2b plus RBV treated patients with HCV. These adverse events most frequently lead to drug discontinuation or to dose modifications. In our previous reports we have observed that L-carnitine improves responses and quality of life and reduces steatosis in patients with HCV treated with interferon^[8-10]. L-carnitine (4-N-trimethyl ammonium 3-hydroxybutyric acid) is a conditionally synthesized nutrient from the amino acids lysine and methionine in the human liver, brain and kidney, but is largely obtained from meat and dairy products. Administration of L-carnitine is an accepted treatment for mitochondrial myopathy and encephalomyopathy, as well as other states of primary and secondary L-carnitine deficiency^[11]. Recently, L-carnitine has been proposed as a potential adjuvant treatment to improve anemia, thrombocytopenia, leukopenia and immunological function^[12-17]. The aim of our study was to evaluate the efficacy of L-carnitine in alleviating anemia, thrombocytopenia and leukopenia, and minimizing dose reductions in patients with chronic hepatitis C virus in treatment with IFN- α plus RBV.

MATERIALS AND METHODS

Patients

Between January 2004 and December 2007, a total of 69 patients with chronic hepatitis (27 women and 42 men, mean age 48 years) aged 32-63 years (median 46 years), were consecutively enrolled in the study (Figure 1).

The patients had to fulfill the following inclusion criteria: alanine aminotransferase (ALT) levels greater than 1.5-fold higher than the upper limit of normal, the presence of anti-HCV antibodies in the serum, HCV-RNA > 1000 copies/mL, and histological modifications in the

liver biopsy. Exclusion criteria were: positive test for serum hepatitis B surface antigen, positive test for serum HIV antibodies, negative for HCV antibodies, alcoholic liver disease (daily alcohol consumptions < 20 g/d), and diabetes. The presence of other causes of hepatopathy, decompensated cirrhosis, pregnancy, and known contraindications for Peg-IFN- α or RBV therapy such as hemoglobinopathies, cardiopathy, hemochromatosis, diabetes mellitus, autoimmune diseases, major depression or other severe psychiatric pathological conditions were considered causes for ruling out.

Study design

The study was a prospective, randomized, open-label trial. Eligible patients were randomly assigned to one of the two study treatments in equal proportions by means of a computer-generated table of random numbers. They were divided into two groups (A and B) and they were stratified by HCV genotype (1 *vs* others) and the viremia. Group A received Peg-IFN- α 2b at a dose of 1.5 μ g/kg per week for 12 mo intramuscularly, plus daily oral RBV, plus L-carnitine 2 g twice a day. The dose of ribavirin was adjusted to body weight: 800 mg for body weight below 60 kg, 1000 mg when it was between 65 kg and 75 kg, and 1200 mg when it was above 75 kg. Group B received Peg-IFN- α and RBV at the same dosage, way and duration. Patients were evaluated before treatment, 6 mo and 12 mo after the initiation of the therapy. A follow up evaluation was performed 6-mo after the end of the planned treatment. A medical interview and a physical examination were realized for all patients included in the study before starting therapy. Guidelines for discontinuing, interrupting or decreasing the dose of study medication because of adverse events and hematologic or biochemical abnormalities were prespecified in the protocol. Study drug was reduced and discontinued when: hemoglobin values of < 10 g/dL and 8.5 g/dL, absolute neutrophil counts of $0.75 \times 10^9/L$ and $0.50 \times 10^9/L$, and platelet counts of < $50 \times 10^9/L$ and < $25 \times 10^9/L$ respectively. The study protocol was approved by the research ethics committee of Cannizzaro Hospital, Catania, Italy and was performed in accordance with the Declaration of Helsinki principles and the Good Clinical Practice Guidelines^[18].

Clinical laboratory tests

A complete routine chemistry (including red cell count, hemoglobin, white cell count, platelets prothrombin time, fasting plasma glucose, blood urea nitrogen, serum creatinine, bilirubin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ -glutamyl transpeptidase and creatine phosphokinase levels) was performed at every medical visit. Anti-HCV antibodies were evaluated by using second generation enzyme-linked immunosorbent assay ELISA (Ortho-Diagnostic Systems, Raritan NJ, United States) and positive samples were confirmed by immunoblotting (RIBA; Chiron Corporation, Emeryville, CA, United States). Serum HCV

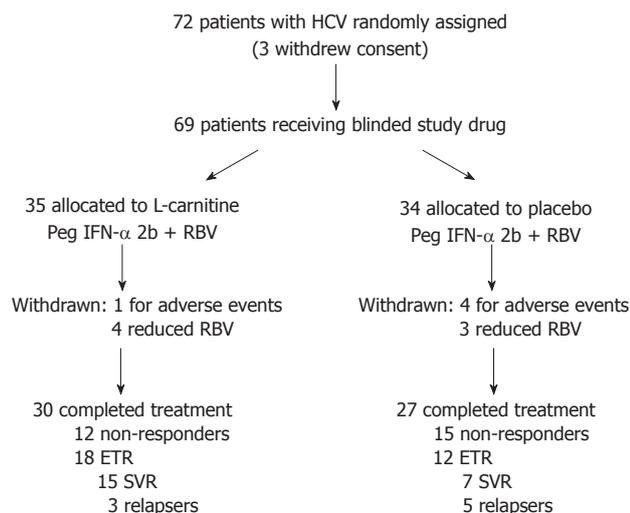


Figure 1 Trial profile of L-carnitine treatment. IFN- α : Interferon α ; HCV: Hepatitis C virus; RBV: Ribavirin; SVR: Sustained virological responders; ETR: End-of-treatment responders.

RNA levels were detected first time by standardized quantitative polymerase chain reaction (PCR) assay, with a lower limit of detection of 50 IU/mL. Then serum HCV RNA levels were measured by standardized quantitative PCR assay with a lower limit of detection of less than 1000 copies/mL, using the amplicor quantitative PCR system (Roche Diagnostic System Inc.-Branchburg, NJ, United States). HCV genotypes and subtypes were identified through a modification of the specific line probe assay (Inno-LiPA system; Innogenetics NV, Zwijnaarde, Belgium) as described by Stuyver *et al*^[19]. The HCV genotypes were designated according to the nomenclature proposed by Simmonds *et al*^[20].

Histology

Liver biopsy was realized in the 6 mo before the initiation of therapy and 6 mo after the end of treatment. It was obtained using a modified Menghini technique. The specimen was fixed in neutral formaldehyde 4% solution for routine histological processing and evaluation. The Knodell and Ishiak Histological activity index (HAI) score was used to assess the histological grading of the disease^[21].

Efficacy and safety assessment

All enrolled patients were included in the intention-to-treat efficacy analysis and patients who received at least one dose of IFN- α plus ribavirin were included in the safety analysis. Data were analyzed by an “intention-to-treat” principle. We considered patients as “sustained virological responders” (SVR) when they showed an undetectable HCV RNA (< 50 IU/mL) in serum at the end of the follow up period. Relapse was defined as undetectable HCV-RNA levels at the end of treatment, but detectable levels during the follow up period. Adverse events were assessed by interviews, laboratory examinations and clinical examinations during treatment. They were graded as mild, moderate and severe on the basis of the WHO score. The treatment was definitively stopped

Table 1 Characteristics of the subjects included in the study (mean \pm SD)

	¹ Group A (n = 35)	² Group B (n = 34)
Mean age (yr)	47.6 \pm 4.9	47.1 \pm 5.4
Sex (M/F)	22/13	20/14
HCV exposure time (yr)	6.44 \pm 4.2	7.08 \pm 4.4
BMI (kg/m ²)	27.1 \pm 3.1	27.4 \pm 2.9
HCV genotype (n)		
1a	3	3
1b	24	25
2a	2	2
3a	6	4
Probable exposure (n)		
Blood transfusion	12	16
Intravenous drug abuse	7	6
Occupational	3	2
Unknown	13	10

There were not significant differences between groups. IFN- α : Interferon α ; HCV: Hepatitis C virus; BMI: Body mass index. ¹Group A: Peg IFN- α + RBV + L-carnitine; ²Group B: Peg IFN- α + RBV.

in the case of severe events, such as hematological toxicity, hepatic failure or no compliance. In moderate and mild cases of adverse effects, a dose reduction of 50% was performed, until the resolution of the event, when a full dose was restarted.

Statistical analysis

Means and standard deviations have been used to describe the distribution of continuous variables. Differences in response rates in the two study groups and histological findings between the initial and follow-up liver biopsy specimens were evaluated by paired *t* test. A *P* value < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics

A total of 69 patients were included in the study (35 in the Peg-IFN- α plus RBV plus L-carnitine group and 34 in the Peg-IFN- α plus RBV group). Baseline demographic characteristics and histological findings in liver biopsy were similar between the two treatment groups.

The mean time since their chronic hepatitis C infection was comparable. The most frequent viral genotype in patients was 1b. Baseline viremia was parallel in the two groups (Table 1). No significant differences were assessed between the two groups for ALT, aspartate aminotransferase (AST) or fasting plasma glucose.

Effects of Peg-IFN- α plus RBV plus L-carnitine

Effects on biochemical pattern: After 6 and 12 mo and at follow up, we observed a significant decrease in AST (*P* < 0.001) and ALT (*P* < 0.001) levels.

Effects on viremia and histological grading of the disease: After 6 and 12 mo and at follow up viremia was significantly reduced (*P* < 0.001). HAI score was significantly reduced after 12 mo (*P* < 0.05).

Table 2 Laboratory parameters of subjects included in the study (mean \pm SD)

	¹ Group A (n = 35)		² Group B (n = 34)	
	Before treatment	After 12 mo	Before treatment	After 12 mo
AST (IU/L)	145 \pm 44.2	36.2 \pm 12.8 ^{a,d}	136 \pm 41.4	59.2 \pm 15.4 ^{b,d}
ALT (IU/L)	182.1 \pm 46.2	44.2 \pm 13.8 ^{b,d}	174.1 \pm 42.2	61.8 \pm 15.4 ^{b,d}
Bilirubin (mmol/L)	10.7 \pm 7.8	10.1 \pm 5.6	10.2 \pm 9.0	10.1 \pm 7.8
Albumin (g/dL)	4.2 \pm 0.8	4.2 \pm 0.8	4.4 \pm 0.8	4.0 \pm 0.8 ^a
Viremia ($\times 10^6$ copies/mL)	5.44 \pm 3.12	1.4 \pm 1.1 ^{b,d}	5.32 \pm 3.21	2.96 \pm 1.9 ^{b,d}
HAI score	10.7 \pm 3.1	8.4 \pm 2.9 ^a	10.5 \pm 2.9	9.0 \pm 3.0 ^a
Hemoglobin (g/dL)	13.1 \pm 1.8	12.1 \pm 2.7 ^d	13.4 \pm 1.9	9.9 \pm 2.7 ^{b,d}
RBC ($\times 10^{12}$ /L)	4.7 \pm 0.4	4.4 \pm 0.7 ^{a,d}	4.9 \pm 0.6	3.8 \pm 0.7 ^{b,d}
WBC ($\times 10^9$ /L)	7.9 \pm 1.8	6.4 \pm 0.8 ^{b,d}	7.8 \pm 1.8	4.8 \pm 0.8 ^{b,d}
Platelets ($\times 10^9$ /L)	384 \pm 22	298 \pm 36 ^{b,d}	412 \pm 21	327 \pm 24 ^{b,d}

^a $P < 0.05$, ^b $P < 0.001$, comparison within group A and within group B according to the values before the treatment; ^d $P < 0.001$, groups A vs B after treatment. RBV: Ribavirin; HAI: Histological activity index; RBC: Red blood cells; WBC: White blood cells; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; IFN- α : Interferon α . ¹Group A: Peg IFN- α + RBV + L-carnitine; ²Group B: Peg IFN- α + RBV.

Effects on hematological pattern: After 6 mo red blood cells (RBCs), white blood cells (WBCs) and platelets were significantly reduced ($P < 0.001$); a significant decrease was also observed after 12 mo in RBCs ($P < 0.05$), WBCs ($P < 0.001$) and platelets ($P < 0.001$). At follow up, the decrease was observed only in platelets ($P < 0.001$).

Effects of Peg-IFN- α plus RBV

Effects on biochemical pattern: After 6 mo there was a significant decrease in albumin ($P < 0.05$). After 6 and 12 mo and at follow up we observed a significant decrease in AST ($P < 0.001$) and ALT ($P < 0.001$) levels.

Effects on viremia and histological grading of the disease: Viremia was significantly reduced after 6 mo ($P < 0.05$), after 12 mo ($P < 0.001$) and at the follow up ($P < 0.001$). The HAI score was significantly reduced after 12 mo ($P < 0.05$).

Effects on hematological pattern: After 6 and 12 mo and at follow up RBCs, WBCs and platelets were significantly reduced ($P < 0.001$).

Comparison between treatments

The comparison between the Peg-IFN- α plus RBV plus L-carnitine group and the Peg-IFN- α plus RBV group showed a significantly greater decrease after 6 mo in Hb 0.7 vs 2.8 (g/dL; $P < 0.05$), RBCs 0.5 vs 1 ($\times 10^{12}$ /L; $P < 0.001$), WBCs 0.9 vs 3.7 ($\times 10^9$ /L; $P < 0.001$) and platelets 82 vs 100 ($\times 10^9$ /L; $P < 0.001$). After 12 mo there were significant improvements in AST 108.8 vs 76.8 (IU/L; $P < 0.001$), ALT 137.9 vs 112.3 (IU/L; $P < 0.001$), viremia 4.04 vs 2.36 ($\times 10^6$ copies/mL; $P < 0.001$), Hb 1 vs 3.5 (g/dL; $P < 0.05$), RBCs 0.3 vs 1.1 ($\times 10^{12}$ /L; $P < 0.001$), WBCs 1.5 vs 3 ($\times 10^9$ /L; $P < 0.001$) and platelets 86 vs 85 ($\times 10^9$ /L; $P < 0.001$). At the end of the follow up, the results confirmed the improvements in AST 90.9 vs 69.6 (IU/L; $P < 0.001$), ALT 125.3 vs 101.9 (IU/L; $P < 0.001$), viremia 3.64 vs 2.34 ($\times 10^6$ copies/mL; $P < 0.001$), Hb 0.3 vs 2 (g/dL; $P < 0.05$), RBCs 0.1 vs 0.8 ($\times 10^{12}$ /L; $P < 0.001$), WBCs 0.3 vs 2.3 ($\times 10^9$ /L; $P < 0.001$)

and platelets 68 vs 56 ($\times 10^9$ /L; $P < 0.001$) (Table 2). The end treatment responders were 18 vs 12 (60% vs 44%) and the non responders were 12 vs 15 (40% vs 50%) (OR 1.65; 95% CI = 0.65-5.37; $P < 0.05$). When comparing the Peg-IFN- α plus RBV plus L-carnitine group and the Peg-IFN- α plus RBV group we observed a significant improvement of SVR in 15 vs 7 patients (50% vs 25%), while the relapsers were 3 vs 5 (10% vs 18%) (OR 3.57; 95% CI = 0.65-19.3; $P < 0.001$).

Adverse events

No serious adverse events (WHO grade 3 or 4) have been reported in the two groups. Five patients of the Peg-IFN- α 2b plus RBV treated group and 2 of the other group showed mild psychological disorders such as anxiety, irritability and depression. Furthermore, other side effects registered in both groups were anorexia (12% in Peg-IFN- α 2b plus RBV patients vs 18% in Peg-IFN- α 2b plus RBV plus L-carnitine), nausea (20% vs 21% respectively), weight loss (14% vs 5%), headaches (44% vs 40%), fatigue (44% vs 25%), myalgia (30% vs 20%), musculoskeletal pain (30% vs 22%), irritability (18% vs 16%), hypertriglyceridemia (34% vs 18%), hypercholesterolemia (24% vs 8%), and hyperglycemia (12% vs 4%). Median Hb concentration significantly falls during the first 3 mo of treatment in the Peg-IFN- α 2b plus RBV group, but remained stable in the Peg-IFN- α 2b plus RBV plus L-carnitine group. The patients treated with Peg-IFN- α 2b plus RBV plus L-carnitine experienced a fall in median hemoglobin concentration from 13.1 g/dL (range 11.8-14.0 g/dL) to 12.1 (range 11.2-14.0 g/dL) at the end of therapy. The patients treated with Peg-IFN- α 2b plus RBV experienced a fall in median hemoglobin concentration from 13.4 g/dL (range 11.4-15.1) to 9.9 g/dL (range 9.1-12.4 g/dL) at the end of therapy. In the Peg-IFN- α 2b plus RBV plus L-carnitine group, 30 patients showed good adherence to their medication and duration of therapy, 2 reduced RBV dose to 1000 mg; 2 reduced RBV dose to 800 mg; and 1 dropped out from the treatment due to headaches. In the Peg-IFN- α 2b plus RBV group, 23 patients showed good adherence to their medication

and duration of therapy; 3 reduced RBV dose to 800 mg; 4 dropped out from the treatment for anemia; 2 reduced RBV dose to 1000 mg; and 2 dropped out from the treatment due to leukopenia.

DISCUSSION

The primary goal of treatment for chronic HCV infection is a sustained virological response. However, a substantial proportion of patients do not have an optimum response to current treatment regimens^[22]. In our study, when comparing the Peg-IFN- α plus RBV plus L-carnitine group versus the Peg-IFN- α plus RBV group we observed a significant improvement of sustained virological response in 50% *vs* 25% of patients. L-carnitine is a necessary nutrient factor in energy production and its deficiency is known to decrease energy availability in vital organs. The carnitine content decreases when endogenous synthesis becomes insufficient, and under such conditions carnitine has been beneficial in restoring the level to normalcy^[23]. L-carnitine is a natural constituent of higher organisms, in particular cells of animal origin, and its deficiency is usually associated with a failure to thrive or due to recurrent infections^[24]. L-carnitine is also reported to inhibit apoptosis and improve the function of the bone marrow progenitors by increasing the number of colony forming units^[25]. In patients treated with a placebo, we observed a significant decrease in Hb, in RBCs, in WBCs and in platelets after 6 and 12 mo and at the end of follow up. Decreased Hb levels represent a common side effect of the IFN- α and RBV combination, or the pegylated IFN- α (Peg IFN- α) and RBV combination used to treat HCV infection, with 29% to 36% of treated patients developing anemia. In a recent study, 54% of patients on this regimen experienced Hb decreases of 3g/dL or more from pre-treatment levels^[26,27]. In a large clinical trial^[28] anemia (defined as Hb < 12 g/dL) resulted in a RBV dose reduction in 22% of patients and, in a community-based setting, anemia resulted in the discontinuation of therapy in 36% of patients. In 3070 patients with HCV genotype 1 treated with RBV plus IFN 2a or 2b the RBV dose was reduced owing to an adverse event in 30.2%^[29]. Between 2.1% and 3.8% of patients met the discontinuation criterion^[29]. Anemia might become evident in the first several weeks of RBV therapy and might continue for the duration of combination therapy^[30,31]. Anemia is a common complication associated with RBV, by both a decrease in erythrocyte precursor production and reduced survival of red blood cells^[32]. In our study we observed a significantly greater decrease in hemoglobin and red blood cells in the Peg-IFN- α plus RBV group than the Peg-IFN- α plus RBV plus L-carnitine group^[33]. In thalassemic patients, El-Beshlawy *et al*^[34] observed a significant increase in blood transfusion interval after L-carnitine therapy. This was previously reported and can be explained by the protective effect of L-carnitine on the red blood cells from oxidative stress and the stabilization to their membranes where latent peroxidative damage has occurred^[35,36]. The

autoxidation of globin chains and iron overload were the suggested mechanisms for the increased oxidative stress. The counteracting effect of antioxidants on lipid peroxidation and their protective effect against oxidative damage of erythrocytes in β -thalassemia were demonstrated^[37]. It has been suggested that L-carnitine may act via stabilization of the red blood cell membrane, thereby increasing the erythrocyte^[38]. Relationships between L-carnitine and erythrocyte osmotic fragility, deformability and membrane fluidity have been demonstrated^[39,40]. Alternatively, the beneficial effects on anemia may be a result of a reduction in lipid peroxidation^[41]. The results of our study showed a greater decrease in white blood cells in the Peg-IFN- α plus RBV group compared to the Peg-IFN- α plus RBV plus L-carnitine group. In 3070 patients with HCV genotype 1, the proportion of patients with neutropenia who met the criterion for Peg IFN dose reduction were: 21.1% receiving Peg IFN- α 2a, 19.4% receiving standard dose Peg IFN- α 2b, and 12.5% receiving low dose Peg IFN- α 2b. Between 2.1 and 5.9% of patients met the discontinuation criterion based on neutropenia^[29]. L-carnitine is known to improve neutrophil and macrophage functions, such as chemotaxis and phagocytosis in aged rats, at lower concentrations^[16]. Sener *et al*^[42] suggested that this compound inhibited leukocyte apoptosis possibly through its free radical scavenging and antioxidant properties. The increase in neutrophil functions, observed after L-carnitine treatment may be attributed to the ability of L-carnitine to increase the activities of glucose-6-phosphate dehydrogenase and myeloperoxidase by L-carnitine^[23]. Moreover, preservation of membrane integrity is a vital phenomenon for efficient phagocytosis as well as chemotaxis. The increase in neutrophil functions and organ cell counts observed in aged animals after L-carnitine treatment may also be related to the property of L-carnitine to preserve membrane integrity. L-carnitine is well known to exhibit membrane modulatory effects and thereby preserve cellular membrane integrity^[43]. In a recent study, Poynard *et al*^[6] reported thrombocytopenia in 20% of HCV patients treated with Peg-IFN- α plus RBV. The results of our study showed a significantly greater decrease in platelet count in the Peg-IFN- α plus RBV group compared to the Peg-IFN- α plus RBV plus L-carnitine group. Some studies indicate that L-carnitine modulates platelet functions and production^[44,46]. L-carnitine acts by reacting with fatty acids and this mechanism may affect the biological function of cells. Pignatelli *et al*^[47] showed that carnitine affects arachidonic acid metabolism and, in turn, blood platelet functions. *In vitro* studies by Saluk-Juszczak *et al*^[17] demonstrated that L-carnitine may modulate platelet activation through antioxidant mechanisms and the inhibition of the arachidonic acid cascade. Arachidonic acid has a key role in the activation of blood platelets and in the formation of free radicals via the stimulation of NADPH oxidase in these cells. L-carnitine interfering with arachidonic acid metabolism has a direct effect on platelet activation and oxidative stress. L-carnitine inhibits platelet superoxide anion formation elicited

by arachidonic acid and collagen, but it has no effect on thrombin-induced platelet aggregation^[47,48]. The well established beneficial effects of L-carnitine (including modulation of cell energy production, fat metabolism, erythropoiesis, leucopoiesis and thrombocytopoiesis) strongly suggests that supplementation of these nutrients may be useful in patients treated for HCV even if our study was conducted on a relatively small sample size. L-carnitine treatment offers the possibility of tailoring treatment to patients and selecting the treatment duration that ensures the best chance of achieving a SVR while preventing overtreatment.

COMMENTS

Background

Interferon α (IFN- α), in combination with ribavirin (RBV), represents the most efficacious therapeutic tool in the management of chronic hepatitis C, but the frequency of hematologic adverse events including thrombocytopenia, anemia and neutropenia during treatment is high. These adverse events most frequently lead to drug discontinuation or to dose modifications.

Research frontiers

Recently L-carnitine has been proposed as a potential adjuvant treatment to improve anemia, thrombocytopenia, leukopenia and immunological function. L-carnitine is also reported to inhibit apoptosis and improve the function of the bone marrow progenitors by increasing the number of colony forming units.

Innovations and breakthroughs

The study showed a significantly greater decrease in platelets, and red and white cells in the Peg-IFN- α plus RBV group compared to the Peg-IFN- α plus RBV plus L-carnitine group. L-carnitine treatment may offer the possibility of tailoring treatment to patients and selecting the treatment duration that ensures the best chance of achieving a sustained virological response while preventing overtreatment.

Applications

The well established beneficial effects of L-carnitine, including modulation of cell energy production, fat metabolism, erythropoiesis, leucopoiesis and thrombocytopoiesis strongly suggested that supplementation of these nutrients may be useful in patients treated for HCV.

Peer review

Although being small in sample number, this is a sound clinical trial with promising results. This is a straight and well conducted investigation. The manuscript is well written.

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S- Editor Tian L L- Editor Rutherford A E- Editor Xiong L

Comparative epidemiology of gastric cancer between Japan and China

Yingsong Lin, Junko Ueda, Shogo Kikuchi, Yukari Totsuka, Wen-Qiang Wei, You-Lin Qiao, Manami Inoue

Yingsong Lin, Junko Ueda, Shogo Kikuchi, Department of Public Health, Aichi Medical University School of Medicine, Aichi 480-1195, Japan

Yukari Totsuka, Division of Cancer Development System, National Cancer Center Research Institute, Tokyo 104-0045, Japan
Wen-Qiang Wei, You-Lin Qiao, Department of Cancer Epidemiology, Cancer Institute/Hospital, Chinese Academy of Medical Sciences, Beijing 100730, China

Manami Inoue, Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center Research Institute, Tokyo 104-0045, Japan

Author contributions: Lin Y, Totsuka Y, and Inoue M contributed to the conception of this review article; Lin Y and Ueda J performed the literature research; Lin Y drafted the article; Totsuka Y, Kikuchi S, Wei WQ, Qiao YL, and Inoue M provided valuable comments and revised the article.

Supported by Grant-in-Aid from the Third Term Comprehensive Control Research for Cancer, the Ministry of Health, Labour and Welfare, Japan

Correspondence to: Yingsong Lin, MD, PhD, Department of Public Health, Aichi Medical University School of Medicine, 21 Yazako, Karimata, Nagakute-cho, Aichi 480-1195, Japan. linys@aichi-med-u.ac.jp

Telephone: +81-561-623311 Fax: +81-561-625270

Received: February 21, 2011 Revised: June 9, 2011

Accepted: June 16, 2011

Published online: October 21, 2011

Abstract

AIM: To clarify the similarities and differences in gastric cancer epidemiology between Japan and China.

METHODS: A comprehensive literature search of the PubMed database was performed. The relevant literature published in China was also been cited. Data on incidence and mortality rates in 2008 were obtained from the Cancer Mondial database, published by International Agency for Research on Cancer at <http://www-dep.iarc.fr/>.

RESULTS: Gastric cancer remains a significant public

health burden in both Japan and China. The prevalence of *Helicobacter pylori* (*H. pylori*) colonization is high in the adult populations of both countries. Accumulating evidence from intervention studies in both countries has shown the effectiveness of *H. pylori* eradication in reducing gastric cancer incidence. There are differences, however, in many aspects of gastric cancer, including patterns of incidence and mortality, trends in the prevalence of *H. pylori* infection, *H. pylori* strains, the magnitude of risk of gastric cancer related to *H. pylori* infection, and associations with dietary habits. Compared with China, Japan has seen a more rapid decline in *H. pylori* infection among adolescents. While Japanese cohort studies have dominated the literature concerning the associations between gastric cancer and dietary habits, numerous case-control studies in China suggest a positive association between a high intake of preserved fish and vegetables and gastric cancer risk. There is a need for a multidisciplinary research approach to understand the interactions between various strains of *H. pylori*, host factors, and other lifestyle and environmental factors in gastric carcinogenesis in both countries.

CONCLUSION: The shared high incidence of gastric cancer and high prevalence of *H. pylori*, as well as differences in many aspects of gastric cancer, provide an excellent opportunity to establish Sino-Japanese collaborations.

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Key words: Gastric cancer; Risk factor; *Helicobacter pylori*; Epidemiology

Peer reviewers: David J McGee, PhD, Associate Professor, Department of Microbiology and Immunology, Louisiana State University Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130, United States; Hikaru Nagahara, Professor, Department of Gastroenterology, Aoyama Hospital, Tokyo Women's Medical University, 2-7-13 Kitaaoyama Minato-ku, Tokyo 107-0061, Japan

Lin Y, Ueda J, Kikuchi S, Totsuka Y, Wei WQ, Qiao YL, Inoue M. Comparative epidemiology of gastric cancer between Japan and China. *World J Gastroenterol* 2011; 17(39): 4421-4428 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4421.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4421>

INTRODUCTION

Gastric cancer is a heterogeneous, multifactorial disease. The incidence and mortality vary geographically, with the highest rates in East Asia (Japan, China and Korea)^[1]. Although a trend of declining incidence has been observed in Japan and China, gastric cancer still represents a tremendous burden in each country. According to the vital statistics released by the Ministry of Health, Welfare and Labor in Japan, approximately 50 000 Japanese men and women die from gastric cancer annually, representing approximately 15% of annual cancer-related deaths over the past four decades^[2]. No systematic national vital statistics exist in China, but a retrospective sampling survey on malignant tumors from 2004 to 2005 found that the mortality rate from gastric cancer ranked third in overall cancer mortality^[3]. Notably, China alone accounts for 42% of all gastric cancer cases worldwide, at least in part because of its large population^[1].

Numerous epidemiologic studies have been conducted in Japan and China to identify environmental and lifestyle factors that contribute to the development of gastric cancer; these studies have identified *Helicobacter pylori* (*H. pylori*) infection as an important risk factor for gastric cancer^[4]. Additionally, high salt intake and exposure to *N*-nitroso compounds significantly increase the risk among *H. pylori* infected individuals^[5]. It is noteworthy that Japan has a strong tradition of gastric cancer research, not only in basic science but also in epidemiology and clinical trials. Seminal papers published during the last three decades have greatly contributed to our understanding of gastric cancer etiology and prevention^[6-9]. However, an increasing number of case-control studies in different regions of China have examined risk factors for gastric cancer, and cohort studies are ongoing to investigate the role of lifestyle in urban and rural areas^[10-12].

In this article, we first summarize the current understanding of gastric cancer etiology on the basis of existing literature. We then compare the burden of gastric cancer between Japan and China in terms of trends in incidence and mortality. Next, we address three of the principal risk factors, based on epidemiologic studies conducted in each country: *H. pylori* infection, cigarette smoking and diet. Finally, we propose three potential avenues for Sino-Japanese collaboration.

MATERIALS AND METHODS

We performed a comprehensive literature search of the

PubMed database using the search terms “risk factors”, “*H. pylori*”, “smoking”, “diet”, “gastric cancer”, “China”, and “Japan”. In addition, relevant literature published in China was also cited. Data on incidence and mortality in 2008 were obtained from the Cancer Mondial database, published by International Agency for Research on Cancer (IARC) at <http://www-dep.iarc.fr/>.

RESULTS

Current knowledge about gastric carcinogenesis

Gastric cancer is a multifactorial disease with a complex interplay between genetics and both lifestyle and environmental factors. Gastric cancer can be classified as intestinal or diffuse. While the triggering factor and the histopathologic changes in the progression of diffuse gastric cancer remain incompletely understood, the progression of intestinal gastric cancer is well characterized^[13]. An individual develops intestinal cancer through a series of histological changes beginning with the transition from normal mucosa to chronic superficial gastritis, which then leads to atrophic gastritis, intestinal metaplasia, and finally dysplasia and adenocarcinoma^[13]. Before the discovery of *H. pylori* in 1983, epidemiologic studies had already suggested an important role for lifestyle in the etiology of gastric cancer. In particular, a high-salt diet and foods rich in *N*-nitroso compounds appeared to be major inducers of gastric cancer. Since the discovery of *H. pylori*, its close association with peptic ulcers and gastric cancer has been supported by numerous studies. Asia-Pacific consensus guidelines on gastric cancer prevention define *H. pylori* infection as necessary but not sufficient for the development of non-cardia gastric adenocarcinoma^[4]. From an epidemiologic perspective, the synergistic interaction between *H. pylori* and diet plays an overriding role in gastric carcinogenesis^[14].

However, current studies have not provided a clear answer as to why only a minority of individuals with *H. pylori* infection develop gastric cancer. One reason is that the interactions of *H. pylori* strains, host factors, and other lifestyle and environmental factors in gastric carcinogenesis are not well defined. Another reason is that few causally linked genes have been found, and the role of genetic and epigenetic changes in gastric carcinogenesis is poorly understood. These two issues need to be addressed in future studies.

Descriptive epidemiology of gastric cancer in Japan and China

Patterns of gastric cancer incidence, mortality and trends: According to Globocan 2008, gastric cancer is the third most frequently diagnosed cancer and the second leading cause of cancer deaths in Japan, with an estimated 102 040 new cases and 50 156 cancer deaths in 2008. The overall estimated age-adjusted incidence rate (standardized for world population) in 2008 was 31.1 per 100 000 people. However, gastric cancer is the second most frequently diagnosed cancer and the third lead-

Table 1 Comparisons of crude and age-standardized incidence rates of gastric cancer between Japan and China (1993-1997)

	Men		Women	
	Crude	ASR	Crude	ASR
Japan				
Hiroshima	113.1	85.5	55.1	33.9
Miyagi	109.2	69	52.2	27.1
Nagasaki	119.9	65.4	56.5	25.6
Osaka	87.7	59.9	42.9	23.8
Saga	115.8	63.6	57	24.9
Yamagata	178.5	91.6	94.1	38.9
China				
Beijing	27.8	19.8	13	8.7
Changle	103.5	145	29.6	34.5
Cixian	55.9	78.1	28	31.9
Jiashan	45.8	38.9	20.3	15.7
Qidong	39.5	37.8	24.2	19
Shanghai	54.6	32.3	29.8	17.6
Tianjin	33.5	26.9	13.9	10
Wuhan	29.3	29.8	17.1	14.5

Source: Cancer incidence in five continents Vol. VIII, IARC scientific publications No. 155. ASR: Age standardized rate, per 100 000 population.

ing cause of cancer death in China, with an estimated 464 439 new cases and 352 315 cancer deaths in 2008. The overall estimated age-adjusted incidence rate in 2008 was 29.9 per 100 000 people in China.

Although China's overall incidence rate is comparable to that of Japan, a wider variation in both crude and age-standardized rates is apparent when cancer registry data (1993-1997) from the 2 countries are compared (Table 1). For example, the incidence in Changle was 145 per 100 000 people, approximately 7 times higher than in Beijing. The highest rates were often found in economically undeveloped rural areas in China, including Gansu, Henan, Hebei, Shanxi, and Shaanxi Provinces^[15]. Although gastric cancer incidence is declining in both rural and urban areas in China^[16-18], the rate of decline may be slower than in developed countries^[19]. The number of new gastric cancer cases has been projected to increase continuously over the next 40 years because of population growth and aging^[19].

Risk factors for gastric cancer in Japan and China

From the large body of literature on gastric cancer etiology in Japan and China, we cite selected epidemiologic studies conducted in each country. Three major risk factors, namely *H. pylori* infection, cigarette smoking and high intake of salt/salty food, are addressed in detail.

H. pylori colonization: Prevalence of *H. pylori* colonization in the general population

Japan: Gastric *H. pylori* infection is common among middle-aged and elderly Japanese people. A seroepidemiological study of *H. pylori* infection among apparently healthy residents of Sapporo found a prevalence of 70%-80% for individuals born before 1950^[20]. For those residents born after 1950, the frequency of *H. pylori* in-

fection increased at approximately 1% per birth year^[20]. The prevalence of *H. pylori* infection, however, has been decreasing over the past several decades. The overall *H. pylori* seropositivity was 72.7% in 1974, 54.6% in 1984, and 39.3% in 1994, based on an assay of serum samples from 1015 healthy people living in several prefectures in central Japan^[21]. As in other developed countries, a clear birth cohort effect has been observed for *H. pylori* infection in Japan, with younger generations experiencing a more rapid decline than older generations^[22,23]. In a 2007 study involving 777 university students with a mean age of 19.6 years, *H. pylori* prevalence was only 14.7%^[24].

China: The Chinese population has a high prevalence of *H. pylori* infection. A 2003 meta-analysis, based on studies published between 1990 and 2002, concluded that the prevalence of *H. pylori* infection for the entire Chinese population was approximately 58%^[25]. Since 2003, numerous studies have also been conducted to examine the prevalence of *H. pylori* in healthy people in different regions of China, with reported prevalence ranging from 40% to 81%^[26-29]. Generally, studies in rural areas found a higher prevalence of *H. pylori* than studies in urban areas. Furthermore, areas with high gastric cancer incidence generally have a higher prevalence of *H. pylori* infection than low-incidence areas.

Because *H. pylori* is acquired during childhood, some surveys of *H. pylori* prevalence in China have focused on children. One recent study reported a prevalence of 37.7% in children aged 10-19 years in Beijing and 25.5% in the same age group in Shandong^[30]. Some studies suggest a downward trend in *H. pylori* seroprevalence in some regions; for example, a significant decrease was observed across age groups in Guangzhou^[31]. Evidence on this subject, however, is fragmentary and inconclusive. In particular, it remains unclear whether the rate of decline is accelerating, especially in the younger segments of the population.

H. pylori colonization: Findings from observational epidemiologic studies addressing the association between *H. pylori* and gastric cancer risk

Japan: Both case-control and cohort studies have been conducted to estimate the degree of gastric cancer risk associated with *H. pylori* infection in the Japanese population. To date, all four prospective studies have shown a positive association, with relative risks (RRs) ranging from 1.0 to 5.1^[32-35] (Table 2). In the prospective study that used the largest dataset (511 cases and 511 control subjects), Sasazuki *et al.*^[35] showed that the adjusted odds ratio (OR) of gastric cancer associated with *H. pylori* infection was 5.1, which is quite similar to the estimate of 5.9 for non-cardia gastric cancer in a combined analysis of 12 case-control studies nested within prospective cohorts^[36]. Based on the substantial evidence from both case-control and cohort studies, it is clear that *H. pylori* infection is causally linked to gastric cancer in the Japanese population.

Table 2 Summary of findings on the associations between *H. pylori* carriage and risk of gastric cancer in prospective studies from Japan and China

Author ^[Ref.] , yr	Country	Study design	Case patients /control subjects	Seroprevalence of <i>H. pylori</i> in cases vs controls (%)	ELISA kit used for measuring seroprevalence of <i>H. pylori</i>	OR (95% CI)
Watanabe <i>et al.</i> ^[32] , 1997	Japan	Nested case-control study	45/225	91.1 vs 75.6	Pirikaplate G <i>Helicobacter</i> enzyme immunoassay (Fujirebio Inc., Tokyo)	3.4 (1.2-9.9)
Sasazuki <i>et al.</i> ^[35] , 2006	Japan	Nested case-control study	511/511	93.5 vs 74.5	E Plate, produced by Eiken Kagaku Co.Ltd., Tokyo	5.1 (3.2-8.0)
Yamagata <i>et al.</i> ^[33] , 2000	Japan	Cohort study	1070 men and 1532 women at baseline	71.5 among men vs 62.4 among women	HM-CAP, Enteric Products Inc, Westbury, NY	Men: RR = 2.9 (1.1-7.4) Women: RR = 1.0 (0.3-3.0)
Yatsuya <i>et al.</i> ^[34] , 2004	Japan	Nested case-control study	202/394	88.6 vs 79.2	HM-CAP, Enteric Products Inc, Westbury, NY	Men: 1.7 (0.5-5.1) Women: 5.1 (1.6-16.5)
Yuan <i>et al.</i> ^[38] , 1999	China	Nested case-control study	188/548	86 vs 85	Locally Developed and Validated Assay	1.8 (1.1-3.1), but 3.7 (1.5-9.3) for subjects followed for 5 or more years
Limburg <i>et al.</i> ^[39] , 2001	China	Nested case-control study	181/192	62.0 vs 52.0	Antibodies to the whole cell antigen	1.6 (1.1-2.5)
Kamangar <i>et al.</i> ^[12] , 2007	China	Case-cohort study	Cardia 582/992 Noncardia 343/992	Cardia 81.0 vs 73.0 Noncardia 80.0 vs 73.0	IgG antibodies to whole-cell antigen	Cardia 1.6 (1.3-2.1) Noncardia 1.6 (1.2-2.1)

OR: Odds ratio; RR: Relative risk; CI: Confidence interval.

China: The majority of epidemiologic studies that examined the association between *H. pylori* infection and gastric cancer in China are retrospective case-control studies. Of 11 case-control studies included in a 2001 meta-analysis, all studies showed a positive association. The ORs ranged from 2.1 to 5.6, with a combined OR of 3.0^[37].

This positive association was also observed in two prospective cohort studies. Yuan *et al.*^[38] reported that the OR was 3.7 for individuals seropositive for *H. pylori* who were followed for 5 or more years, on the basis of a nested case-control study within a cohort of Shanghai residents. A prospective, nested case-control study in Linxian, one of the highest-incidence regions in China, found that *H. pylori* seropositivity results in an approximately 2-fold increased risk of gastric cancer^[39]. This result was confirmed by a 2007 case-cohort study, in which *H. pylori* was associated with a 1.6-fold increased risk of both cardia and non-cardia gastric adenocarcinomas^[12].

***H. pylori* colonization: Findings from clinical studies, including both non-intervention and intervention studies**

Japan: Several recent clinical studies have greatly improved our understanding of the role of *H. pylori* in the development of gastric cancer. Umemura *et al.*^[7] (2001) found that gastric cancer developed in 36 of 1246 *H. pylori*-infected patients but none of the 280 uninfected patients in a prospective study involving 1526 Japanese patients with peptic ulcers, gastric hyperplasia or non-ulcer dyspepsia. The results are convincing because *H. pylori* colonization was confirmed by a combination of tests, including endoscopy, biopsy, histology, a rapid urease test, and serologic testing. This seminal study thus offers compelling evidence that *H. pylori* infection is associated with the development of both intestinal and diffuse gastric cancers. Another important study, a multi-center, open-label randomized controlled trial followed

544 patients who underwent endoscopic resection of early gastric cancer, half of whom underwent eradication of colonizing *H. pylori*^[8]. Eradication decreased the risk of developing metachronous gastric cancer by approximately 65%, even though these patients had already been diagnosed with early gastric cancer.

China: To determine whether *H. pylori* eradication reduces the incidence of gastric cancer at the population level in high-risk areas in China, Wong *et al.*^[40] (2004) conducted a randomized, placebo-controlled trial, using subjects without precancerous lesions. Unfortunately, however, this study was restricted by a short follow-up period and did not address whether those subjects with precancerous lesions experience a similar reduction in gastric cancer risk.

Cigarette smoking

Japan: Numerous epidemiologic studies over the past several decades have examined the association between cigarette smoking and gastric cancer risk in Japan, with the majority showing a significantly increased risk in current smokers when compared with those subjects who have never smoked. According to a systematic review and meta-analysis conducted by the Research Group for the Development and Evaluation of Cancer Prevention Strategies in Japan in 2006, the summary RR for current smokers were estimated to be 1.8 (95% CI: 1.5-2.1) in men and 1.2 (1.1-1.4) in women^[41]. Based on these results, the research group concluded that there is convincing evidence that tobacco smoking moderately increases the risk of gastric cancer in the Japanese population. Approximately 28.4% of gastric cancers are related to cigarette smoking, according to data from the Hisayama Study, a population-based prospective study of the combined influence of cigarette smoking and *H. pylori* infection^[42]. That study found that cigarette smoking is sig-

nificantly associated with increased risk of gastric cancer independent of *H. pylori* infection.

Although cigarette smoking is associated with an increased risk of gastric cancer, it remains unclear whether the observed positive association is homogeneous in terms of histologic type or anatomic location; such information has not been included in most previous studies. A cohort study was designed to address this question, incorporating complete histologic data. The results suggest that smoking significantly increases the risk of differentiated, but not undifferentiated, distal gastric cancer^[43].

China: The association between cigarette smoking and gastric cancer has been investigated in a number of epidemiologic studies, including both case-control and cohort studies, but the results are inconsistent^[44]. No association was found in a cohort study involving 9351 middle-aged adults in urban Shanghai^[45]. Another cohort study showed a non-significant increase in risk, with an RR of 1.4 for current smokers^[46]. In contrast, a recent prospective study of men in Shanghai showed that among nondrinkers, smokers have an 80% greater risk of gastric cancer, suggesting that cigarette smoking and alcohol consumption exert independent effects on gastric cancer risk^[47].

High intake of salt/salty food and food sources of nitrosamines

Japan: Collective evidence from epidemiologic and experimental studies over the past several decades strongly suggests that high intake of salt/salty food is associated with an increased risk of gastric cancer in Japanese populations^[9]. Japanese cohort studies dominate the published literature on gastric cancer epidemiology; of the 11 cohort studies included in a recent meta-analysis of salt consumption and gastric cancer risk, six of these studies came from Japan^[10]. In four of these Japanese studies, a statistically significant association was observed, with the RR ranging from 2.2 to 5.4 at the highest intake level.

The positive association observed between salt/salty food intake and gastric cancer risk in epidemiologic studies is also supported by experimental evidence. Using chemical carcinogens such as *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), Tatematsu *et al*^[6] reported the first experimental model of gastric carcinogenesis in *H. pylori*-infected Mongolian gerbils. Experiments with this model demonstrated that sodium chloride (NaCl) enhances the carcinogenic effects of MNNG and 4-nitroquinoline-1-oxide. Another notable finding is that salt and *H. pylori* act synergistically to promote the development of gastric cancer in Mongolian gerbils^[48].

No cohort or case-control studies in Japan have published results on nitrite or nitrosamine intake in relation to gastric cancer risk; however, the association between gastric cancer and dietary intake of exogenous and/or endogenous nitrosamine, including meat, processed meat, preserved fish, and preserved vegetables, was ex-

amined in 11 cohort studies published between 1985 and 2005^[49]. The results are inconsistent, but most studies show no statistically significant association.

China: Of the 45 case-control studies and 11 cohort studies that were included in a 2009 meta-analysis of salt consumption and gastric cancer risk, 13 case-control studies, but no cohort studies, focus on Chinese populations^[10]. The associations of gastric cancer with intake of salt, salty fish, salty vegetables, pickled vegetables, salted and fermented soya paste, and other salted foods have been examined, with ORs for individuals at the highest intake level ranging from 1.1 to 2.6. Overall, these findings indicate that high intake of salt and salty food increase the risk of gastric cancer.

Neither cohort nor case-control studies have been conducted to examine the risk of gastric cancer in relation to nitrite or nitrosamine intake in China. One cohort study, however, found no significant association between processed meat consumption and gastric cancer risk^[49]. Additionally, a number of case-control studies have found that high intake of preserved fish and preserved vegetables is significantly associated with increased risk of gastric cancer^[49].

DISCUSSION

We aimed to clarify the similarities and differences in gastric cancer epidemiology in Japan and China by closely examining patterns of incidence, mortality rates and risk profiles. It can be difficult to compare data from two different countries because of differences in genetic susceptibility; environmental exposure; lifestyle; and the way each country defines, reports and interprets data. Commonalities emerge, however, when the data are carefully compared. First, gastric cancer still poses a tremendous health burden in both countries. Second, the prevalence of *H. pylori* remains high in adults, and *H. pylori* infection significantly increases the risk for gastric cancer. The magnitude of positive association may have been underestimated in studies from both countries if only conventional IgG enzyme-linked immunosorbent assay (ELISA) serology was used to detect past *H. pylori* exposure. Because atrophy of the gastric mucosa progresses with time, seroreversion may result from loss of infection. In an analysis restricted to early diffuse cancer, a very strong association with *H. pylori* infection was observed among all age groups^[50]. In a 2001 Swedish study that combined IgG ELISA with CagA immunoblot to detect *H. pylori* exposure, the adjusted OR for noncardia gastric cancer was 21.0 (8.3-53.4) among *H. pylori*-positive subjects^[51]. Third, almost all *H. pylori* strains are CagA-positive, and CagA plays a central role in *H. pylori*-induced gastric carcinogenesis^[52]. Fourth, in addition to *H. pylori* infection, cigarette smoking and high intake of salt/salty food are two important risk factors for gastric cancer. Fifth, clinical studies have provided important insights into the effects of *H. pylori* eradication on the

development of gastric cancer.

Despite these similarities, there are significant differences in many aspects of gastric cancer epidemiology between Japan and China, including patterns in mortality and the prevalence of *H. pylori* infection, *H. pylori* strains, the magnitude of gastric cancer risk related to *H. pylori* infection, and associations with diet. Studies in China have found a wider variation in patterns of incidence and mortality than have studies in Japan. Because the highest rates of gastric cancer are often seen in economically undeveloped rural areas in China, reduction of the mortality rate in these high-risk areas should be given top priority. Because of the pivotal role of *H. pylori* infection in gastric carcinogenesis, trends in infection prevalence likely affect the incidence of gastric cancer. The decline in *H. pylori* prevalence may have occurred faster in Japan than in China. Furthermore, in Japan, the observed decrease in gastric cancer was more marked than in China, especially among 20-39 years old subjects, suggesting a clear cohort effect. Further research is required to determine whether such an effect has been or will be occurring in China. Although it is unclear why only a small percentage of individuals infected with *H. pylori* develop gastric cancer, differences in *H. pylori* strains (i.e., virulence factors), inflammatory responses, and environmental exposure may be important factors in determining individual susceptibility to gastric cancer. In an analysis of 419 *H. pylori* strains from Japanese subjects and 65 *H. pylori* strains from Chinese subjects, East Asian CagA type accounted for 94% and 93%, respectively, of the detected strains^[51]. This result suggests that almost all Japanese and Chinese *H. pylori* strains are CagA-positive; however, differences in other virulence factors, such as VacA and OipA, also warrant further study.

Diet is commonly believed to play an important role in the development of gastric cancer^[53]. Because of the complexity of diet and the limitations of questionnaire-based surveys, clarifying its precise role remains a major challenge in epidemiologic studies. Compared to China, Japanese cohort studies dominate the literature on the associations of gastric cancer with diet; in particular, salt/salty food and dietary *N*-nitroso compounds are associated with gastric cancer incidence. There is substantial evidence suggesting that high intake of salt/salty food significantly increases the risk of gastric cancer in the Japanese population. Similarly, numerous case-control studies in China strongly suggest a positive association between high intake of preserved fish and vegetables and gastric cancer risk. The role of *N*-nitroso compounds is also crucial in gastric carcinogenesis, but epidemiologic studies from both Japan and China do not provide a clear picture of this role, at least in part because the intake of *N*-nitroso compounds is notoriously difficult to measure.

There is a need to accelerate the reduction of gastric cancer incidence and mortality in both countries and to determine the most effective strategy for the prevention of gastric cancer. Cost-effective prevention strategies have been extensively discussed in Japan^[54]; one dif-

ficult issue is whether to adopt a test-and-treat policy for asymptomatic individuals. The available data do not provide a clear picture of the optimal timing for *H. pylori* eradication to achieve the maximum benefit while doing the least harm. In China, tobacco control could confer substantial public health benefits. With 20% of the world's population, China produces and consumes about 30% of the world's cigarettes and suffers about a million deaths a year from tobacco^[55]. Efforts to promote tobacco control and decrease salt consumption should effectively reduce incidence and mortality from gastric cancers.

We propose the following three avenues for potential collaborative work based on the comparison of gastric cancer epidemiology between Japan and China. First, data comparisons on *H. pylori* genotyping are useful for identifying those people at increased risk of neoplastic transformation. Second, because the prevalence of premalignant disease states in the general population is currently undefined, it is important to estimate the prevalence of precancerous lesions, such as chronic atrophic gastritis and gastric intestinal metaplasia, and their associations with *H. pylori* infection, on the basis of endoscopic findings and serologic tests. Third, a multidisciplinary study is needed to address the role of *N*-nitroso compounds in the development of gastric cancer because epidemiologic studies are limited by difficulties in the precise measurement of *N*-nitroso compound intake. It is a challenge to find common ground for international collaboration. However, the similarities between these two countries, namely a high incidence of gastric cancer and a high prevalence of *H. pylori* infection, along with differences in many aspects of gastric cancer epidemiology, provide an excellent opportunity for Sino-Japanese collaboration. Such collaborations will facilitate a more complete understanding of gastric cancer etiology and the development of more effective interventions to reduce the mortality and incidence of gastric cancers. Given the pivotal role of *H. pylori* in gastric carcinogenesis, screening strategies in both countries based on *H. pylori* infection status would be very powerful for developing appropriate and cost-effective screening programs.

COMMENTS

Background

Japan and China have the highest incidences of gastric cancer in the world. Although a trend of declining incidence has been observed over the past several decades, gastric cancer still poses a tremendous burden to populations in each country. Although numerous gastric cancer studies have been conducted in each country, this article is the first to clarify the similarities and differences in gastric cancer between Japan and China by closely examining both epidemiologic features, such as patterns of incidence and mortality rates, and risk profiles on the basis of extensive published literature.

Research frontiers

To address why only a minority of individuals with *Helicobacter pylori* (*H. pylori*) colonization develop gastric cancer, the authors need to elucidate the interacting roles of various strains of *H. pylori*, host factors, and other lifestyle/environmental factors in gastric cancer. The authors also need more evidence on the optimal timing for *H. pylori* eradication in the interest of preventing gastric cancer.

Innovations and breakthroughs

The authors found differences in many aspects of gastric cancer between Japan and China, including patterns of mortality, trends in prevalence of *H. pylori* infection, *H. pylori* strains, and risk profiles. Due to the pivotal role of *H. pylori* infection in gastric carcinogenesis, trends in its population prevalence are likely to affect gastric cancer incidence. The decline in *H. pylori* prevalence may have occurred faster in Japan than in China. In Japan, a more marked decrease in gastric cancer was observed, especially among those people aged 20-39 years old; this evidence suggests a clear cohort effect. It will therefore be intriguing to see whether such a cohort effect has occurred in China or will do in the future.

Applications

This article has implications for future collaborative studies between Japan and China. First, comparing data on *H. pylori* genotyping is useful for identifying those patients at increased risk of neoplastic transformation. Second, it is important to estimate the prevalence of precancerous lesions, such as chronic atrophic gastritis and gastric intestinal metaplasia, and to evaluate the distribution of *H. pylori* in high-risk populations, on the basis of endoscopic findings and serologic tests. Third, a multidisciplinary study is needed to address the role of *N*-nitroso compounds in the development of gastric cancer. Specifically, screening strategies based on *H. pylori* negativity or positivity in both countries would be very powerful in terms of developing appropriate and cost-effective screening programs.

Terminology

H. pylori colonizes the human stomach, and individuals with *H. pylori* infection have an increased risk of developing gastric cancer.

Peer review

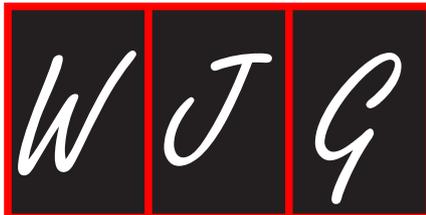
This article compares factors associated with the risk for developing gastric cancer in *H. pylori*-infected individuals from China or Japan. It summarizes a large body of literature on the rates of *H. pylori* infection along with the links between smoking, high salt and nitrate diets and gastric cancer rates. This article should be well received, especially in Asian countries, where the prevalence of *H. pylori* infection and gastric cancer remains unacceptably high.

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S- Editor Zhang SJ L- Editor O'Neill M E- Editor Zhang DN



Improvement of clinical parameters in patients with gastroesophageal reflux disease after radiofrequency energy delivery

Hai-Feng Liu, Jian-Guo Zhang, Jun Li, Xiao-Guang Chen, Wei-An Wang

Hai-Feng Liu, Jian-Guo Zhang, Jun Li, Xiao-Guang Chen, Wei-An Wang, Department of Gastroenterology, General Hospital of Chinese People's Armed Police Forces, Beijing 100039, China

Author contributions: Liu HF, Zhang JG, Li J, Chen XG and Wang WA performed the experiments. Liu HF designed the study and wrote the manuscript.

Correspondence to: Hai-Feng Liu, Professor, Department of Gastroenterology, General Hospital of Chinese People's Armed Police Forces, Beijing 100039, China. haifengliu333@163.com
Telephone: +86-10-57976547 Fax: +86-10-57976168

Received: March 20, 2011 Revised: August 11, 2011

Accepted: August 18, 2011

Published online: October 21, 2011

Abstract

AIM: To evaluate the efficacy of Stretta procedure with gastroesophageal reflux disease (GERD) based on symptom control, medication changes and oesophagitis grade.

METHODS: Ninety patients with a history of GERD underwent Stretta procedure from June 2007 to March 2010. All patients with GERD diagnosed by the presence of endoscopically evidenced oesophagitis or abnormal esophageal pH testing. We evaluated GERD-health-related quality of life, satisfaction, medication use and endoscopy at baseline, 6, 12 mo after treatment. Complications of the procedure were analyzed.

RESULTS: We found that patients experienced significant changes in symptoms of GERD after Stretta procedure. The onset of GERD symptom relief was less than 2 mo (70.0%) or 2 to 6 mo (16.7%). The mean GERD-HRQL score was 25.6 (baseline), 7.3 (6 mo, $P < 0.01$), and 8.1 (12 mo, $P < 0.01$). The mean heartburn score

was 3.3 (baseline), and 1.2 (12 mo, $P < 0.05$). The percentage of patients with satisfactory GERD control improved from 31.1% at baseline to 86.7% after treatment, and patient satisfaction improved from 1.4 at baseline to 4.0 at 12 mo ($P < 0.01$). Medication usage decreased significantly from 100% of patients on proton pump inhibitors therapy at baseline to 76.7% of patients showing elimination of medications or only as needed use of antacids/H₂-RA at 12 mo. An improvement in endoscopic grade of oesophagitis was seen in 33 of the 41 patients. All patients had either no erosions or only mild erosive disease (grade A) at 6 mo.

CONCLUSION: The experience with Stretta procedure confirms that it is well tolerated, safe, effective and durable in the treatment of GERD. The Stretta procedure provides the drug-refractory patients with a new minimally invasive method.

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Key words: Gastroesophageal reflux disease; Clinical parameters; Stretta procedure; Radiofrequency

Peer reviewers: Salvatore Leonardi, Assistant Professor, Department of Pediatrics, University of Catania, Via S. Sofia 78, 95100, Catania, Italy; Richard A Awad, Professor, Experimental Medicine and Motility Unit, Mexico City General Hospital, Dr. Balmis 148, Mexico DF, 06726 Mexico; Cesare Tosetti, MD, Department of Primary Care, Health Care Agency of Bologna Via Rosselli 21, 40046 Porretta Terme (BO), Italy

Liu HF, Zhang JG, Li J, Chen XG, Wang WA. Improvement of clinical parameters in patients with gastroesophageal reflux disease after radiofrequency energy delivery. *World J Gastroenterol* 2011; 17(39): 4429-4433 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4429.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4429>

INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most common illnesses of the gastrointestinal tract, with a large proportion of the population affected. Patients report adverse effects on their quality of life because of symptoms such as heartburn, regurgitation, or dysphagia. GERD is associated with severe health-related quality-of-life impairment, which is comparable to patients afflicted with diabetes mellitus, congestive heart failure or arthritis^[1]. Although GERD is known as a chronic disease requiring life-long treatment, many patients have inadequate symptom control with medication, usually proton pump inhibitors (PPIs). About 20% of patients will have breakthrough heartburn and regurgitation causing detrimental effects on the quality of life. GERD treatment using a minimally invasive endoluminal method to deliver low-level radiofrequency energy to the gastroesophageal junction (Stretta procedure) is a new method^[2-4]. Several clinical trials showed that the Stretta procedure improves GERD symptoms, quality of life, esophageal acid exposure, and eliminates the need for antisecretory drugs in most patients^[5-8]. This study reports our experience using the Stretta procedure in patients with persistent GERD symptoms in China.

MATERIALS AND METHODS

Patients

Fifty-seven men and 33 women (ages 31-72 years, 22.2% hiatal hernia, Table 1) were treated using the Stretta procedure. These patients had a history of GERD symptoms ranging from 1 to 26 years. They were either self-referred or recruited from the outpatient endoscopy unit from June 2007 to March 2010. All patients had significant GERD with persistent symptoms of heartburn and regurgitation in spite of the use of PPI drugs taken everyday. All patients had the diagnosis of GERD confirmed by finding erosive esophagitis at upper endoscopy (Los Angeles grade A or higher) or abnormal acid contact time detected at ambulatory esophageal pH testing. They were excluded if they were under 18 or over 80 years of age, pregnant, achalasia, or if they had a sliding hiatal hernia > 2 cm, collagen vascular disease, or severe uncontrolled medical illness.

Stretta procedure

Radiofrequency energy was delivered using the Stretta system (Curon Medical Inc., Sunnyvale, Calif., United States) in an outpatient endoscopy unit. The Stretta procedure catheter uses a balloon basket assembly to deploy 4 nitinol needle electrodes into the muscular layer of the esophageal wall. Radiofrequency energy delivered by the needle electrodes causes a thermal reaction in the LES with controlled temperature elevation to 85 °C, while continuous mucosal irrigation with chilled water prevents the development of stricture or ulceration. Deploying the needle electrodes at 5 mm levels above and below the squamocolumnar junction (SCJ) produces 56 thermal lesions. After informed consent, the patient is prepared

Table 1 Population baseline characteristics

Characteristics	
No. of patients (n)	90
Gender (n)	
Male	57 (63.3%)
Female	33 (36.7%)
Age (yr)	
mean ± SD	51 ± 13
Range	31-72
Years with GERD	
mean ± SD	6.7 ± 6.0
Range	1-26
0-2	21.1%
3-5	38.9%
6-8	10.0%
8-11	14.4%
> 12	15.6%
Patients with hiatal hernia	
None	77.8%
< 2 cm	22.2%
Patients with esophagitis	
None	54.4%
A	13.3%
B	28.9%
C	3.3%

GERD: Gastroesophageal reflux disease.

for upper endoscopy with conscious sedation by using midazolam or fentanyl in the usual manner^[9]. Diagnostic upper endoscopy is used to carefully inspect the esophagus and the cardia, and to determine the SCJ location. The Stretta catheter is passed over the guidewire, and introduced into the esophagus, where it is positioned 1 cm above the SCJ. After appropriate balloon inflation, the treatment elements are deployed 1 to 2 mm into the lower esophageal sphincter muscle, where energy is delivered in a series of thermal treatments at 4 levels in 2 positions (distal esophagus) and at 2 levels in 3 positions (gastric cardia). Constant monitoring and feedback of temperature and impedance ensures that each treatment element is maintained safely within target tissues. The mucosa was cooled during this procedure to prevent ulceration or stricture. After completion of the procedure and catheter removal, the diagnostic endoscopy procedure is repeated to verify that there have been no complications such as bleeding or perforation and to document the appropriate site of treatment. All pre-Stretta medication is maintained for 6 to 8 wk after the procedure to maintain baseline and allow time for complete healing.

Procedure at baseline and after treatment

GERD symptom assessment has been described as the most appropriate measure for the definition of treatment success, because it directly pertains to patients and the clinicians who provide their care. Before undergoing the Stretta procedure, patients underwent symptom assessment with the GERD health-related quality-of-life questionnaire (rated 0-50, with scores less than 10 considered normal)^[10]. This is a validated questionnaire assessing specific GERD symptoms, followed by questions about heartburn severity on a scale of 0 to 5, with higher scores

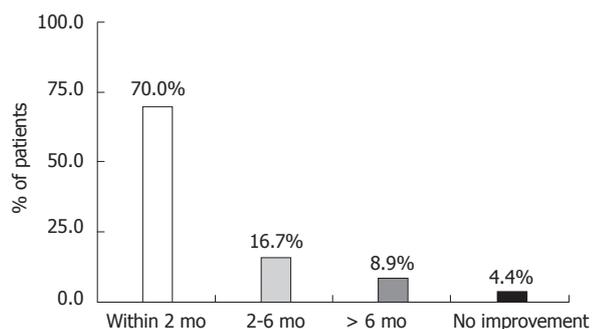


Figure 1 Time interval for onset of gastroesophageal reflux disease. Symptom relief after Stretta procedure, demonstrating significant improvement of gastroesophageal reflux disease-related symptoms within 6 mo.

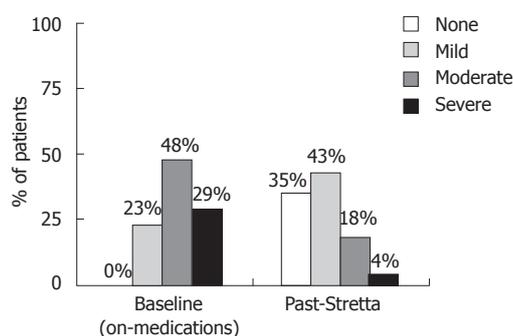


Figure 2 Improvement in gastroesophageal reflux disease symptom severity after Stretta procedure. The percentage of patients with gastroesophageal reflux disease symptom severity is shown at baseline on medications and 12 mo after Stretta procedure.

indicating more severe symptoms, and with questions about patient satisfaction on a scale of 0 to 5, with higher scores indicating better satisfaction/quality of life^[11]. This testing was repeated 6 and 12 mo after the Stretta procedure. Assessment of medication usage was performed at baseline and 6 mo with the assistance of patient diaries and detailed questions addressing the use of all GERD medications such as PPIs, histamine-2 blockers, antacids, and prokinetic agents.

Statistical analysis

The date of the experiment was represented in the form of mean \pm SD. Variance analysis and *t* test for non-match data were performed by a professional SPSS statistical program.

RESULTS

Complications

Minor complications occurred after the procedure, including 5 cases of dyspepsia (5.6%), 9 transient chest pain (10%), 2 superficial mucosal injury (2.2%), 3 mucosal bleeding (3.3%), 2 low-grade fever after procedure (2.2%). These complications resolved within 1 wk, without sequelae. No serious complications were noted after the procedure.

Table 2 Comparison of clinical parameters at baseline and after Stretta procedure

Parameters	Baseline	6 mo	12mo	<i>P</i> value
GERD health-related quality-of-life questionnaire	25.6 \pm 9.0	7.3 \pm 4.1	8.1 \pm 3.9	< 0.01
Heartburn	3.3 \pm 1.3	1.0 \pm 0.9	1.2 \pm 1.1	< 0.01
Satisfaction	1.4 \pm 1.1	4.3 \pm 1.3	4.0 \pm 0.9	< 0.01
Percent without PPI	0	78.9	76.7	< 0.05

GERD: Gastroesophageal reflux disease; PPI: Proton pump inhibitors.

Improvement of GERD symptoms

The onset of GERD symptom relief was reported as within 2 mo by the majority (70%) of patients, 2 to 6 mo by 16.7%, after 6 mo by 8.9%, and no improvement by 4.4% (Figure 1). At baseline, 77% of patients had moderate or severe symptoms despite PPI therapy. After Stretta, however, this number fell to 22%, indicating that 78% of patients had good control after treatment (Figure 2). The mean GERD-HRQL scores improved from 25.6 \pm 9.0 at baseline to 7.3 \pm 4.1 at 6 mo, and 8.1 \pm 3.9 at 12 mo (*P* < 0.01). The mean heartburn scores improved from 3.3 \pm 1.3 at baseline to 1.0 \pm 0.9 at 6 mo, and 1.2 \pm 1.1 at 12 mo (*P* < 0.05) (Table 2).

Improvement of quality of life

At baseline, only 31.1% of patients reported that they were satisfied with GERD symptom control on twice-daily PPI, whereas after Stretta treatment, 86.7% reported satisfactory GERD symptom control. This is also reflected in the satisfaction score, which improved from 1.4 \pm 1.1 at baseline to 4.3 \pm 1.3 at 6 mo, and 4.0 \pm 0.9 at 12 mo (*P* < 0.01) (Table 2).

Improvement in medication use

Medication usage decreased significantly (*P* < 0.05) (Table 2). At baseline, 100% of patients were receiving twice-daily PPI therapy. After Stretta treatment, 76.7% of patients showed elimination of medications or only as needed use of antacids/H2-RA at 12 mo.

Improvement in endoscopic grade of oesophagitis

Six months after Stretta treatment, an improvement in endoscopic grade of oesophagitis was seen in 33 of the 41 patients, 80.5% patients had no erosions, and only 19.5% patients had mild erosive disease (grade A).

DISCUSSION

GERD is a common disease that significantly impairs the quality of life of patients, and it is associated with pathophysiologic alterations such as transient lower esophageal sphincter relaxations and the presence of a hiatal hernia. Although antisecretory medications such as PPIs are considered as the mainstay of GERD treatment, they do not address any of the underlying pathophysiologic derangements. It is not surprising, therefore, that up to 20% of

patients do not have adequate symptom control despite these drugs effectively control GERD in most patients by reducing acid reflux. Moreover, long-term use of these medications may provide a significant and lifelong economic expense. These patients may seek antireflux surgery, which has high success rates but may wane over time, with 50% of patients requiring medications to control recurrent reflux symptoms^[12]. The invasiveness, high costs, and risks associated with surgery, and the dependence and long-term costs of medical management have caused patients and physicians alike to pursue a minimally invasive, effective, and durable treatment alternative that actually addresses the underlying pathophysiologic problems of GERD^[7,13].

The Stretta endoscopic antireflux procedure was introduced in 2000 for the treatment of patients with GERD who had suboptimal symptom control with GERD therapy. The Stretta procedure has been found to have multiple mechanisms of action, including mechanical alteration of the gastroesophageal junction, with increased gastric yield pressure^[14]; neural modulation of transient lower esophageal sphincter relaxation^[15]; and normalization of delayed gastric emptying^[14]. The Stretta procedure uses temperature-controlled delivery of radio-frequency energy to the lower esophageal sphincter to address the underlying pathophysiology. Production and healing of these lesions causes inflammation, subsequent collagen deposition^[16,17], and muscular thickening^[18], whereas efferent/afferent vagal nerve ablation causes a decrease in total lower esophageal sphincter relaxations and thus a decrease in esophageal acid exposure^[19,20].

The Stretta procedure is an endoluminal radiofrequency energy delivery system to the gastroesophageal junction. Several clinical trials showed that the Stretta procedure improved GERD-related symptoms, decreased symptom scores and heartburn scores, increased the satisfaction. Meier *et al*^[21] reported the results of an European multicenter, open-label, prospective study. At 12 mo after treatment, 75% of the patients were more satisfied with their symptom control and had statistically significantly fewer GERD symptoms. GERD-HRQL score decreased from 19.2 to 6.6 ($P < 0.0001$) and overall physical and mental health also improved significantly. This is also reflected in the satisfaction score, which improved significantly from 2 to 4 ($P < 0.0001$). Wolfsen *et al*^[22] reported the multicenter Stretta registry study of 558 patients. The percentage of patients with satisfactory GERD control improved from 26.3% at baseline (on drugs) to 77.0% after treatment ($P < 0.0001$). Median baseline symptom control on drugs was 50%, compared with 90% at follow-up ($P < 0.0001$). Baseline patient satisfaction on drugs was 23.2%, compared with 86.5% at follow-up ($P < 0.0001$). Subgroup analysis showed a superior effect on symptom control in these patients beyond 1 year of follow-up, supporting procedure durability. Noar *et al*^[9] reported on a series of 109 consecutive patients treated with the Stretta procedure who have reached 4-year follow-up. Heartburn scores decreased from 3.6 to 1.2 ($P < 0.001$), GERD-HRQL score decreased from 27.8 to

7.1 ($P < 0.001$), and patient satisfaction improved from 1.4 to 3.8 ($P < 0.001$). In our study, we found significant changes in symptoms of GERD. After treatment, onset of GERD symptom relief was less than 2 mo (70.0%) or 2 to 6 mo (16.7%). The GERD-HRQL score decreased from 25.6 to 8.1 ($P < 0.01$). The mean heartburn score decreased from 3.3 to 1.2 ($P < 0.05$). The percentage of patients with satisfactory GERD control improved from 31.1% at baseline to 86.7% after treatment, and patient satisfaction improved from 1.4 to 4.0 ($P < 0.01$).

The need for medication for GERD symptoms improved significantly one year after Stretta treatment compared to baseline. In our study, about 76.7% of the patients studied needed no medication or less medication after Stretta treatment. This is in line with results from Tam *et al*^[15]. They report that at 12 mo after treatment, 75% of the patients are off all medication. Noar *et al*^[9] presented 4-year of follow-up in 109 patients treated with the Stretta procedure. In their follow-up, medication usage decreased significantly from 100% of patients on twice-daily PPI therapy at baseline to 75% of patients showing elimination of medications or only as-needed use of antacids/over-the-counter PPIs. In a European study, Meier *et al*^[21] report that at 6 mo after treatment, 45% and at 12 mo 38% of the patients are off all medication. Perhaps the lower percentage of patients that were off medication in Meier's study was caused by the patient population having slightly different characteristics. 8.5% of their population had hiatal hernia > 2 cm, while other studies only included patients with hiatal hernia < 2 cm.

The Stretta procedure produced significant improvements in oesophagitis grade. Despite most patients being off therapy at 6 mo, only mild erosive reflux oesophagitis (grade A) was noted in 19.5% of the patients while remainder had no visible mucosal breaks. This is in line with results from Tam *et al*^[23]. They report that at 6 mo after treatment, half of the patients had no macroscopic mucosal breaks and half of the patients had mild erosive reflux oesophagitis (Los Angeles grade A).

Taken together, the patients in this study had statistically significant improvement and a sustained effect in all parameters. In these patients, the observed improvement was superior to that achieved with escalated PPI therapy above baseline dosing. The Stretta procedure is a viable, well tolerated, minimally invasive endoluminal procedure^[24,25]. This procedure should be considered for patients who are not satisfied with pharmacologic therapy and who are considering anti-reflux surgery.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is one of the most common illnesses of the gastrointestinal tract, with a large proportion of the population affected. GERD is associated with severe health-related quality-of-life impairment, which is comparable to patients afflicted with diabetes mellitus, congestive heart failure, or arthritis. Although antisecretory medications such as PPIs are considered as the mainstay of GERD treatment, up to 20% of patients do not have adequate symptom control despite these drugs effectively control GERD in most patients by reducing acid reflux. These patients may seek antireflux surgery,

which has high success rates but may wane over time, with 50% of patients requiring medications to control recurrent reflux symptoms. The invasiveness, high costs, and risks associated with surgery, and the dependence and long-term costs of medical management have caused patients and physicians alike to pursue the minimally invasive, effective, and durable treatment methods.

Research frontiers

The devices of endoluminal minimally invasive treatment of gastro-oesophageal reflux disease, which included endoluminal fundoplication (EndoCinch, NDO Plicator), biopolymer injection (Enteryx), and Stretta procedure, have gained popularity over the last several years. Endoluminal minimally invasive treatment of gastroesophageal reflux disease has been shown to be safe and effective in recent studies.

Innovations and breakthroughs

The experience with Stretta procedure confirms that it is well tolerated, safe, effective, and durable in the treatment of GERD. It has produced significant improvements in symptom control and oesophagitis grade and decreased medication usage. Stretta procedure is a new minimally invasive endoluminal therapeutic technique.

Applications

The Stretta procedure provides the drug-refractory patients with a new minimally invasive method. The Stretta procedure improves GERD symptoms, quality of life, esophageal acid exposure, and eliminates the need for antisecretory drugs in most patients.

Terminology

Refractory GERD: Refractory GERD is a patient-driven phenomenon. What constitutes refractory GERD remains an area of controversy. Many investigators suggest that only patients who exhibit incomplete or lack of response to PPI, twice daily, should be considered as PPI failures. Others believe that a lack of symptomatic response to PPI once a day is sufficient to consider patients as PPI failures.

Peer review

This is an interesting work. The study is useful in order to take advantages from a new therapeutic technique, above all for such a widespread disease like the gastroesophageal reflux. Moreover the possibility to avoid the more invasive surgery would be a desirable outcome for patients with severe GERD.

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Effects of octreotide on glucose transporter type 2 expression in obese rat small intestine

Na Wei, Rui Liu, Yan Ou, Xian Li, Ou Qiang, Wei Guo, Cheng-Wei Tang

Na Wei, Rui Liu, Yan Ou, Xian Li, Ou Qiang, Wei Guo, Cheng-Wei Tang, Division of Peptides Related to Human Disease, National Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Na Wei, Yan Ou, Cheng-Wei Tang, Department of Gastroenterology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: Liu R and Tang CW developed the concept of the study; Liu R participated in its design and coordination, applied for funding; The majority of experiments were performed by Ou Y, Li X, Qiang O, Guo W and Wei N; Wei N analyzed the data, wrote the manuscript; and Liu R revised the manuscript; All authors read and approved the final manuscript. Supported by Grants from the National Natural Sciences Foundation of China, No. 30870919; Sichuan Provincial Department of Science and Technology, No. 2010SZ0176

Correspondence to: Dr. Rui Liu, Division of Peptides Related to Human Disease, National Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China. lru60@yahoo.com.cn

Telephone: +86-28-85164011 Fax: +86-28-85164013

Received: February 14, 2011 Revised: April 5, 2011

Accepted: April 12, 2011

Published online: October 21, 2011

Abstract

AIM: To investigate the effects of the somatostatin analogue, octreotide, on maltase and sucrase activities and expression of glucose transporter type 2 (GLUT2) in obese rat intestinal mucosa.

METHODS: We divided 49 Sprague-Dawley rats into a group of 31 high fat diet-induced obese rats and a group of 18 normal controls. The obese rats were separated into an octreotide treated group of 16 rats and an obese group of 15. The intervention group was injected with octreotide at 40 µg/kg body weight every 12 h for 8 d. Rat body weight was measured weekly to calculate Lee's index. After euthanization, maltase and sucrase activities in the small intestine were measured

by activity assays, and the fasting plasma glucose level was measured. The expression of GLUT2 in small intestinal mucosa was analyzed by immunohistochemistry, reverse transcriptase polymerase chain reaction and Western blotting assays.

RESULTS: Body weight, Lee's index, fasting plasma glucose level, maltase activity in small intestinal mucosa, mucosa and apical GLUT2, GLUT2 mRNA and protein expression levels were all significantly higher in the obese group than in the normal control group (605.61 ± 141.00 vs 378.54 ± 111.75 , 337.61 ± 10.82 vs 318.73 ± 20.10 , 8.60 ± 1.38 vs 7.33 ± 0.70 , 156.01 ± 58.81 vs 50.43 ± 30.49 , $390\ 744.2 \pm 62\ 469.21$ vs $170\ 546.50 \pm 50\ 646.14$, $26\ 740.18 \pm 3809.60$ vs 354.98 ± 57.19 , 0.26 ± 0.11 vs 0.07 ± 0.02 , and 2.08 ± 0.59 vs 1.27 ± 0.38 , respectively, all $P < 0.01$). Sucrase activity did not differ between the two groups. Octreotide intervention significantly decreased the body weight and fasting plasma glucose level of obese rats (508.27 ± 94.39 vs 605.61 ± 141.00 , 7.58 ± 1.51 vs 8.60 ± 1.38 , respectively, all $P < 0.05$). The intestinal mucosa and apical GLUT2, expression of GLUT2 mRNA and protein were also significantly lower in the octreotide intervention group than in the obese group ($269\ 975.2 \pm 53\ 730.94$ vs $390\ 744.2 \pm 62\ 469.21$, 3758.06 ± 364.51 vs $26\ 740.18 \pm 3809.60$, 0.08 ± 0.02 vs 0.26 ± 0.11 , and 1.31 ± 0.27 vs 2.08 ± 0.59 , respectively, all $P < 0.01$).

CONCLUSION: High fat diet-induced obesity is associated with elevated intestinal maltase activity, GLUT2 expression, and permanent apical GLUT2 in the small intestinal mucosa of rats. Octreotide can inhibit these effects.

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Key words: Glucose transporter type 2; High fat diet; Maltase; Obesity; Octreotide; Rat; Small intestinal absorption

Peer reviewer: Akio Inui, MD, PhD, Professor, Department of Behavioral Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, 8351 Sakuragaoka, Kagoshima 8908520, Japan

Wei N, Liu R, Ou Y, Li X, Qiang O, Guo W, Tang CW. Effects of octreotide on glucose transporter type 2 expression in obese rat small intestine. *World J Gastroenterol* 2011; 17(39): 4434-4439 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4434.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4434>

INTRODUCTION

Recently, obesity has become a worldwide health issue, as it increases the risk of a variety of human medical consequences^[1] such as type 2 diabetes, cardiovascular and cerebrovascular disease. Nutritional obesity is a metabolic disorder involving chronic imbalance of energy. Feeding a high fat diet to rodents normally results in disorders of glucose metabolism, and leads to impaired glucose tolerance or diabetes^[2].

Carbohydrates are the main source of energy in rats. With the exception of monosaccharides, which can be absorbed directly into the intestinal mucosa, carbohydrates and their intermediate metabolites must be hydrolyzed to disaccharides in the gastrointestinal tract. The disaccharides then broken down to glucose through maltose and sucrose on the surface of intestinal villi. There are two mechanisms of intestinal glucose absorption^[3]. When the luminal glucose level is lower than the blood glucose level, absorption occurs *via* the sodium-dependent glucose transporter 1 (SGLT-1) through classical active transport. Once the glucose concentration in the intestine is beyond the transport saturation of SGLT-1, the diffusion is mediated primarily through the transient insertion of glucose transporter type 2 (GLUT2) into the apical membrane^[3].

Somatostatin (SST), a multifunctional gut peptide synthesized and released by intestinal endocrine cells (D cells), regulates the physiological function of intestinal epithelial cells and immune cells, as well as gastric motor activity^[4,5]. Somatostatin's effects are mediated by the SST receptor (SSTR) which is located in the cell membrane. Somatostatin secreted from the intestine can thus suppress replication of gastrointestinal epithelial cells. The clinical utility of somatostatin is limited by its short half-life of about two minutes^[6]. Octreotide is an artificial synthetic analogue of SST that is more convenient to use and has a longer half-life, duration of activity and fewer side effects.

The expression of SGLT-1 in intestinal mucosa is significantly elevated in high-fat-diet-induced obese rats, and octreotide can dramatically inhibit the expression of SGLT-1 (unpublished results). However, it is unknown whether obesity induced by a high fat diet is associated with maltase and sucrase activities and expression of GLUT2. It is also unknown whether octreotide can influence maltase and sucrase activities and GLUT2 expression. Thus, the authors conducted an experiment to investigate these questions.

MATERIALS AND METHODS

Experimental animals and study design

All experiments were approved by the Institutional Animal Care and Use Committee of Sichuan University (Chengdu, China).

We used 66 healthy male 21-d-old Sprague-Dawley (SD) rats which had been weaned for 3 d. All animals were obtained from the Animal Center of Sichuan University. After adaptive feeding for 3 d, rats were placed into two groups, one group of 18 (the normal control group) fed with standard chow (290 kcal/100 g, in line with the People's Republic of China National Standard GB 14924-2001), and another group of 48 fed with high-fat chow (430 kcal/100 g).

Food and water were supplied *ad libitum*, and the animals were housed in independently ventilated cages on a 12:12-h light: dark schedule and kept at 20 °C-25 °C. They were weighed and measured for body and tail length each week for 24 wk. After 24 wk, rats from the high-fat chow group with body weight at least 1.4 times the mean body weight of the normal control group were selected as obese rats. Obese rats were then placed into two groups, an obese group of 16 rats, and an octreotide-treated group of 15 rats. The octreotide-treated group was injected subcutaneously with octreotide (40 g/kg body weight) every 12 h for 8 d. Rat body weight was measured weekly to calculate Lee's index [body weight (g)^{1/3} × 1000/body length (cm)].

Sample preparation

At the end of the experiment, after fasting for 12 h, rats were euthanized intraperitoneally with 2% sodium pentobarbital. The small intestine was removed from each rat. The mucosa from 15 cm of the small intestine was collected by scraping with a glass slide and kept at -80 °C for measurement of sucrase and maltase activities and Western blot analysis. Another 1-cm section of small intestine was kept at -140 °C until total RNA extraction. Plasma and serum were collected for blood glucose measurement. Blood glucose levels were measured by the enzymatic method. Each specimen was measured in duplicate.

Sucrase and maltase activities measurement

Sucrase and maltase activities were measured using a sucrase and maltase activity assay kit (Jiancheng Bio-Engineering Institute, Nanjing, China) according to the manufacturer's instructions. The sucrase and maltase activity unit was defined as the nanomoles of disaccharide hydrolyzed per milligram of protein in 1 min (U/mg protein, 37 °C, pH = 6.0), and activity was calculated as follows:

$$\text{activity (U/mg protein)} = \frac{\frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{standard}} - A_{\text{blank}}} \times \text{standard concentration (5.55 mmol/L)}}{\text{reaction time (20 min)} \times \text{protein concentration (mg protein/mL)}} \times 1000$$

The small intestine mucosa was homogenized in phosphate buffered saline (PBS, pH = 7.4) with a homogenizer. After centrifugation at 1050 × *g*, 4 °C for 5 min, the supernatant was used to measure sucrase and maltase activi-

ties. The protein concentrations in the homogenate were determined by a BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, United States) according to the manufacturer's instructions.

Immunohistochemistry of small intestine mucosa

For the immunohistochemical detection of GLUT2, a paraffin-embedded tissue section was deparaffinized and antigen retrieval was performed at high pressure. For nonspecific blocking, 10% goat sera was added, each section was incubated for 20 min at 37 °C, then GLUT2 goat anti-rat polyclonal antibody was added (1:400; Santa Cruz Biotechnologies, Santa Cruz, CA, United States). After incubating overnight at 4 °C and rewarming to 37 °C, each section was stained with a ready-to-use streptavidin-catalase immunohistochemical reagent system for detection. The color reaction was developed with diaminobenzidine (DAB; Zhongshan Bioagent Company, Beijing, China). A semiquantitative immunohistochemical analysis of raw data with Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD, United States) was used to score integrated optical density (IOD) for positive reaction area.

Reverse transcriptase polymerase chain reaction analysis

Total RNA was extracted from the frozen small intestine using Trizol reagent (Takara Bio-Engineering Co., Ltd., Kyoto, Japan). First-strand cDNA was synthesized from 2 mg of total RNA for each sample using reverse transcription kits (MBI, Fermentas Life Sciences Inc., Vilnius, Lithuania). The GLUT2 sense and antisense PCR primers were 5'-TGCTGGAAGAAGCGTAT CAG-3' and 5'-GGCCAAGTAGGATGTGCCAG-3', respectively; the GAPDH sense and antisense primers were 5'-CATGAC-CACAGTCCATGCCA-3' and 5'-CACCCCTGTTGCT CTAGCCATATTC-3', respectively^[7]. The PCR reaction was catalyzed using Taq DNA polymerase (MBI, Fermentas Life Sciences Inc., Vilnius, Lithuania). The thermal cycling parameters consisted of an initial denaturation step of 5 min at 94 °C, followed by 23 cycles of 1 min at 94 °C, 1 min at 63 °C, and 1 min at 72 °C. The final extension step lasted 7 min at 72 °C. PCR products were resolved by 2% agarose gel electrophoresis, and visualized by ethidium bromide staining. Densitometry was carried out using a Bio-Rad GelDoc image acquisition system and Quantity One (v4.3) quantitation software (Bio-Rad, Hercules, CA, United States).

Western blotting analysis

Small intestinal mucosa homogenate was prepared in lysis buffer containing protease inhibitor and phosphatase inhibitor (KeyGEN Biological Company, Nanjing, China). The protein concentrations were determined by a BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, United States). The extracted protein (60 µg) was incubated in loading buffer and heated at 100 °C for 5 min. Samples were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel, then transferred electronically to poly-

vinylidene difluoride membranes (Millipore, Bedford, MA, United States). The membranes were incubated with a 1:2000 dilution of rabbit polyclonal GLUT2 antibody (Millipore, Bedford, MA, United States) at 4 °C overnight. The membranes were then washed three times in blocking solution and incubated with a secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, United States, 1:50000). The signals were developed using Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, United States). Band densities were quantified using Quantity One software 4.3.1 (Bio-Rad, Hercules, CA, United States). Each value was expressed as the ratio of the IOD of the GLUT2 band to that of β-actin.

Statistical analysis

Data are presented as means and standard deviations. Groups were compared using analysis of variance or the *t* test. All tests were two-tailed and $P \leq 0.05$ was considered statistically significant. All data met the assumptions of the tests used to analyze them. All data were analyzed by statistical software SPSS 13.0 (SPSS Inc., United States).

RESULTS

Octreotide treatment and parameters associated with obesity and activities of disaccharidases

The rats with high-fat-diet-induced obesity showed typical features of obesity, such as higher body weight, Lee's index and blood glucose levels than those in the normal control group ($P < 0.01$) (Table 1). The octreotide-treated group had significantly lower body weight and blood glucose levels than those in the obese comparison group ($P < 0.05$) (Table 1). Maltase activity in the small intestinal mucosa of the obese group was significantly higher than that in the normal control group ($P < 0.01$) (Table 1).

Octreotide treatment and GLUT2 expression

GLUT2 distribution and expression in the intestinal mucosa were measured by immunohistochemistry. GLUT2 resided mainly in the basolateral membrane (BLM) in the small intestinal mucosa (Figure 1). GLUT2 expression in the obese group was significantly higher than that in the normal control group ($P < 0.01$). In the octreotide-treated group, GLUT2 expression was significantly lower than that in the obese group ($P < 0.01$) (Figure 1 and Table 1).

Apical GLUT2 expression in the obese group was significantly higher than that in the normal control group ($P < 0.01$). After octreotide intervention, the level of apical GLUT2 was significantly lower than that in the obese group ($P < 0.01$) (Figure 1 and Table 1).

Reverse transcriptase polymerase chain reaction and Western blotting showed that GLUT2 mRNA and protein expression levels in the intestinal mucosa of the obese group were significantly higher than those in the normal control group ($P < 0.01$). GLUT2 mRNA and protein expression levels were significantly lower in the octreotide-treated group than in the obese rats ($P < 0.01$) (Figures 2 and 3, Table 1).

Table 1 Obesity and metabolic indicators in normal control and obese rats, and in obese rats treated with octreotide

	Control (<i>n</i> = 18)	Obese (<i>n</i> = 16)	Octreotide treated (<i>n</i> = 15)
Body weight (g)	378.54 ± 111.75	605.61 ± 141.00 ^b	508.27 ± 94.39 ^{b,c}
Lee's index	318.73 ± 20.10	337.61 ± 10.82 ^b	334.67 ± 16.56 ^a
Glucose (mmol/L)	7.33 ± 0.70	8.60 ± 1.38 ^b	7.58 ± 1.51 ^c
Sucrase activity (U/mg protein)	53.84 ± 17.98	48.90 ± 13.95	35.85 ± 21.31
Maltase activity(U/mg protein)	50.43 ± 30.49	156.01 ± 58.81 ^b	112.85 ± 51.86 ^a
Mucosa GLUT2 (IOD)	170546.5 ± 50646.14	390744.2 ± 62469.21 ^b	269975.2 ± 53730.94 ^{b,d}
Apical GLUT2 (IOD)	354.98 ± 57.19	26740.18 ± 3809.60 ^b	3758.06 ± 364.51 ^{a,d}
GLUT2 mRNA (IOD)	0.07 ± 0.02	0.26 ± 0.11 ^b	0.08 ± 0.02 ^{b,d}
Western-blotting (IOD)	1.27 ± 0.38	2.08 ± 0.59 ^b	1.31 ± 0.29 ^d

^a*P* < 0.05, ^b*P* < 0.01 *vs* normal control group; ^c*P* < 0.05, ^d*P* < 0.01 *vs* obese group. GLUT2: Glucose transporter type 2; IOD: Integrated optical density.

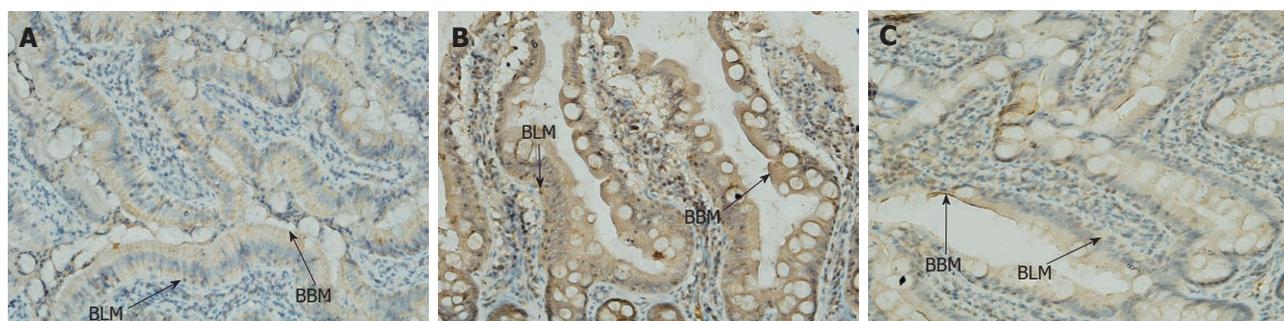


Figure 1 Expression of glucose transporter type 2 and apical glucose transporter type 2 in the small intestinal mucosa. A: In normal control rats, glucose transporter type 2 (GLUT2) mainly resides at the BLM (arrows); B: In obese rats, the GLUT2 and apical GLUT2 expression was increased (arrows) compared to the controls; C: In octreotide-treated rats, GLUT2 and apical GLUT2 expression was significantly decreased (arrows) compared to non-treated obese rats. Magnification: × 400.

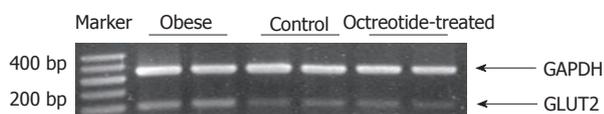


Figure 2 Expression of glucose transporter type 2 mRNA in the small intestinal mucosa. Obese rats showed increased mRNA levels of glucose transporter type 2 (GLUT2) when compared with the controls, whereas octreotide-treated rats showed decreased levels of GLUT2 mRNA, when compared with obese rats. Total RNA was analyzed by reverse transcriptase polymerase chain reaction. House-keeping gene *GAPDH* was used as an internal control.

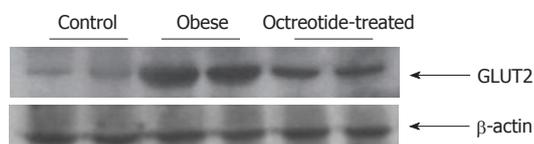


Figure 3 Expression of glucose transporter type 2 protein by Western blotting. Obese rats showed increased protein levels of glucose transporter type 2 (GLUT2) when compared with the controls, whereas the octreotide-treated rats showed decreased levels of GLUT2 protein, when compared with obese rats. β-actin was used as an internal loading control.

DISCUSSION

Starch is the major source of food glucose, and its digestion to maltose requires amylases in the gastrointestinal tract. Disaccharides, such as maltose, must be hydrolyzed to glucose by disaccharidase before being absorbed direct-

ly. As the most essential disaccharides, maltose and sucrose are broken down in the top of the intestinal brush border membrane (BBM)^[8], which hydrolyzes sugar to glucose and fructose and plays a vital role in the utilization of carbohydrate.

Maltase and sucrase activities are regulated by diet, and are enhanced by increasing substrate concentration^[9]. Our finding that maltose activity was markedly higher in the obese group suggests there is more robust intestinal digestion of starch in obese rats. Ferraris^[10] reported that sucrase activity increased 68% when sucrose was added to the diets of mice. We fed rats a high fat diet with some sucrose to establish an obese model. In contrast with Ferraris's results, we found no significant difference in sucrase activity between the obese and normal control groups. A possible explanation for this is that a high fat diet can stimulate secretion of trypsin, which reduces sucrase activity by degrading sucrose^[11]. Thus, the obese rats' high fat diet may account for the lack of variability in sucrase activity between the two groups.

According to Kellett's review^[3], there are two mechanisms of intestinal glucose absorption. When the luminal glucose level is lower than the blood glucose level, absorption occurs *via* SGLT-1 through classical active transport. Once the glucose concentration in the intestine is beyond the transport saturation of SGLT-1, the diffusion is mediated primarily by transient insertion of GLUT2 into the apical membrane (the apical membrane is op-

posed to the BLM). Lostao *et al.*^[12] reported that the diffusive component was three to five times greater than the active component at high luminal glucose concentrations. Therefore, GLUT2 is a key transport protein for glucose in the intestine.

We found that SGLT-1 and GLUT2 expression in the intestinal mucosa, as well as fasting plasma glucose levels, were all higher in obese rats than in non-obese rats. These findings are consistent with those of others^[2,13]. A prolonged high-fat diet may downregulate muscle insulin receptors, inducing insulin resistance or hyperinsulinemia^[3,13], and eventually leading to higher fasting plasma glucose levels. Sterol response element-binding protein (SREBP)-1c may exert beneficial effects in transporting glucose out of hepatocytes into blood, contributing to hyperglycemia^[14]. SREBP-1c may mediate glucose-stimulated GLUT2 gene expression upregulation by binding to the -84/-76 region to activate the GLUT2 promoter^[14]. In diabetic rats, GLUT2 expression increased hyperglycemia in the liver^[15,16], intestine, and pancreatic β -cells^[16]. Our results suggest that along with the increase in fasting plasma glucose levels, GLUT2 mRNA and protein expression are enhanced in the intestinal mucosa of obese rats, which results in greater glucose digestive ability and eventually obesity.

We observed that obese rats fed a high fat diet showed greater insertion of apical GLUT2 than did non-obese controls. It has been proposed that GLUT2 normally resides at the BLM but it can insert within min into the apical membrane of the intestine in response to high glucose concentrations; this facilitated component of absorption is called the apical GLUT2 pathway^[3]. GLUT2 translocates to the apical membrane by at least two signaling pathways: one related to calcium absorption *via* the nonclassical neuroendocrine L-type voltage-dependent calcium channel (Cav1.3), and the other activated by sweet taste receptors stimulated by natural sugars and artificial sweeteners^[17].

After the activation of protein kinase C (PKC) β II, a large number of GLUT2 proteins are inserted into the apical membrane to transport glucose^[3]. As glucose is absorbed and its concentration in the intestine decreases, the signaling system is reversed, and GLUT2 leaves the apical membrane to restore the condition of low luminal glucose concentrations^[18]. However, permanent apical GLUT2 insertion in a pathological state may result in increased sugar absorption and eventually lead to obesity, insulin resistance and diabetes^[3,17]. All rats in our experiment were denied food for 12 h before being euthanized to avoid high glucose concentrations in the intestine. Thus, we speculate that the apical GLUT2 in obese rats was a kind of pathological permanent insertion^[3]. Insulin binding to its enterocyte receptor (IR) inhibits the insertion of apical GLUT2, resulting in rapid trafficking of GLUT2 away from both the apical membrane and the BLM into the cell^[19]. This function of insulin could be impaired by a prolonged high fat diet, leading to the permanent insertion of apical GLUT2.

Octreotide, a type of octopeptide, is an artificial syn-

thetic analogue of SST. It has a longer half-life than natural SST. It binds to SSTR2 with high affinity, and has intermediate affinity for SSTR3 and SSTR5^[4]. According to the work of Aydede *et al.*^[20], octreotide was safe and effective when administered subcutaneously (at 100 μ g/kg 12 h apart) for 2 wk in rats with portal hypertensive colopathy. Thus, octreotide at 40 μ g/kg 12 h apart for 8 d in our experiment seems reasonable. Some studies reported that children with hypothalamic obesity and hyperinsulinaemic obese adults received octreotide at a dosage of 5-15 μ g/kg per day or long-acting release octreotide 40 mg/28 d for 6 mo, respectively, could decrease body weight by inhibiting β -cell insulin secretion^[21,22]. In the present study, we also observed decreased body weight in obese rats after octreotide treatment. Lustig *et al.*^[21] found that almost half of children with hypothalamic obesity developed gallstone or sludge formation after treatment with octreotide for about 6 mo. However, ursodeoxycholic acid could reverse these problems^[23].

Our experiments show that in comparison to the untreated obese rats, the octreotide-treated obese rats had much lower fasting plasma glucose levels. It may be that octreotide has a high affinity for SSTR2, which inhibits the release of glucagon by pancreatic α -cells^[24] and results in a decrease in fasting plasma glucose levels. Moreover, decreased GLUT2 mRNA and protein levels were found in the octreotide-treated obese rats, which is consistent with the idea that glycemia is a systemic factor that can affect GLUT2 expression^[25].

We found that the octreotide-treated group had significantly lower apical GLUT2 levels than the obese group. A possible explanation for this is that octreotide tightly binds to SSTR2, and SSTR2 binds with $G_{\alpha 02}$ to inhibit the L-type calcium channel and reduce Ca^{2+} transportation^[4,26], which in turn suppresses the translocation of GLUT2 by depressing the activation of PKC β II^[3].

In conclusion, our results indicate that high fat diet-induced obesity in rats is associated with increased fasting plasma glucose levels, intestinal maltase activity, GLUT2 expression, and permanent apical GLUT2 in small intestinal mucosa. After treatment with octreotide, an analogue of somatostatin, fasting plasma glucose levels, expression of GLUT2, and insertion of apical GLUT2 were inhibited, and eventually the obese rats lost weight. Therefore, the use of an analogue of somatostatin, such as octreotide, to suppress glucose absorption in intestinal mucosa may provide a novel approach in the pharmacological treatment of obesity.

COMMENTS

Background

Obesity induced by a high fat diet can result in disorders of glucose metabolism, and lead to impaired glucose tolerance or diabetes. Maltase and sucrase are the most important disaccharidases in sugar absorption. In addition, Glucose transporter type 2 (GLUT2) is a key transport protein for glucose in the small intestine. It is unknown whether obesity induced by a high fat diet is associated with glucose absorption. The relationship between the somatostatin analogue, octreotide, and glucose absorption in obesity has not yet been reported.

Research frontiers

Although diet is an important way of treating nutritional obesity, many patients

fail to lose weight with diet control. It is not known whether octreotide, a somatostatin analogue, which has an important role in the regulation of physiological functions in the small intestine, is able to inhibit glucose absorption. In the present study, the authors demonstrated that octreotide can inhibit glucose absorption and the potential mechanisms involved in obese rats.

Innovations and breakthroughs

Recently, several studies have shown the change in GLUT2 expression in diabetes or insulin resistance. Other researchers focused on the role of somatostatin analogues by suppressing β -cell insulin secretion in obesity in humans. This is the first study to provide evidence that the somatostatin analogue, octreotide, can inhibit glucose absorption in obese rats.

Applications

Since this study suggested that octreotide may be effective in inhibiting glucose absorption, octreotide might have potential applications as an alternative medicine in the treatment of obesity.

Terminology

GLUT2 is normally thought to reside at the basolateral membrane in the small intestine, and it can be inserted within minutes into the apical membrane *in vivo* in response to high glucose concentrations. Somatostatin is a 14- or 28-amino-acid peptide, which mainly inhibits exocrine and endocrine secretion, motility and absorption via different somatostatin receptors in the gastrointestinal tract. Octreotide, a type of octapeptide, is an artificial synthetic analogue of somatostatin which is more convenient to use and has a longer half-life and duration of activity and fewer side effects.

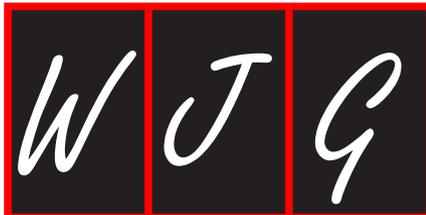
Peer review

The authors well demonstrated that high fat feeding produces permanent apical GLUT2 expression in addition to increased maltase activity, and octreotide improves all of them leading to decreased body weight and blood glucose levels compared to controls. The authors indicated the clinical significance of their findings. The authors need to discuss on the dosage of octreotide used in this and human studies. The potential side effects of octreotide such as gallstone should also be considered.

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S- Editor Tian L L- Editor Webster JR E- Editor Xiong L



Intracranial hemorrhage in patients treated with bevacizumab: Report of two cases

Takeshi Nishimura, Makoto Furihata, Hideyuki Kubo, Masao Tani, Senichiro Agawa, Ryuhei Setoyama, Tomikatsu Toyoda

Takeshi Nishimura, Makoto Furihata, Hideyuki Kubo, Masao Tani, Senichiro Agawa, Ryuhei Setoyama, Department of Surgery, the Mutual Aid Association for Teachers and Officials Sanraku Hospital, 2-5 Kandasurugadai, Chiyoda-ku, Tokyo 101-8326, Japan

Tomikatsu Toyoda, Department of Neurosurgery, Tokyo Koseinenkin Hospital, 5-1 Tsukudo-cho Sinjuku-ku, Tokyo 162-8543, Japan

Author contributions: Nishimura T and Furihata M contributed equally to this work, and wrote the manuscript; Kubo H, Tani M, Agawa S, Setoyama R, Toyoda T examined previous literature regarding complications of bevacizumab treatment.

Correspondence to: Takeshi Nishimura, MD, Department of Surgery, the Mutual Aid Association for Teachers and Officials Sanraku Hospital, 2-5 Kandasurugadai, Chiyoda-ku, Tokyo 101-8326, Japan. tnishimura-mie@umin.ac.jp

Telephone: +81-03-32923981 Fax: +81-03-32925023

Received: January 28, 2011 Revised: March 29, 2011

Accepted: April 5, 2011

Published online: October 21, 2011

metastases are a risk factor for intracranial hemorrhage during anti-vascular endothelial growth factor (VEGF) therapy. We report a 64-year-old man and a 65-year-old man with recurrent colorectal cancer without brain metastases; these patients developed multifocal and solitary intracranial hemorrhage, respectively, after the administration of bevacizumab. Our findings suggest that intracranial hemorrhage can occur even if the patient does not have brain metastases prior to bevacizumab treatment and also suggest that brain metastases are not a risk factor for intracranial hemorrhage with bevacizumab treatment. These findings also question the necessity of excluding patients with brain metastases from clinical trials on anti-VEGF therapy.

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Key words: Anti-vascular endothelial growth factor therapy; Bevacizumab; Central nervous system; Colorectal cancer; Intracranial hemorrhage

Peer reviewer: Dr. Jose Perea, Department of Surgery, 12 De Octubre University Hospital, Rosas De Aravaca, 82a, Madrid 28023, Spain

Nishimura T, Furihata M, Kubo H, Tani M, Agawa S, Setoyama R, Toyoda T. Intracranial hemorrhage in patients treated with bevacizumab: Report of two cases. *World J Gastroenterol* 2011; 17(39): 4440-4444 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4440.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4440>

Abstract

Treatment with bevacizumab, an antiangiogenic agent, in patients with metastatic or unresectable colorectal cancer was approved less than 4 years ago in Japan. Bevacizumab improves the survival of patients with metastatic colorectal cancer; however, it may lead to complications such as bleeding, which are sometimes fatal. Bevacizumab should be administered only after careful consideration because the potential risks of therapy outweigh its benefits. Therefore, pharmaceutical companies do not recommend bevacizumab therapy for patients with brain metastases. While some reports support the cautious use of bevacizumab, others report that it is not always necessary to prohibit its use in patients with metastases to the central nervous system (CNS), including the brain. Thus, bevacizumab therapy in colorectal cancer patients with brain metastases is controversial, and it is unclear whether brain

INTRODUCTION

Bevacizumab (Avastin[®], Chugai, Tokyo, Japan) is a recombinant humanized monoclonal antibody that selectively binds to and neutralizes the biological activity of vascular endothelial growth factor (VEGF) receptor^[1-4].

It reduces tumor vascularization and inhibits tumor growth, and has been shown to be efficacious in metastatic breast, colorectal, non-small cell lung, and renal cell carcinoma in combination with other agents^[1,5-9]. Thus, therapeutically bevacizumab can greatly enhance overall survival and progression-free survival in cases where patients are undergoing treatment for cancer metastasis or recurrence of colorectal cancer.

In spite of its efficacy, bevacizumab sometimes causes complications such as hypertension, gastrointestinal perforation, wound healing complications, arterial thromboembolic events, and bleeding, which are associated with mechanism-based adverse events. In 1977, it was reported that treatment with bevacizumab in a patient with undiagnosed brain metastasis from hepatocellular carcinoma caused fatal cerebral hemorrhage; this led to the suggestion that patients with brain metastases should not be treated with bevacizumab^[9]. Conversely, several investigators have reported that there is no evidence that anti-VEGF therapy, including bevacizumab, results in an increased risk of intracranial hemorrhage (ICH), regardless of the presence of central nervous system (CNS) metastases^[1,10]. Bhaskara *et al.*^[11] suggested that bevacizumab therapy is promising in the case of primary brain malignancies as well as metastatic disease. Therefore, whether cases with metastases to the CNS should be treated with combination chemotherapy including bevacizumab has provoked an ongoing discussion in this field^[1,10]. Only a limited number of cases of CNS bleeding associated with bevacizumab treatment have been reported, offering little, if any, strong argument for limiting the use of bevacizumab as a combination therapy in cancer.

In this study, we report 2 patients with recurrent colorectal cancer in the absence of brain metastases who underwent bevacizumab treatment; severe multifocal or solitary ICH was observed after this treatment.

CASE REPORT

Case 1

A 64-year-old man with lower rectal carcinoma underwent abdominoperineal excision with lymph node dissection in January 2004. According to tumor-node-metastasis (TNM) classification, the pathological stage of the carcinoma was T2N0M0 (stage II). He underwent adjuvant chemotherapy with oral 5-fluorouracil (5-FU) for 6 mo. Approximately 2 years after surgery, the serum carcinoembryonic antigen (CEA) level started to increase; however, radiological examination failed to show whether the cancer had recurred. The patient was afraid of recurrence and wished to receive an oral anticancer agent; therefore, S-1 treatment was provided. At the 3-year follow-up visit, computed tomography (CT) of the pelvis and the lateral segment of the liver revealed the first recurrence sites. He was subsequently prescribed FOLFOX-4 [oxaliplatin/5-FU/leucovorin (LV)] every 2 wk over 5 courses. Partial hepatectomy was

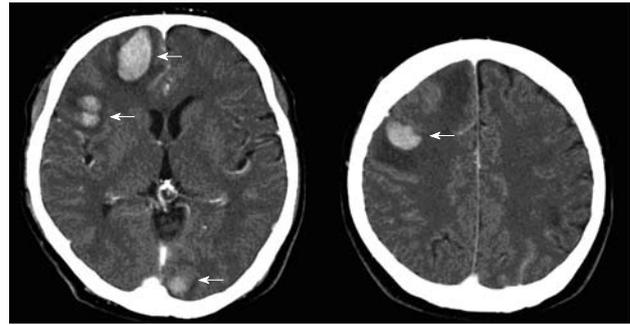


Figure 1 Contrast-enhanced computed tomography scan shows multifocal hemorrhage in the right frontal lobe, right temporal lobe, left occipital lobe, and right parietal lobe (white arrows).

performed for the solitary liver metastasis. Subsequently, FOLFOX-4 was repeated for approximately 12 mo; however, because of augmentation of the intrapelvic recurrence, bevacizumab was added in February 2010. After 4 courses of FOLFOX-4 in combination with bevacizumab (5 mg/kg), the patient suddenly developed dysarthria on day 2 of the 5th course, although he had tolerated the regimen fairly well until then. Brain CT revealed massive multifocal intracranial hemorrhage in the right frontal lobe, right temporal lobe, left occipital lobe, and right parietal lobe, although brain CT performed before bevacizumab administration revealed that there was no disorder in the brain, including metastasis (Figure 1). He had no history of head injury, but had hypertension before and after administration of bevacizumab, which was well controlled with medication. Hematological testing revealed slight thrombopenia (platelet count, $8.5 \times 10^4/\mu\text{L}$). These data together with other laboratory data for this patient met the criteria for the administration of FOLFOX-4 with bevacizumab. A neurosurgeon was immediately consulted, and an urgent craniotomy was performed to judge whether the multiple hemorrhages were accompanied by brain metastases. A hematoma was removed from the right frontal lobe of the brain. The removed hematoma did not show any histological signs of tumor components microscopically, which suggested that the multiple ICHs might be caused by bevacizumab. The patient soon recovered, and follow-up brain CT revealed the complete disappearance of multiple ICHs 6 wk after craniotomy. We discontinued chemotherapy, which included FOLFOX-4 with bevacizumab, and the patient was placed under another regimen; FOLFIRI (irinotecan/5-FU/LV), which the patient had not previously undergone, and was restarted within 8 wk after craniotomy. The serum CEA level gradually leveled off; intrapelvic recurrence has been uncontrollable since we discontinued the combination therapy of FOLFOX-4 with bevacizumab.

Case 2

A 65-year-old man with ascending colon carcinoma and liver metastases underwent right hemicolectomy with lymph node dissection, and partial hepatectomy in

Table 1 Summary of 8 cases of bevacizumab-associated central nervous system bleeding

Study	Yr	Age	Gender	Primary location	Bevacizumab dose (mg/kg)	Bleeding site	Combination drug	Comorbidity	Survival status ¹
Chen <i>et al</i> ^[15]	2006	NR	NR	Colorectum	5	NR	FU, LV	NR	NR
Nguyen <i>et al</i> ^[13]	2007	72	Female	Fallopian tube	NR	Pontine	GEM	Upper extremity Venous thrombosis	NR
Giantonio <i>et al</i> ^[16]	2007	59	Female	Peritoneum	NR	Subarachnoid	IRI, CDDP	Pulmonary emboli	NR
		NR	NR	Colorectum	10	NR	None or FOLFOX	NR	Dead
Tanvetyanon <i>et al</i> ^[14]	2009	52	Male	Lung	15	Large vein draining to the superior sagittal sinus	None or FOLFOX	NR	Dead
		NR	NR	Colorectum	10		PTX, CBDCA	AVM	Dead
Our cases	2011	64	Male	Rectum	5	Right frontal lobe, right temporal lobe, Left occipital lobe, right parietal lobe	FOLFOX	Hypertension	Alive
		65	Male	Colon	5	right thalamus	FOLFIRI	Hypertension	Alive

¹At time of publication. 5-FU: 5-fluorouracil; LV: Leucovorin; GEM: Gemcitabine; IRI: Irinotecan; CDDP: Cisplatin; CBDCA: Carboplatin; PTX: Paclitaxel; FOLFOX: Oxaliplatin/5-FU/LV; FOLFIRI: IRI/5-FU/LV; AVM: Arteriovenous malformation; NR: Not reported.

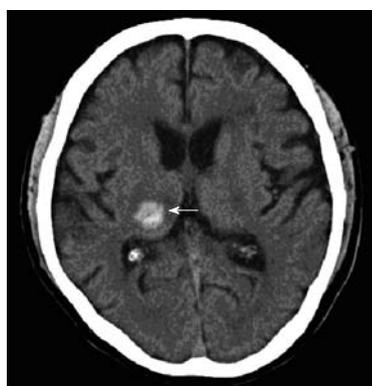


Figure 2 Non-contrast computed tomography scan shows a single right thalamic hemorrhage (white arrow).

February 2008. According to TNM classification, the pathological stage of the carcinoma was T2 N0 M1 (stage IV). He was treated with an intra-arterial infusion of 5-FU/intravenous infusion of LV for 5 mo. Subsequently, pulmonary metastasis became obvious in the 6-mo follow-up CT scan. Chemotherapy with oral 5-FU/LV was then prescribed for 6 mo, but the metastatic lesion progressed. He subsequently received FOLFOX-4 which was repeated for 15 courses over 12 mo. However, the lesion developed further. For the solitary pulmonary metastasis, partial lung resection was performed in March 2010. After that, a course of FOLFIRI with bevacizumab 5 mg/kg was started in July 2010. After 2 courses of FOLFIRI in combination with bevacizumab every 3 wk, which was tolerated fairly well, the patient suddenly developed left hemiplegia on day 11 of the 3rd course. Brain CT revealed right thalamus hemorrhage, although brain CT performed before starting chemotherapy showed no brain disorder, including metastasis, similar to case 1 (Figure 2). He had no history of head injury, but had hypertension that was well controlled with antihypertensive agents before and after administration of bevacizumab. His hematological results met the criteria for the administration of FOLFIRI with bevacizumab. Because of the right thalamic hemorrhage, combination chemotherapy of FOLFIRI with bevacizumab was discontinued.

DISCUSSION

Although minor bleeding, for example, grade 1 or 2 epistaxis, is a well-known complication of bevacizumab, serious bleeding such as hematemesis or hemoptysis (grade 3 and 4) occurs only in 3% of the patients treated with bevacizumab^[10,12]. As there is a possibility that bevacizumab administration can increase the risk of bleeding, it is advised that patients with CNS metastases should be excluded from treatment with bevacizumab. If bleeding into brain metastases occurs even once, it can have devastating complications that could develop into serious morbidity and mortality. To our knowledge, 8 cases of bevacizumab-associated CNS bleeding have been reported^[13-16]. Table 1 shows the characteristics of these cases. There have been 2 case reports that raise caution regarding ICH in patients without brain metastases under bevacizumab administration due to the use of anticoagulant agents^[13,14]. The first reports 2 cases where bevacizumab was accompanied by low-molecular-weight heparin. It draws attention to the fact that a full dose of anticoagulation agent with bevacizumab may increase the risk of ICH, regardless of whether a patient has any underlying parenchymal or intracranial vascular lesions^[13]. The second study reports a case of arteriovenous malformation (AVM)^[14]. These 3 patients did not have brain metastases, but presented with factors related to bleeding such as anticoagulation therapy and AVM. In addition to these 3 cases and the 2 cases presented here, 3 other cases of CNS bleeding have been reported by Chen *et al*^[15] and Giantonio *et al*^[16]. However, these 2 reports did not mention whether the patients had brain metastases^[16].

In contrast, more information on the safety of bevacizumab therapy in patients with CNS metastases is being reported. There has been several reports affirming the use of bevacizumab in treating patients with CNS metastases or primary brain tumors^[1,17-20]. Bhaskara *et al*^[11] presented a challenging case of a treatment-naïve patient with colorectal cancer and brain metastases. They emphasized the importance of weighing the risks and benefits of a treatment course when selecting treat-

ment options, and demonstrated the feasibility of bevacizumab in the treatment of metastatic brain tumors from colorectal cancer. Labidi *et al*^[21] showed no tumor-associated cerebral hemorrhage in patients with metastases from breast carcinoma treated with bevacizumab and paclitaxel. Moreover, Besse *et al*^[11] explored whether the general exclusion of patients with CNS metastases from bevacizumab treatment is justified, and concluded that patients with CNS metastases from advanced or metastatic breast cancer, non-small cell carcinoma, renal cell carcinoma, and colorectal cancer should not generally be excluded from bevacizumab therapy because such patients are at a similar risk of developing cerebral hemorrhage, independent of bevacizumab therapy.

The mechanism of bevacizumab is mainly thought to inhibit angiogenesis through VEGF. VEGF is an important mediator in new blood vessel formation in both physiological and pathological settings^[2]. Among the multitude of factors driving tumor angiogenesis, VEGF is the most potent, exerting myriad effects on vascular pruning and sprouting, permeability, network formation, proliferation, and cell death. Despite the initial unimpressive clinical performance of anti-VEGF antibodies, such as bevacizumab, as a cancer monotherapy, clear improvements in clinical outcomes following combination therapy of bevacizumab with chemotherapy regimens, and multi-targeted VEGF receptor tyrosine kinase inhibitors, such as sorafenib and sunitinib, in selected tumor types have established VEGF-targeted agents as an effective means of controlling cancer growth^[22]. Anti-VEGF therapeutic agents have been shown to have clinical benefits, although induction of bleeding is a recognized side effect that can sometimes occur and become troublesome during therapy.

The recent safety information on anti-VEGF therapy has been encouraging. Carden *et al*^[10] reviewed the current literature to determine whether there is a high incidence of significant bleeding due to anti-VEGF therapy. They identified 57 trials examining the safety of anti-VEGF therapy including bevacizumab in a total of 10 598 patients. In their studies, phase I and II clinical trials of anti-VEGF agents that excluded or included brain metastases did not show any significant differences (2 out of 1711; < 1% *vs* 1 out of 524; < 1%). Trials of anti-VEGF therapy, such as sunitinib and sorafenib, which included patients treated for active brain metastases revealed no episodes of ICH. Thus, the authors concluded there was no evidence of anti-VEGF therapy conferring an increased risk of cerebral hemorrhage, regardless of CNS metastases.

As well as metastatic lesions, data are available from bevacizumab trials in primary malignant brain tumors. For instance, Vrendenburgh *et al*^[23] reported the results of a phase II trial of bevacizumab and irinotecan treatment every 2 wk in recurrent malignant glioma, and showed that anti-VEGF therapy was not associated with an increased risk of ICH. Studies of anti-VEGF agents in patients with high-grade gliomas have been collective-

ly presented by Carden *et al*^[10]. They collated 5 studies of anti-VEGF agents including bevacizumab, vatalanib, and cediranib used in the treatment of high-grade gliomas^[10]. The limited data currently available on these studies showed no evidence that anti-VEGF therapy for high-grade gliomas increased the risk of ICH^[23,24].

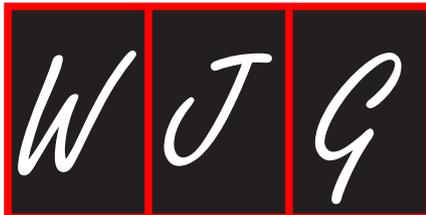
Collectively, the existing clinical literature and the cases presented here, suggest that patients with metastatic or primary brain tumors need not necessarily be excluded from treatment with anti-VEGF agents, rather that anti-VEGF therapy needs to be prescribed only after careful examination of the patient's history. This is because at least 6 out of 8 cases had CNS bleeding without CNS metastasis^[13-15]. Although, we cannot deny that ICH in the 2 cases presented here could have occurred by chance, they both have commonalities in their clinical presentation. Both had high-blood pressure, even though it was well controlled with medication. Although only 2 such cases are presented here, we think that rather than examining whether primary or metastatic brain tumors are present or absent when considering risk factors for ICH during anti-VEGF treatment (including bevacizumab), precautions should be taken when treating patients who have thrombocytopenia or are undergoing therapy with anticoagulation or antihypertensive agents. The 2 cases presented here and recent available reports do not confirm that anti-VEGF therapy for patients with metastatic brain tumors from colorectal cancer is a safe treatment, but suggest that anti-VEGF agents, including bevacizumab, are an alternative for patients with metastatic colorectal cancers, even in the presence of brain metastases. Epidemiological prospective studies should be carried out in order to identify the risk factors for ICH in patients treated with anti-VEGF agents.

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S- Editor Sun H L- Editor Webster JR E- Editor Zhang DN



Non-alcoholic fatty liver disease and metabolic syndrome in obese children

Mehmet Emre Atabek

Mehmet Emre Atabek, Selcuk University, School of Medicine, Department of Pediatric Endocrinology, 42080 Konya, Turkey

Author contributions: Atabek ME is solely responsible for this manuscript.

Correspondence to: Mehmet Emre Atabek, MD, Selcuk University, School of Medicine, Department of Pediatric Endocrinology, 42080 Konya, Turkey. meatabek@hotmail.com

Telephone: +90-332-2237264 Fax: +90-332-2236181

Received: March 22, 2011 Revised: June 27, 2011

Accepted: July 4, 2011

Published online: October 21, 2011

Abstract

I read with great interest the article of Fu *et al* who investigated whether non-alcoholic fatty liver disease (NAFLD) is an early mediator for prediction of metabolic syndrome, and whether liver B-ultrasound could be used for its diagnosis, in a study involving 861 obese children (6-16 years old). In this study, it was reported that NAFLD is not only a liver disease, but also an early mediator that reflects metabolic disorder, and that liver B-ultrasound can be a useful tool for metabolic syndrome (MS) screening. The authors reported that NAFLD and MS were present in 68.18% and 25.67% of obese children, respectively. Moreover, they observed that the prevalence of MS in NAFLD children was 37.64%, which was much higher than that in the non-NAFLD group. Criteria analogous to those of the Adult Treatment Panel III definition for MS were used for children in this study. The reported prevalence data on MS in the young has varied markedly, in large part because of disagreement among the variously proposed definitions of MS. Therefore, in my opinion, a study aiming to assess the association between MS components and NAFLD in obese children has to take into account a simple, easy-to-apply clinical definition proposed by the international diabetes federation for MS. Interpretation of the results of the Fu *et al* study are limited by

another major caveat: that the diagnosis or exclusion of NAFLD was based on liver enzymes and ultrasound imaging, but was not confirmed by liver biopsy. Indeed, it is known that liver enzymes may be within the reference interval in up to 70% of patients with diagnosed NAFLD and that the full histopathological spectrum of NAFLD may be present in patients with normal liver enzymes, which therefore cannot be reliably used to exclude the presence of NAFLD.

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Key words: Non-alcoholic fatty liver disease; Metabolic syndrome; Obese children

Peer reviewers: Michelle Lai, MD, MPH, Instructor in Medicine, Harvard University, Department of Medicine, Division of Gastroenterology/Hepatology, Beth Israel Deaconess Medical Center, 110 Francis Street, Suite 4A, Boston, MA 02215, United States; Dr. Michelangelo Foti, PhD, MBA, Department of Cellular Physiology and Metabolism, Faculty of Medicine, University of Geneva, Centre Médical Universitaire, Geneva 1211, Switzerland

Atabek ME. Non-alcoholic fatty liver disease and metabolic syndrome in obese children. *World J Gastroenterol* 2011; 17(39): 4445-4446 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i39/4445.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i39.4445>

TO THE EDITOR

I read with great interest the article of Fu *et al*^[1], who investigated whether non-alcoholic fatty liver disease (NAFLD) is an early mediator for prediction of metabolic syndrome, and whether liver B-ultrasound can be used for its diagnosis, in a study of 861 obese children (6-16 years old). In this study, it was reported that NAFLD is not only a liver disease, but also an early mediator that reflects metabolic disorder, and that

liver B-ultrasound can be a useful tool for metabolic syndrome (MS) screening. The authors reported that NAFLD and MS were present in 68.18% and 25.67% of obese children, respectively. Moreover, they observed that the prevalence of MS in NAFLD children was 37.64%, which was much higher than that in the non-NAFLD group. Criteria analogous to those of the adult treatment panel (ATP) III definition for MS were used for children in this study.

Since De Ferranti *et al*^[2] demonstrated different prevalence rates of MS by using slightly different modifications of the ATP III criteria in the same population using the same dataset, there has been a need for standardization of MS in children. For example, De Ferranti *et al*^[2] analyzed the same population which consisted of 2430 adolescents aged 12-19 years who had been previously evaluated by Cook *et al*^[3] using slightly different criteria. Changing the criteria for the diagnosis increased the prevalence of the MS from 4.2% to 9.3% in the total population and from 28.7% to 31.2% among the obese population in the total cohort.

In our previous study^[4], we have reported that MS defined according to modified WHO criteria adapted for children was found in 27.2% of young people, with a significantly higher rate among obese adolescents aged 12-18 years (37.6%) than among obese children aged 7-11 years (20%) ($P < 0.001$). Several studies have revealed race/ethnic differences in the prevalence of MS in children as well as in adults.

In 2007, recognizing how difficult it is to have multiple working definitions of the MS, the international diabetes federation (IDF) published its definition for children^[5]. The new IDF definition is ranked according to age groups 6 to < 10 years, 10 to < 16 years, and ≥ 16 years. This was believed to be necessary because of the developmental challenges presented by age-related differences in children and adolescents. Children < 6 years were excluded as a result of insufficient data in this age group. The IDF suggests that below 10 year of age, the MS should not be diagnosed, and a strong message for weight reduction should be delivered to parents and caregivers of those with abdominal obesity.

As described, the reported prevalence data on MS in the young has varied markedly, in large part because of disagreement among the variously proposed definitions of MS. Therefore, in my opinion, a study aiming to assess the association between MS components and NAFLD in obese children has to take into account the simple, easy-to-apply clinical definition proposed by the IDF for MS. Moreover, the statement: "There were significantly higher incidences concerning every component of MS in obese children with NAFLD compared with

the non-NAFLD group" is unclear and probably wrong since data for MS were derived from a prepubertal age group most of whom were below 10 years of age. The major concern originates from the assumption that in order to validate such a result it is mandatory to consider a wide population of adolescents. The authors have not referenced other studies evaluating MS in children in this paper, but they offer a new observation.

Interpretation of the results of the Fu *et al*^[1] study are limited by another major caveat that the diagnosis or exclusion of NAFLD was based on liver enzymes and ultrasound imaging, but was not confirmed by liver biopsy. Indeed, it is known that liver enzymes may be within the reference interval in up to 70% of patients with diagnosed NAFLD and that the full histopathological spectrum of NAFLD may be present in patients with normal liver enzymes, which therefore cannot be reliably used to exclude the presence of NAFLD^[6]. Moreover, although liver ultrasonography is widely used for diagnosing NAFLD, this imaging method has good sensitivity and specificity only for detection of moderate and severe hepatic steatosis, but its sensitivity is reduced when hepatic fat infiltration on liver biopsy is < 33%^[7]. Only liver biopsy can be used for diagnosing NAFLD and accurately determining the histological severity and prognosis of liver damage^[6].

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S- Editor Sun H L- Editor Logan S E- Editor Zhang DN

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.

Fikri M Abu-Zidan, Professor, Department of Surgery, Faculty of Medicine, UAE University, POBox 17666, Al-Ain, United Arab Emirates

Hussein M Atta, MD, PhD, Department of Surgery, Faculty of Medicine, Minia University, Mir-Aswan Road, El-Minia 61519, Egypt

Paolo Angeli, MD, PhD, Department of Clinical and Experimental Medicine, University of Padova, Padova Via Giustiniani 2, Padova, cap. 35100, Italy

John Beynon, BSc, MB BS, MS, FRCS (ENG), Consultant Colorectal Surgeon, Singleton Hospital, Sketty Lane, Swansea, SA2 8QA, United Kingdom

Mark Bloomston, MD, FACS, Assistant Professor of Surgery, Division of Surgical Oncology, N924 Doan Hall, 410W.10th Avenue, Columbus, Ohio 43082, United States

Radan Bruha, MD, PhD, Associate Professor, 4th Department of Internal Medicine, General Teaching Hospital, Charles University, U Nemocnice 2, 128 08 Prague 2, Czech Republic

Dr. Herwig R Cerwenka, Professor, Department of Surgery, Medical University of Graz, Auenbruggerplatz 29, A-8036 Graz, Austria

Parimal Chowdhury, Professor, Department of Physiology and Biophysics, College of Medicine University of Arkansas for Medical Sciences, 4301 W Markham Street Little Rock, Arkansas 72205, United States

Yeun-Jun Chung, MD, PhD, Professor, Director, Department of Microbiology, Integrated Research Center for Genome Polymorphism, The Catholic University Medical College, 505 Banpo-dong, Socho-gu, Seoul 137-701, Korea

A M El-Tawil, MSc, MRCS, PhD, Department of Surgery, University Hospital of Birmingham, East Corridor, Ground Floor,

Birmingham, B15 2TH, United Kingdom

Francesco Feo, Professor, Department of Biomedical Sciences, Section of Experimental Pathology and Oncology, University of Sassari, Via P, Manzella 4, 07100 Sassari, Italy

Linda A Feagins, MD, Assistant Professor of Internal Medicine, Division of Gastroenterology and Hepatology, UT Southwestern Medical Center / Dallas VA Medical Center, 4500 S. Lancaster Rd., MC 111B1, Dallas, TX 75216, United States

Yasuhiro Fujino, MD, PhD, Director, Department of Surgery, Hyogo Cancer Center, 13-70 Kitaoji-cho, Akashi, 673-8558, Japan

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

Shiu-Ming Kuo, MD, University at Buffalo, 15 Farber Hall, 3435 Main Street, Buffalo, NY 14214, United States

Eric CH Lai, MB, ChB(CUHK), MRCS(Ed), FRACS, FCSHK, FHKAM(Surgery), Department of Surgery, Pamela Youde Nethersole Eastern Hospital, 3 Lok Man Road, Chai Wan, Hong Kong, China

Alan C Moss, MD, FACP, Assistant Professor of Medicine, Director of Translational Research, Center for Inflammatory Bowel Disease, Beth Israel Deaconess Medical Center, Harvard Medical School, Rose 1/East, 330 Brookline Ave, Boston, MA 02215, United States

Huanbiao Mo, PhD, Associate Professor, Department of Nutrition and Food Sciences, Texas Woman's University, POBox 425888, Denton, TX 76204, United States

Pradyumna Kumar Mishra, MS, PhD, Professor, Division of Translational Research, Tata Memorial Centre, ACTREC, Navi Mumbai 410 210, India

Richard A Rippe, Dr, Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7038, United States

Scott Steele, MD, FACS, FASCRS, Chief, Colon and Rectal Surgery, Dept of Surgery, Madigan Army Medical Center, Fort Lewis, WA 98431, United States

Shun-Fa Yang, PhD, Associate Professor, Institute of Medicine, Chung Shan Medical University, No. 110, Sec.1 Chien-Kuo N. Road, Taichung 402, Taiwan, China

Events Calendar 2011

- January 14-15, 2011
 AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States
- January 20-22, 2011
 Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States
- January 27-28, 2011
 Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany
- January 28-29, 2011
 9. Gastro Forum München, Munich, Germany
- February 4-5, 2011
 13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany
- February 13-27, 2011
 Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia
- February 17-20, 2011
 APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver Bangkok, Thailand
- February 22, 2011-March 04, 2011
 Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada
- February 24-26, 2011
 Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland
- February 24-26, 2011
 2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil
- February 24-26, 2011
 International Colorectal Disease Symposium 2011, Hong Kong, China
- February 26-March 1, 2011
 Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada
- February 28-March 1, 2011
 Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates
- March 3-5, 2011
 42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States
- March 7-11, 2011
 Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States
- March 14-17, 2011
 British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom
- March 17-19, 2011
 41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany
- March 17-20, 2011
 Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States
- March 18, 2011
 UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States
- March 25-27, 2011
 MedicRes IC 2011 Good Medical Research, Istanbul, Turkey
- March 26-27, 2011
 26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States
- April 6-7, 2011
 IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States
- April 7-9, 2011
 International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy
- April 15-16, 2011
 Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany
- April 18-22, 2011
 Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States
- April 20-23, 2011
 9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea
- April 25-27, 2011
 The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia
- April 25-29, 2011
 Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States
- April 28-30, 2011
 4th Central European Congress of Surgery, Budapest, Hungary
- May 7-10, 2011
 Digestive Disease Week, Chicago, IL 60446, United States
- May 12-13, 2011
 2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom
- May 19-22, 2011
 1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain
- May 21-24, 2011
 22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venice, Italy
- May 25-28, 2011
 4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina
- June 11-12, 2011
 The International Digestive Disease Forum 2011, Hong Kong, China
- June 13-16, 2011
 Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy
- June 14-16, 2011
 International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia
- June 22-25, 2011
 ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain
- June 29-2, 2011
 XI Congreso Interamericano de Pediatría "Monterrey 2011", Monterrey, Mexico
- September 2-3, 2011
 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany
- September 10-11, 2011
 New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States
- September 10-14, 2011
 ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States
- September 30-October 1, 2011
 Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium
- October 19-29, 2011
 Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia
- October 22-26, 2011
 19th United European Gastroenterology Week, Stockholm, Sweden
- October 28-November 2, 2011
 ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States
- November 11-12, 2011
 Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan
- December 1-4, 2011
 2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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Name of journal

World Journal of Gastroenterology

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 Identifier, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 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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World Journal of Gastroenterology®

Volume 17 Number 40
October 28, 2011



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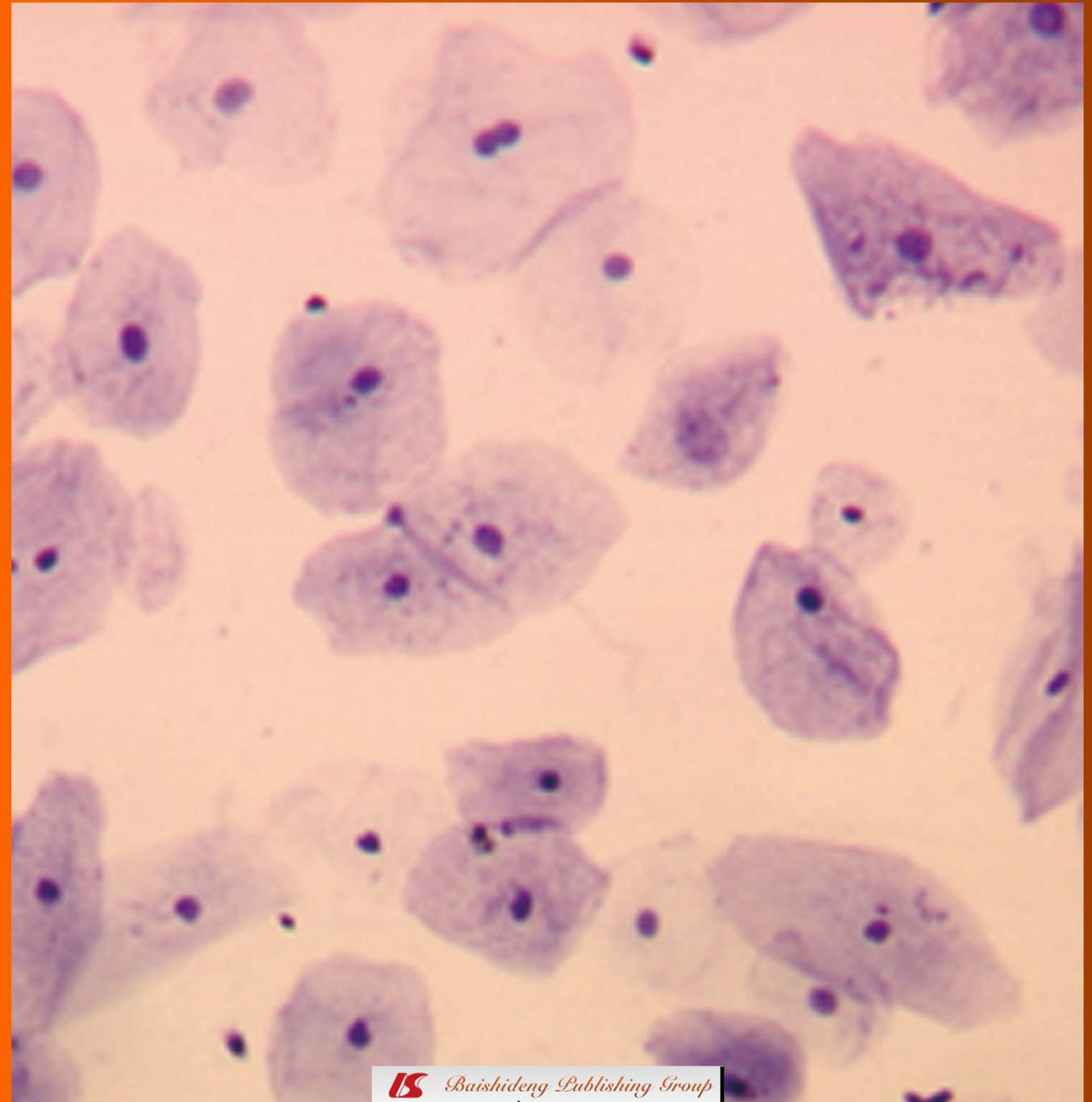
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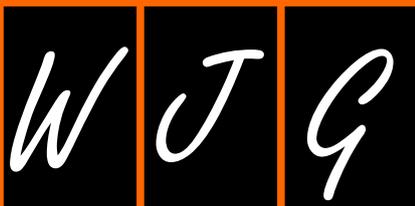
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ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

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Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
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PUBLISHING
Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building, No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
E-mail: baishideng@wjgnet.com
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SUBSCRIPTION
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
October 28, 2011

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

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Chronic proctalgia and chronic pelvic pain syndromes: New etiologic insights and treatment options

Giuseppe Chiarioni, Corrado Asteria, William E Whitehead

Giuseppe Chiarioni, Division of Gastroenterology of the University of Verona, Azienda Ospedaliera Universitaria Integrata di Verona, Valeggio sul Mincio Hospital, 37067 Valeggio sul Mincio, Verona, Italy

Corrado Asteria, Department of Surgery and Orthopedics, General Surgery Unit, Azienda Ospedaliera Carlo Poma di Mantova, Asola General Hospital, 46041 Asola, Mantova, Italy

William E Whitehead, Division of Gastroenterology and Hepatology, Division of Urogynecology, and Center for Functional Gastrointestinal and Motility Disorders, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7080, United States

Author contributions: Chiarioni G and Whitehead WE contributed equally to the conceiving of the designing and the drafting of the manuscript; Asteria C provided criticism and suggestion of high intellectual content.

Supported by In part by Grant R01 DK031369 from the NIDDK

Correspondence to: Giuseppe Chiarioni, MD, Division of Gastroenterology of the University of Verona, Azienda Ospedaliera Universitaria Integrata di Verona, Valeggio sul Mincio Hospital, 37067 Valeggio sul Mincio, Verona, Italy. chiarioni@tin.it

Telephone: +39-045-6338548 **Fax:** +39-045-7950188

Received: March 15, 2011 **Revised:** June 3, 2011

Accepted: June 10, 2011

Published online: October 28, 2011

Abstract

This systematic review addresses the pathophysiology, diagnostic evaluation, and treatment of several chronic pain syndromes affecting the pelvic organs: chronic proctalgia, coccygodynia, pudendal neuralgia, and chronic pelvic pain. Chronic or recurrent pain in the anal canal, rectum, or other pelvic organs occurs in 7% to 24% of the population and is associated with impaired quality of life and high health care costs. However, these pain syndromes are poorly understood, with little research evidence available to guide their diagnosis and treatment. This situation appears to be changing: A recently published large randomized,

controlled trial by our group comparing biofeedback, electrogalvanic stimulation, and massage for the treatment of chronic proctalgia has shown success rates of 85% for biofeedback when patients are selected based on physical examination evidence of tenderness in response to traction on the levator ani muscle—a physical sign suggestive of striated muscle tension. Excessive tension (spasm) in the striated muscles of the pelvic floor appears to be common to most of the pelvic pain syndromes. This suggests the possibility that similar approaches to diagnostic assessment and treatment may improve outcomes in other pelvic pain disorders.

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Key words: Biofeedback; Chronic pelvic pain; Chronic proctalgia; Coccygodynia; Levator ani syndrome; Pudendal neuralgia

Peer reviewers: Guang-Yin Xu, MD, PhD, Assistant Professor, Division of Gastroenterology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77555-0655, United States; Bhupendra Kumar Jain, Dr., MS, Professor of Surgery and Head, Department of Surgery, GTB Hospital and University College of Medical Sciences, Delhi 110 095, India

Chiarioni G, Asteria C, Whitehead WE. Chronic proctalgia and chronic pelvic pain syndromes: New etiologic insights and treatment options. *World J Gastroenterol* 2011; 17(40): 4447-4455 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4447.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4447>

INTRODUCTION

Chronic or frequently recurring pain in the anal canal, rectum, or pelvis is a prevalent symptom that affects an estimated 6.6% of the population^[1]. Although only 1/3 of people with such pains consult physicians, they nev-

ertheless report significant impairment in quality of life, work absenteeism, and psychological distress. However, despite its prevalence and impact, relatively little research has been published which addresses its epidemiology, pathophysiology, and treatment; and pelvic and rectal pain is widely considered frustrating to diagnose and treat. This may soon change: Our group recently reported a randomized controlled trial^[2] in which we compared different approaches to treating chronic proctalgia and showed that the results were excellent 85% success rate if the patients were appropriately screened. This study also provided new insights into the pathophysiology of chronic proctalgia and stimulated renewed interest in anorectal pain syndromes. The aims of this review are to critically assess what is known about the diagnosis and treatment of the most common forms of anorectal and pelvic pain, namely chronic proctalgia, chronic pelvic pain, coccygodynia, and pudendal neuralgia. This review is meant to help Gastroenterologists and Colorectal Surgeons when dealing with complex consultation on pelvic pain syndromes. It is mostly focused on chronic proctalgia and issues of differential diagnosis with other pelvic pain syndromes.

One of the challenges in caring for patients with anorectal and pelvic pain is that a number of inflammatory and structural etiologies must be considered. The organic diseases that are most commonly involved in chronic anorectal and pelvic pain are cryptitis, fissure, abscess, hemorrhoids, solitary rectal ulcer, inflammatory bowel disease, and rectal ischemia^[3]. One should also consider chronic prostatitis and pelvic endometriosis as potential contributors to chronic pelvic pain^[3]. Although the differential diagnosis is large and unfortunately poorly standardized, our experience^[2] suggests that no organic disease explanation will be found in approximately 85% of patients presenting to gastroenterologists with chronic anorectal or pelvic pain. We screened 227 patients referred for unremitting, chronic rectal pain with a diagnostic evaluation that included digital rectal examination, colonoscopy, pelvic ultrasound and surgical consultation in all patients, plus gynecology and urology referrals in selected cases^[2]. This extensive work-up identified only 33 patients (15%) with a probable organic disease accounting for their symptoms. Thus, for most patients with chronic anorectal or pelvic pain, the origin of the pain is uncertain and the relevant pathophysiological mechanisms are unclear. These are commonly defined as “functional” chronic anorectal and pelvic pain syndromes since no structural and anatomical disease was found. These functional pain syndromes constitute the main subject of this review.

CHRONIC PROCTALGIA

Chronic proctalgia is a general term for chronic or recurring pain in the anal canal or rectum^[3]. Other names considered synonymous with chronic proctalgia are levator ani syndrome, puborectalis syndrome, chronic

idiopathic perineal pain, piriformis syndrome, and pelvic tension myalgia. Thiele, one of the first researchers to investigate this pain syndrome, called it coccygodynia, although he acknowledged that the pain was not in the coccyx^[4]. To provide greater consistency in the diagnosis and labeling of anorectal pain syndromes, the Rome III criteria^[3] define chronic proctalgia as chronic or recurrent rectal pain or aching lasting at least 20 min, in the absence of structural or systemic disease explanations for these symptoms^[3]. Pain duration of at least 20 min is a key feature since shorter episodes of pain are suggestive of proctalgia fugax, which is defined as a sudden, severe pain in the anorectal region lasting less than 20 min and then disappearing completely^[3]. Proctalgia fugax may recur, but episodes are rare. Proctalgia fugax is believed to have a different etiology to chronic proctalgia, although there is no consensus on what causes it. Its consideration is beyond the scope of this review, which is intended to deal with chronic unremitting diseases.

Chronic proctalgia is further divided by the Rome III criteria into two subtypes—levator ani syndrome (LAS) and unspecified functional anorectal pain—based on the presence or absence of a sensation of tenderness when the levator muscle is palpated during digital rectal examination. This classification updates the previous Rome II classification in which LAS was designated as “highly likely” if traction on the pelvic floor produced a report of tenderness and only “possible LAS” if no tenderness was elicited^[5]. Subgrouping patients with chronic proctalgia is consistent with clinical experience of different response to treatment, but distinct epidemiology and pathophysiology data are lacking^[5]. Therefore, data provided mostly refer to chronic proctalgia patients as a whole.

Pathophysiology

Chronic tension or spasm of the striated muscles of the pelvic floor is commonly assumed to be the pathophysiological basis for chronic proctalgia^[3,5-7], although there is no definitive evidence for this hypothesis. Inflammation of the levator or arcus tendon of the levator ani muscle has also been suggested as a cause of chronic proctalgia, since tenderness on palpation is most commonly found on the left side where the muscle inserts into the pubic ramus of the pelvis. However, contrary to this tendinitis hypothesis, local steroid injection has not been shown to be an effective treatment for chronic proctalgia^[8]. In retrospective studies, many patients reported prior pelvic surgery, anal surgery and even spinal surgery as significant in the development of their pain syndrome^[6,9]. Childbirth can be another precipitating factor^[9]. In addition, high rates of anxiety disorders, depression, and stress are frequently reported in chronic proctalgia, and may act as significant precipitating factors in some patients^[6,10].

Except for the exclusion of organic diseases, tests of anorectal physiology and imaging studies were traditionally considered to be of little diagnostic or prognostic

value^[11,12]. Increased anal canal resting pressures tested by anorectal manometry were sometimes reported, but results were inconsistent. Grimaud and coworkers reported that LAS was associated with anal sphincter hypertonia and disordered defecation on dynamic proctography in a study of 12 patients, but this was not confirmed in a larger prospective study of 60 patients by Ger and coworkers^[11,13]. Ger *et al.*^[11] reported that LAS was associated with paradoxical contraction of the pelvic floor muscles on straining as evidenced by anal electromyography or defecography. However, all these studies were potentially biased by small size, mixed patient population, and poor patient selection^[3,5]. In addition, a number of structural disorders (descending perineum, rectocele, mucosal prolapse and pelvic floor dyssynergia) have been reported in small studies^[6,11-13].

In a recent study, Hompes *et al.*^[14] reported on 59 patients referred to a Pelvic Floor Clinic for chronic functional anorectal pain who were tested by means of defecating proctography, anorectal manometry, anal ultrasound, and in selected cases, rectal examination under anesthesia. The same diagnostic protocol was applied to 543 rectal prolapse patients complaining of obstructed defecation and to a control group of patients with fecal incontinence. In the control group with fecal incontinence, pain was reported in 50% of patients but was a non-dominant symptom. Anorectal manometry failed to show any difference among groups. Rectal morphology examinations demonstrated high grade internal rectal prolapse in 59% of pain patients, which was often associated with symptoms of obstructed defecation. The authors concluded that rectal prolapse commonly underlies chronic proctalgia, particularly when obstructed defecation is present. However, the severity of prolapse did not correlate with pain intensity, leaving pain pathophysiology unclear^[14]. In addition, chronic idiopathic rectal pain is sometimes reported as a complication of corrective surgery for rectal prolapse^[14].

An innovative pathophysiology explanation for chronic proctalgia was recently reported by our group in a large, prospective, randomized controlled trial comparing biofeedback, electrogalvanic stimulation (EGS), and digital massage of the levator muscles for the treatment of chronic proctalgia. In this study, 157 patients with chronic proctalgia (confirmed by Rome II criteria) were studied by anorectal manometry and a balloon evacuation test at baseline and again after 3 mo of treatment^[2]. Based on a priori exclusion criteria, patients reporting symptoms consistent with either irritable bowel syndrome or functional constipation were not enrolled in the study. In patients reporting tenderness on palpation of the levator muscles (Rome II: highly likely LAS, Rome III: LAS), physiologic features of dyssynergic defecation (i.e., paradoxical contraction or failure to relax the pelvic floor on straining) were seen in approximately 85% of subjects in the absence of symptoms of constipation. Conversely, in patients who denied tenderness when the levators were palpated during digital rectal

examination, inability to relax pelvic floor muscles when straining was an uncommon finding (19%). Dyssynergic defecation was a strong predictor of successful treatment outcome. These observations led us to conclude that the physiologic mechanisms responsible for LAS and dyssynergic defecation are similar^[2].

This study also showed that the inability to relax pelvic floor muscles when straining to defecate may occur without symptoms of constipation, even though it is commonly assumed that dyssynergic defecation invariably results in obstructed defecation. Factors that interact with pelvic floor physiology to determine which symptoms develop—either pain or constipation—are left unanswered by our study and deserve further investigations. Also, we were not able to provide a physiological explanation for unspecified functional anorectal pain (i.e., anorectal pain without tenderness on digital palpation), which may represent a heterogeneous group of patients. Our study suggests, however, that adding a simple balloon evacuation test with a disposable Foley catheter to the diagnostic work up of chronic proctalgia patients enables one to select subjects that are more likely to benefit from pelvic floor rehabilitation.

Clinical presentation

Chronic proctalgia is often described by patients as a dull ache or pressure sensation in the rectum that is exacerbated by prolonged sitting and relieved by standing or lying down^[3,5]. This pain rarely occurs at night; rather, it usually begins in the morning and increases in severity throughout the day. The pain may be precipitated by long-distance car travelling, stress, sexual intercourse and defecation^[6,7]. During digital rectal examination, the examining finger is moved from the coccyx posteriorly to the symphysis pubis anteriorly^[5,7]. For unexplained reasons, tenderness is often non-symmetric, being greater on the left side than on the right^[5]. When performing digital rectal examination, the examiner should pause after inserting their finger into the rectum before applying traction on the levator muscles to avoid false positive results. In our experience, repeating the posterior traction on the levator muscle on the same exam is also useful to check for reproducibility and to avoid false positive results.

Diagnostic assessment

Although our recent study provides new insights into the pathophysiology of chronic proctalgia, these observations require validation by other laboratories before they can be incorporated as diagnostic criteria. The diagnosis of chronic proctalgia still relies on (1) clinical symptoms of recurring or chronic pain or aching in the anal canal or rectum with episodes lasting 20 min or longer^[3], and (2) exclusion of alternative disease explanations for these symptoms by multiple diagnostic tests and consultations by other specialists. In addition, digital rectal examination should be performed to ascertain whether the patient reports tenderness when traction is applied to the levator ani muscles because this diagnostic sign

is a strong predictor of whether the patient is likely to benefit from treatments directed at relaxing pelvic floor muscles.

Treatment

No single treatment has been reported to be consistently effective in chronic proctalgia^[3,7], and management can be a frustrating endeavor for both patients and physicians^[11]. The first-line treatment most commonly provided is reassurance that the pain is of benign origin and is not suggestive of malignancy^[7,9]. No data are available on the impact of reassurance, but education and counseling are often incorporated as a component of treatment.

Digital massage of the puborectalis sling, intended to relax tense muscles, was one of the first treatments proposed for chronic proctalgia^[9]. Massage of the puborectalis muscle should be performed in a firm manner with the affected side massaged up to 50 times, depending on the patient's tolerance. Some claim that if the massage is not uncomfortable to the patient while being performed, it may not be effective^[9]. Massage of the levator ani muscle is rarely performed as the sole therapy, with the most common adjunctive treatments being hot sitz baths or a short-term course of oral Diazepam, both of which are assumed to have myorelaxant properties. Earlier open-label studies suggested that digital massage combined with hot sitz baths and/or Diazepam were effective for relieving pain in 68% of 316 chronic proctalgia patients^[15]. However, benefits seemed to fade away during long-term follow-up, and the addictive potential of Diazepam discourages long-term treatment^[7].

Electrogalvanic stimulation, traditionally used by physiatrists to treat muscle spasticity^[9], has also been advocated for the treatment of LAS when conservative therapy is ineffective. A low frequency oscillating current applied to the pelvic floor muscles through an anal probe, induces fasciculation and prolonged fatigue, which breaks the spastic cycle and may produce sustained symptom relief. Low frequency current has no thermal effect. No side effects have ever been reported other than mild worsening of pain on the first days of treatment. Sohn and coworkers were the first to test EGS in an open study of 80 chronic proctalgia patients^[16]. They recommended a pulse frequency of 80 cycles per second with the voltage being gradually increased from zero to the point of discomfort (250-300 Volts according to patient's tolerance). Recommended treatment duration is one hour per day for 3 sessions in a ten-day period. In the Sohn study^[16], 91% of patients reported good to excellent pain relief from EGS in the short-term, but no long-term follow-up was reported. This high percentage of success was never replicated by subsequent open label studies, although approximately two-thirds of patients did report short-term pain relief. Treatment protocols varied widely in terms of number and duration of sessions. Authors claimed that non-responders showed features of psychology disturbances,

but no evidence was provided on the issue. However, three additional studies that investigated the long-term benefits of EGS treatment in chronic proctalgia found that only 25%-38% of patients reported persistent pain improvement^[17-19].

Biofeedback treatment of LAS was first described in 1991 by Grimaud and coworkers^[13]. They treated 12 patients with biofeedback techniques focused on voluntary relaxation of external anal sphincter tone. Pain disappeared in all patients after a mean of eight sessions. Subsequent studies using biofeedback were not able to replicate these results, with success rates varying from 35% to 87.5%^[6,11,19]. All studies were small, none was controlled, and treatment modalities varied.

Botulinum Toxin A (BoTox A) was tested in a randomized controlled trial run in 12 patients, and no differences in rectal pain were observed between patients injected with active BoTox *versus* those injected with saline^[20]. The average amount of time required to defecate a rectal balloon was actually increased after BoTox injection. The tendinitis (inflammation) hypothesis for chronic proctalgia was tested by steroid caudal block and by pelvic tender point injection of a mixture of Triamcinolone Acetonide and Lidocaine with negative results^[8,11]. Sacral nerve stimulation was also reported to be beneficial in an open study involving 27 chronic proctalgia patients. However, when benefits were assessed by intent to treat analysis, pain relief was reported in less than 50% of subjects^[21].

A major drawback in assessing the literature on chronic proctalgia treatment is the huge variation in inclusion criteria, outcome criteria, and follow-up intervals. Additional limitations are small sample sizes and lack of an appropriate control group. The few quasi-randomized studies had control groups that included subjects who received more than one treatment and patients not responding to a former therapy^[7]. To overcome these limitations, Chiarioni and coworkers recently reported a prospective, randomized controlled trial of 157 chronic proctalgia patients to investigate the comparative effectiveness of the 3 most commonly prescribed treatments: biofeedback to teach pelvic floor muscle relaxation, EGS, and digital massage of the levator muscles^[2]. A physiological assessment including manometry and balloon defecation was carried-out at baseline and at 1-3 mo follow-up. In addition, self-reported stool frequency was assessed at baseline and at 6-mo follow-up. The primary outcome was subjective reporting of adequate pain relief by the patient. Secondary outcomes included subjective pain improvement on an ordinal scale, number of days per month with rectal pain, and visual analog scale ratings of pain. According to Rome II criteria, proctalgia patients were subgrouped into highly likely LAS and possible LAS based on the presence or absence of levator tenderness at digital rectal exam, and randomization to treatment groups was stratified so that each treatment group contained a similar number of patients with a highly likely diagnosis of LAS.

At one-month follow-up, biofeedback was significantly more effective than EGS and massage by intent-to-treat analysis, with adequate relief of pain reported by 59.6% *vs* 32.7% *vs* 28.3% for biofeedback, EGS, and massage, respectively. Benefits were maintained throughout follow-up (12 mo) and no side effects were reported with any treatment. When results were further investigated in subgroups of patients, no treatment was effective in possible LAS patients (Rome III unspecified functional anorectal pain). However, among patients with highly likely LAS (Rome III levator ani syndrome) adequate relief was reported by 87% for biofeedback, 45% for EGS and 22% for massage at 1 mo follow-up. Improvements were maintained for the whole follow-up. The superiority of biofeedback was supported by all the secondary outcome measures including number of days per month with pain, which decreased from 14.7 per month to 3.3 per month for biofeedback, 8.9 for EGS, and 13.3 for massage^[2].

Physiological measurements revealed that the mechanism for achieving adequate pain relief was an improvement in pelvic floor function from being unable to relax anal canal pressures on straining to being able to do so and/or an improvement on the balloon evacuation test from being unable to pass a 50 mL balloon to being able to do so^[2]. This interpretation of the mechanism of action was confirmed by a post-hoc analysis showing that 94.2% of those who improved pelvic floor dysfunction on one or both of these measures reported adequate pain relief, while only 13.6% of those who did not improve pelvic floor function reported positive therapy outcome regardless of the treatment provided. In addition, stool frequency increased from baseline to post-treatment in responders, even in the absence of a former complaint of constipation. This study led us to conclude that biofeedback is an effective treatment for LAS, and EGS is somewhat effective. However, the minority of proctalgia patients affected by unspecified functional anorectal pain are still left without a satisfactory treatment option. In this regard, depression and anxiety are both frequently reported in non-responsive proctalgia patients^[6,10]. Brain processing of pain may be altered in functional gastrointestinal disorders, but data in proctalgia patients are lacking^[22]. In addition, no trial has actually evaluated the effect of either psychotherapy intervention or psychotropic drugs in proctalgia patients. Finally, there is no evidence that surgery can help these severely disabled patients. Invasive interventions should be avoided in the absence of a clearer etiologic understanding of non-responsive proctalgia patients^[3].

COCCYGDYNIA

Coccygodynia is defined as pain arising in or around the coccyx, usually triggered by prolonged sitting on hard surfaces^[23]. The pain is considered chronic when it lasts more than two months and it is commonly reported after repetitive trauma or childbirth^[23,24]. Coccygodynia may also be of idiopathic origin or secondary to lumbar

disc degeneration^[23-25]. It is also rarely reported as a complication of epidural injection of anesthetic or of various rectal and spine surgery^[25].

Pathophysiology

It is up to five times more common in women than in men, and obesity seems to be a predisposing factor due to the associated pelvic rotation^[23-25]. The female pelvic anatomy may also predispose to coccygodynia by leaving the coccyx more exposed to traumatic injury. The exact etiologic mechanism/s associated with coccygodynia are still obscure. Chronic spasm of the pelvic floor exerting a painful tension on a stiff coccyx has been traditionally considered a relevant etiologic factor, with accidental trauma acting as a trigger^[23-25]. However, instability of the coccyx potentially correlated with symptom severity was then discovered in a high percentage of patients by dedicated X-Ray examination^[26]. In addition, it is unclear whether pre-existing spine alterations play a role by predisposing patients to develop post-traumatic coccygodynia^[26,27]. Inflammation of structures (i.e., bursitis) in close proximity to the spine has also been described as a causative factor in a minority of patients complaining of coccygodynia^[27]. Depression and anxiety disorder have been reported to amplify coccygeal pain symptoms^[23]. Some authors do not diagnose coccygodynia when there is an ongoing medicolegal litigation, even if it occurs following a traumatic injury^[24,25].

Clinical presentation

Pain in the coccyx and in close anatomical regions (sacrum, perineum, anorectum) is the main reported symptom^[23]. Epidemiologic data on coccygodynia in the general population are lacking, but coccygodynia is considered to be a rare disorder. Retrospective data suggest that coccygodynia accounts for less than 1% of all reported cases of lower back pain^[24,25]. Diagnosis of coccygodynia relies heavily on history and clinical exam. Questioning the patient about previous trauma to the coccyx or childbirth trauma is a must, since according to Salvati the absence of a previous trauma makes the diagnosis unlikely^[9]. In addition, patients should report worsening of pain by prolonged sitting, bending, lifting or having a restricted poor posture for long intervals^[23-25]. Some patients may report that standing from a sitting position triggers the pain^[23].

Diagnostic assessment

Reproducing the usual pain by pressure or manipulation of the coccyx is key to diagnosis^[23]. Patients may also report mild tenderness on puborectalis posterior traction on digital rectal examination and a differential diagnosis of chronic proctalgia needs to be entertained^[9,28]. However, this maneuver should never be able to provoke the usual pain. Abnormal movement of the coccyx on palpation is an additional sign to confirm the clinical suspicion of coccygodynia^[25]. Dynamic X-Ray investigation may support the clinical diagnosis. The standard lateral

X-Ray investigation of the coccyx in the standing position should be supplemented with a second film taken while the patient is sitting on a hard surface possibly in a posture worsening the pain^[26]. More than 50% of patients would show features of coccyx instability (either exaggerated flexion or luxation) that seem to correlate with pain severity and previous traumatic events. An additional 15% of coccygodynia patients would show features of an abnormal bone spur at the end of the tailbone (so called spicule)^[26]. Spine magnetic resonance imaging (MRI) could be performed to exclude tumors or disc disease, but do not seem to add significantly to the diagnosis in coccygodynia^[25].

Treatment

The initial treatment of coccygodynia is focused on avoiding potentially offending factors and includes sitting on a donut-shaped pillow or a gel cushion to reduce pressure, posture ameliorating interventions, sitz bath and on demand nonsteroidal anti-inflammatory drugs^[23,24]. This treatment is commonly applied for 6-8 wk. No controlled study has investigated the therapeutic outcome of these simple measures. When initial treatment fails most authors recommend adding digital manipulation of the coccygeal ligaments as well as intrarectal manipulation of the pelvic floor muscles. Various massage and manipulation techniques have been described in open studies to decrease coccygeal pain in up to 85% of patients, particularly when combined with local steroid injection or physiotherapy^[29]. A recent prospective, randomized, controlled study aimed to compare intrarectal pelvic floor muscles manipulation (3 sessions) *vs* placebo physiotherapy (sacral short wave magnetic field applied at marginal power) in 102 chronic coccygodynia patients^[30]. Primary outcome was subjective decrement of more than 50% in pain intensity on a visual analog scale score at follow-up intervals of 1-6 mo. At 1 mo follow-up, 22% of patients in the manipulation group reported a significant pain decrement compared to only 12% of patients in the placebo group. Benefits persisted throughout follow-up in both groups. Manipulation was more effective in recent onset coccygodynia of post-traumatic origin not associated with instability of the coccyx. Psychosocial factors seemed to predict a poorer treatment outcome. The authors concluded that intrarectal manipulation is at least mildly effective in chronic coccygodynia and suggested either to increase the number of therapeutic sessions or to add local steroid injection to improve outcome. However, no randomized study has actually evaluated both treatment options for coccygodynia. In selected patients with severe and unresponsive coccygodynia, surgery may be considered^[31].

A recent review on surgical treatment of coccygodynia reported on 24 studies, but 22 of them were retrospective case series^[32]. Surgery was a treatment option in a minority of patients (approximately 19%), but mean satisfaction rate for pain relief was high (over 80% of treated patients). Some series reported a satisfactory out-

come of just 54% which was attributed to patient selection bias. Mean overall complication rate was 10.9% with wound infection being the most commonly reported complication. Surgeon expertise seemed to play a role since the smallest series reported the highest procedure-related complication rates (up to 50%). The type of surgery chosen was either total or partial removal of the coccyx and this did not seem to influence outcome. However, the worst outcomes were reported in patients with a history of rectal or spinal diseases and ongoing compensation issues^[32].

PUDENDAL NEURALGIA

Pathophysiology

Pudendal neuralgia is a chronic pain in the perineal area secondary to entrapment and injury to the pudendal nerve in its musculo-osteo-aponeurotic tunnel between the sacrotuberal and sacrospinal ligaments, in the absence of organic diseases that may explain this symptom^[23]. Pudendal neuralgia has been rarely described as secondary to herpetic neuropathy, stretch neuropathy, and post-radiotherapy neuropathy, but pudendal nerve entrapment is by far the most common etiology^[33,34]. Pudendal neuralgia is also called Alcock's canal syndrome, or pudendal canal syndrome^[23].

Clinical presentation

It is commonly described as a superficial pain, burning sensation, numbness, or paresthesia in the gluteal, perineal, and/or genital areas^[23]. It may be homolateral or bilateral, radiate to the pelvis and the thighs, and be associated with deep pelvic discomfort^[33,34]. Pain may be worsened by sexual intercourse and initially reported as sciatic pain^[53,34]. The epidemiology of pudendal neuralgia in the general population is unknown. The diagnosis is rarely considered except in highly focused Pelvic Floor Units or in specialized Urogynecologist practices. It is usually considered to be a rare entity, but it may be overdiagnosed due to the functional comorbidities associated with pudendal nerve dysfunction^[33,34]. Recently, a multidisciplinary Committee reported that pudendal neuralgia may be simply diagnosed by default in the presence of pelvic, perineal, and buttock pain without evidence of organic disease at diagnostic workup^[35]. Particularly controversial is its association with rectal pain, the presence of which requires differential diagnosis with chronic proctalgia^[23].

Diagnostic evaluation

Clinical neurophysiology has improved our knowledge of this disorder, but a definitive diagnostic test is still not available. As in many neuropathic pain syndromes, the diagnosis of pudendal neuralgia remains primarily clinical and should be reviewed in the light of the course of the disease. In 2006, a multidisciplinary working party on pudendal neuralgia held in Nantes, France, concluded that only the operative finding of nerve entrapment

and post-operative pain relief can formally confirm the diagnosis, provided the placebo effect of surgery is excluded^[35]. However, this panel of experts identified four domains of diagnostic criteria for pudendal neuralgia: (A) essential criteria, (B) complementary diagnostic criteria, (C) exclusion criteria, (D) associated signs not excluding the diagnosis. Essential criteria are particularly relevant and will be discussed in detail. (1) Pain should be limited to the innervation territory of the pudendal nerve. This excludes any pain that is limited to the coccygeal, pelvic or gluteal areas; (2) Pain is predominantly experienced while sitting, in accordance with the nerve compression etiology hypothesis. In long-standing pudendal neuralgia, pain may become continuous, but it is still worsened by the sitting position; (3) The pain rarely awakens the patient at night; (4) On clinical examination, no objective sensory impairment can be found even in the presence of paresthesia. The presence of a sensory defect should prompt investigations to exclude diseases of the sacral nerve roots and the cauda equina; and (5) Pain should be relieved by anesthetic infiltration of the pudendal nerve. This is an essential criterion, but it lacks specificity as pain related to any perineal disease may be relieved by pudendal nerve block. Moreover, a negative block does not exclude the diagnosis of pudendal neuralgia because it may have been performed inadequately (e.g., too distally). The complementary diagnostic criteria include the sensation of a rectal foreign body and the worsening of pain during defecation, both of which should prompt the physician to entertain the differential diagnosis of chronic proctalgia. Exclusion criteria for pudendal neuralgia are pain in a territory unrelated to the pudendal nerve, symptomatic pruritus instead of paresthesia, exclusively paroxysmal pain, and imaging abnormalities that could explain the symptom^[35].

Treatment

Pudendal neuralgia is treated by pudendal nerve block, which is both diagnostic and therapeutic. However, data on the long-term benefits of pudendal nerve block are lacking^[35]. In addition, only the operative demonstration of nerve entrapment and post-operative pain relief can formally confirm the diagnosis of pudendal neuralgia secondary to it, except for a potential placebo effect of surgery^[35].

CHRONIC PELVIC PAIN IN WOMEN

Chronic pelvic pain (CPP), which is diagnosed only in women, is commonly defined as noncyclic, nonmalignant pain in any organs related to the pelvis, in the absence of pregnancy and inflammatory bowel disease, that has lasted for at least six months^[36]. Pain occurring exclusively in association with menstruation (dysmenorrhea) and sexual intercourse (dyspareunia) are generally not considered to be CPP, but general agreement is lacking. Other definitions include a pain severity sufficient to cause functional disability or to require medical care^[36]. Since

the definition of CPP varies, it is difficult to ascertain its exact prevalence. However, the prevalence of CPP in the general population assessed by mail questionnaires among women aged 18-50 has been reported to be as high as 15% in the United States and 24% in the United Kingdom^[37,38]. CPP has been estimated to account for 10% of all outpatient referrals to gynecologists and 40% of diagnostic laparoscopies, so it constitutes a significant economic burden^[39,40]. No organic disease is found on laparoscopy in at least a third of women with CPP^[40]. In the community, 32% of patients who consult for this symptom report high rates of anxiety and quality of life impairment as measured by the SF-36^[37,38]. Consulting behavior is directly influenced by the severity of pain^[39].

Pathophysiology

The etiology of CPP is considered to be complex and multifactorial^[36,40]. Some gynecological diseases such as endometriosis, pelvic inflammatory disease, and interstitial cystitis may cause CPP, but gastrointestinal comorbidities are also reported in up to 1/3 of CPP patients in primary care^[38,40]. A psychosomatic component of pain has also been hypothesized^[36,40]. The common association of CPP with irritable bowel syndrome has led some to question whether these two diseases are actually a single clinical entity that is diagnosed differently according to the specialist consulted^[41]. The etiology of CPP is poorly understood.

Diagnostic assessment

Initial evaluation should include a history and physical examination to narrow the differential diagnosis^[40]. When this examination does not identify another explanation for the pain, limited laboratory testing and transvaginal ultrasound scanning is often employed to rule out organic disease and reassure the patient^[40]. The laboratory workup should include: complete blood count, beta human chorionic gonadotropin level, erythrocyte sedimentation rate, vaginal swabs for Chlamydia and Gonorrhea, and urinalysis with urine and culture^[40]. Additional magnetic resonance imaging should be considered when in doubt for organic disease and diagnostic laparoscopy may be eventually performed in selected cases^[40]. A tense pelvic floor is often reported during vaginal examination in CPP and spasm of the pelvic floor muscles is considered a relevant etiologic factor^[36]. In addition, up to 60% of patients may report symptoms of either voiding dysfunction or dyschezia^[42].

Treatment

Physiotherapy to relax the pelvic floor is often prescribed as first-line treatment for CPP, but randomized, controlled trials to confirm its effectiveness are lacking. Vaginal electrical stimulation was retrospectively reported to decrease pain in 52% of 66 chronic pelvic pain patients when coexistent levator ani spasm was also diagnosed by clinical exam^[43]. Benefits were generally sustained during a 30-wk follow-up. Nonetheless, few studies have evalu-

ated pelvic floor function in women with CPP. Abbott and coworkers reported pelvic floor myalgia in 68 out of 118 patients referred for long-standing, unresponsive CPP^[44]. They diagnosed pelvic floor myalgia based on objective evidence of contracted, painful pelvic muscles on palpation and elevated resting intraluminal pressures as measured by vaginal manometry. This study was a double blind, placebo controlled trial to test the efficacy of BoTox A in patients who had CPP with pelvic floor spasm. BoTox injection was associated with a significant reduction in vaginal resting pressure compared to placebo, but pain was only partially relieved and was not significantly different between BoTox and placebo. The authors concluded that pelvic floor spasm can cause CPP and that improvement in some symptoms occurs following reductions in muscle spasm^[44]. In open studies, a number of treatment modalities have been reported to be effective for decreasing symptoms in CPP. These options include either oral or intramuscular hormone therapy, levator ani trigger point steroid injections, and sacral neuromodulation^[36,40,42,45]. Most studies are open, retrospective, single center experiences with poor generalizability of treatment outcome.

Tricyclic antidepressants and Sertraline seem to work no better than placebo in CPP^[40]. Surgery should be limited to patients with an organic cause for pelvic pain^[36,40]. In non-responsive, severely disabled patients a multidisciplinary approach is advocated to fit with a biopsychosocial model of pain^[40].

CONCLUSION

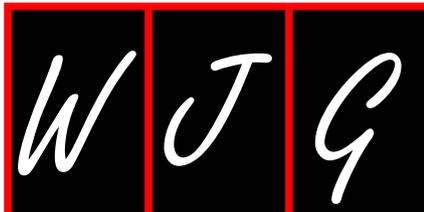
Chronic anorectal and pelvic pain syndromes receive little research attention despite the fact that they are prevalent, often disabling pain syndromes which are associated with significant health care costs and quality of life impact. Their frequency in the general population may be as high as 24% for chronic pelvic pain in women and 6.6% for chronic proctalgia. It is common for these patients to be referred to multiple specialists. Etiology is poorly defined, but chronic tension (spasm) in the striated muscles of the pelvic floor is often considered to be the pathophysiological mechanism for most of them. A recent randomized, controlled trial provided evidence that dyssynergic defecation (i.e., paradoxical contraction or failure to relax the pelvic floor muscles when straining to defecate) is the primary cause of pain for the majority of patients with chronic proctalgia, even for patients who do not complain of constipation. Biofeedback to treat dyssynergic defecation was an effective treatment for the subset of patients with chronic proctalgia who reported tenderness when traction was applied to the levator ani muscles during digital rectal exam (a sign of excessive tension in these muscles). This finding should prompt researchers to look for features of dyssynergic defecation in other pelvic pain syndromes and to try a similar treatment. A multidisciplinary and tailored approach to treat anorectal and pelvic pain patients without pelvic floor dysfunction is strongly suggested.

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S- Editor Tian L L- Editor Webster JR E- Editor Zhang DN



Multiphoton microscopy: An introduction to gastroenterologists

Hye Jin Cho, Hoon Jai Chun, Eun Sun Kim, Bong Rae Cho

Hye Jin Cho, Hoon Jai Chun, Eun Sun Kim, Institute of Digestive Disease and Nutrition, Department of Internal Medicine, Korea University College of Medicine, 136-705 Seoul, South Korea
Bong Rae Cho, Department of Chemistry, Korea University, 136-705 Seoul, South Korea

Author contributions: Cho HJ, Chun HJ, Kim ES designed research and performed research; Cho BR contributed new reagents; Cho HJ analyzed data and wrote the paper.

Correspondence to: Hoon Jai Chun, MD, PhD, AGAF, Professor, Institute of Digestive Disease and Nutrition, Department of Internal Medicine, Korea University College of Medicine, 136-705 Seoul, South Korea. drchunhj@chol.com

Telephone: +82-2-9206555 Fax: +82-2-9531943

Received: November 24, 2010 Revised: March 2, 2011

Accepted: March 9, 2011

Published online: October 28, 2011

Abstract

Multiphoton microscopy, relying on the simultaneous absorption of two or more photons by a fluorophore, has come to occupy a prominent place in modern biomedical research with its ability to allow real-time observation of a single cell and molecules in intact tissues. Multiphoton microscopy exhibits nonlinear optical contrast properties, which can make it possible to provide an exceptionally large depth penetration with less phototoxicity. This system becomes more and more an inspiring tool for a non-invasive imaging system to realize "optical biopsy" and to examine the functions of living cells. In this review, we briefly present the physical principles and properties of multiphoton microscopy as well as the current applications in biological fields. In addition, we address what we see as the future potential of multiphoton microscopy for gastroenterologic research.

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Key words: Multiphoton microscopy; Optical biopsy; Gastrointestinal disease

Peer reviewer: Kevin J Spring, Dr., PhD, Conjoint Gastroenterology Laboratory, The Queensland Institute of Medical Research, the Bancroft Centre, Rm H07, PO Royal Brisbane Hospital, Herston, QLD 4029, Australia

Cho HJ, Chun HJ, Kim ES, Cho BR. Multiphoton microscopy: An introduction to gastroenterologists. *World J Gastroenterol* 2011; 17(40): 4456-4460 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4456.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4456>

INTRODUCTION

A remarkable evolution of biological science has induced the need to visualize cells in an intact whole organism. To date, most applications using microscopy are limited to fixed cells or excised tissues. However, characterization of morphological features and establishment of cell function of native tissues is important for the diagnosis of disease in the early stage and has improved understanding of the associated pathophysiological processes. Therefore, the need for real-time observation of cells and their subcellular components in intact tissues is of great interest and imaging techniques have been developed to pursue this goal.

One of these techniques is fluorescence imaging. Although the combination of microscopy with fluorescent labeling has improved sensitivity, this approach provides only a two-dimensional view of samples. The use of confocal microscopy allows for the observation of subcellular material with three-dimensional resolution. However, confocal microscopy is limited by the effective imaging depth of typically less than 100 μm and phototoxicity, which is caused by using a short wavelength laser^[1].

Recent advances in nonlinear optical processes of multiphoton microscopy compensate single photon-linear microscopy technologies such as confocal microscopy by the capacity for deeper tissue penetration with

clear images and the reduction of direct ultraviolet damage^[2]. Thus, multiphoton microscopy has been applied to various parts of the imaging task and has now become the technique of choice for subcellular observations of thick tissues and in living animals^[3].

In addition, endoscopists often want to know the relationship between the gross endoscopic findings and the microscopic diagnosis during routine endoscopy. Although a mucosal biopsy is the standard method for histopathological diagnosis of an abnormal mucosal lesion, this approach is limited by sampling error, bleeding risk and the time lag for results. Therefore, endoscopists would like to have the ability to directly observe and promptly identify pathology of cellular and/or subcellular structures without biopsy. Multiphoton microscopy has the full potential to achieve this goal because it can provide thin optical sections from thick specimens.

In this article, the principles of multiphoton microscopy and its applications in bioscience are reviewed, as well as the prospects for clinical use.

MULTIPHOTON MICROSCOPY

Early in the development of quantum mechanics, the theoretical concept was first proposed by Göppert-Mayer in 1931. Multiphoton excitation is based on the probability that fluorophore molecules are excited by multiple low energy photons that can arrive “simultaneously” at the fluorophore and interact with it. The fluorophore molecule absorbs the sum of the energy from each photon, and an electron in the fluorophore is transferred to the excited state, which can induce an electronic transition similar to a single high-energy photon^[4]. Soon after, the molecule in the excited state falls back to the ground state with emission of fluorescence, which has most, but not all, of the initial energy, owing to non-radiative relaxation (Figure 1).

Because the energy of a photon is inversely proportional to its wavelength (λ), the emitted fluorescence is a longer wavelength than the exciting light. However, in the case of multiphoton excitation, the fluorophore molecule almost simultaneously absorbs the energy from multiple photons, each of which contributes a part of the total energy required to induce the fluorescent emission. Thus, the emitted fluorescent photon has a shorter wavelength than each of the photons involved in excitation. For this reason, multiphoton microscopy can induce fluorescence equal to the energy of single photon excitation microscopy by low energy photons.

However, multiphoton excitation requires enormously high light intensities that, if continuous, would almost instantly vaporize the specimen. Therefore, to generate enough fluorescence practical for multi-photon microscopy, a pulsed laser source is needed. In other words, using a laser that produces extremely brief pulses (femtosecond laser, about 10^{-5}) at a high repetition rate, thus generating high instantaneous energy but low average energy^[5].

Multiphoton microscopy exhibits nonlinear optical contrast properties that are predicated upon second

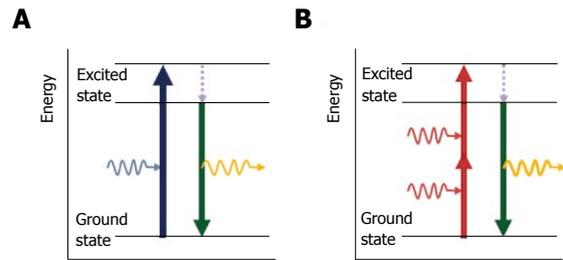


Figure 1 Multiphoton excitation. A: Single-photon excitation. Individual photons of high-energy blue light (wavelength, $\lambda = 480$ nm) excite fluorophores in the sample. After an electron in the fluorophore is transferred to the excited state (blue arrow), it loses energy rapidly owing to non-radiative relaxation (dashed arrow). Subsequently, fluorescence emission (yellow curved arrow) occurs at a longer wavelength than the excitation light as the electron falls back to the ground state (green arrow); B: Two-photon excitation. Two infrared photons ($\lambda = 780$ nm) are absorbed simultaneously (red arrows) to excite the fluorophore and light is emitted in the same manner as for single-photon excitation (green arrow) with emission of fluorescein.

and third-order nonlinear interactions between light and particles^[6]. The nonlinear optical effects are proportional to the square or cube of the fundamental light intensity; this gives multiphoton microscopy the intrinsic characteristics of 3-dimensional images. This is because the photon density is high at the focal point, and it falls off steeply from the focal point^[7]. This eliminates out-of-focus contributions and allows multiphoton microscopy to obtain high resolution images from the scattered photons of the fluorophore emission used to produce the image. In addition, photobleaching is restricted to a narrow region around the plane of focus.

To date, the most widely used imaging modalities associated with multiphoton microscopy are multiphoton excitation with fluorescence, second harmonic generation, multiphoton fluorescence lifetime imaging microscopy, and spectral lifetime imaging microscopy^[1]. For example, with two-photon microscopy, a fluorophore molecule is excited by the nearly simultaneous absorption of two photons, each twice the wavelength required for a single photon excitation^[8]. A molecule of fluorescein can be excited by two photons of near-infrared light ($\lambda \approx 780$ nm), each of which has approximately half the energy of a single blue photon ($\lambda \approx 480$ nm), and then emit a photon of green light, in the same manner as for standard (one-photon) excitation with blue light^[7]. According to nonlinear excitation, fluorescent emission from fluorophore molecules is proportional to the square of the excitation intensity. This intensity-squared dependence of two-photon microscopy provides “optical sectioning” capability, without using an adjustable pinhole aperture in front of the detector to reject out-of-focus fluorescence like confocal microscopy^[9].

Multiphoton microscopy has several advantages over confocal microscopy. Most of all, the use of long excitation wavelengths has major advantages. Since light scattering declines rapidly with an increasing wavelength, deeper penetration can be achieved by using a longer wavelength of light than with single-photon confocal microscopy. In addition to an increase in the penetration

Table 1 Comparison of two-photon microscopy with confocal microscopy

	Confocal microscopy	Two-photon microscopy
Excitation wavelength	Short (ultraviolet light)	Long (infra-red light)
Tissue imaging depth	About 50-100 μm	About 400-1000 μm
Spatial resolution	nm (3D-resolution with pinhole aperture)	nm (3D-resolution with inherent optical sectioning)
Photodamage and photobleaching	High	Low

depth, a longer wavelength of light, such as infrared light (700-1000 nm) used for multiphoton microscopy, has much less energy than confocal microscopy, and therefore causes negligible photodamage and phototoxicity to cells and tissues. Cells and molecules deep inside living tissues can be observed for long periods of time. Moreover, because excitation and emission take place only at the focal plane, multiphoton microscopy reduces the photobleaching outside of the focal plane, unlike confocal microscopy. Therefore, it results in high fluorescence collection efficiency and thus greater signal intensity at any given tissue depth.

Table 1 details comparative differences in excitation wavelength, tissue imaging depth, resolution and photo damage/bleaching between two-photon microscopy and confocal microscopy.

BIOLOGICAL APPLICATIONS

Multiphoton microscopy is a powerful tool for visualizing cellular and subcellular events within living tissue with its inherent “optical sectioning” capability, deeper penetration and minimal phototoxicity and photobleaching. Multiphoton microscopy can capture whole organisms or embryos on a large scale. Though transparent organisms such as the zebra fish and drosophila are ideal candidates for such studies, the development of the hamster embryo model has allowed for observations over long periods of time, for several days^[10].

In addition to morphological studies, multiphoton microscopy can be used for dynamic and functional cellular imaging with the development of various fluorescent probes. For example, two-photon microscopy of the calcium sensitive fluorophore allows for the collection of subcellular spatial and temporal information on (Ca^{2+}) ion entry through voltage-gated channels or release from intracellular stores within a single myocyte at depths of up to 200 μm below the epicardial surface. Therefore, two-photon microscopy is well suited to determine the functional state of donor cells following intracardiac transplantation^[11].

Neuroscientists use multiphoton microscopy for the observation of neuronal plastic changes within brain slices, measuring ionized-calcium dynamics deep in brain tissues^[12]. The dendritic spines, which are a major functional component of the nervous system associated with learning and memory activated by chemical and electrical

transmission mechanisms, are very tiny structures. Since neurons are very sensitive to phototoxicity and brain tissue is highly scattered, it has been difficult to visualize these dynamic processes in live tissues^[13,14]. However, multiphoton microscopy overcomes these obstacles by using long wavelength light and providing high resolution deep imaging without causing injury to the living material^[15]. It allows visualization of fine structures of the brain in the head and neck area, including unique signaling and dynamic motility of the dendritic spines 300-400 μm into the brain tissue^[16,17].

Multiphoton microscopy enables imaging of dynamic and heterogeneous immune processes at the cellular and molecular levels deep within intact organs of living animals. Due to the depth of penetration and minimal photodamage, multiphoton microscopy permits six-dimensional (x, y, z, time, intensity, wavelength) imaging of intact lymphoid organs and can be used to observe naïve lymphocytes for hours without loss of viability or motility^[18]. Dynamic movements and cellular interactions of viable T- and B-cells can be revealed, as well as the antigen presenting cells in the *in vivo* setting^[7,19].

Multiphoton microscopy is also a preferred imaging technique for cancer research, for example in studies on angiogenesis and metastasis *in vivo*^[20,21]. Tumor micro-invasion and metastasis involves complex interactions between cells and extracellular matrix proteins, most notably collagen^[22]. Due to the ability of imaging more deeply in tissues with less toxicity, multiphoton microscopy facilitates imaging of tumor-stroma interactions and thus facilitates improved understanding of the processes of cell migration, metastasis, and tumor progression with direct observation *in vivo*^[23].

Gastrointestinal endoscopists have to rely on visual inspection for the diagnosis of disease. Therefore, multiphoton excitation imaging may be helpful in the diagnosis and offer additional diagnostic benefit. Indeed, a pilot study of multiphoton microscopy to diagnose gastric cancer has been reported recently^[24]. The results of the study showed that multiphoton microscopy can be used to diagnose gastric cancer by optical biopsy. Multiphoton microscopy has proved to be a promising tool for real-time histological diagnosis. Recent developments in imaging technology now make this possible.

Multiphoton microscopy also has the ability to penetrate deeper inside the tissue and excite endogenous autofluorescence molecules such as intracellular nicotinamide adenine dinucleotide phosphate (reduced form), flavin, melanin and lipofuscin, instead of using fluorescent dyes which must be used for *in vivo* confocal laser microscopy^[25]. It provides the ability to detect cellular and subcellular details of the gastrointestinal mucosa without fixation or staining. Multiphoton imaging of intact human gastrointestinal mucosa *ex vivo* provides improved cellular detail compared to confocal imaging, without the need for fluorescent dyes^[2].

Suitable indicators for two-photon microscopy are required in order to get a clearer image. Recently, our col-

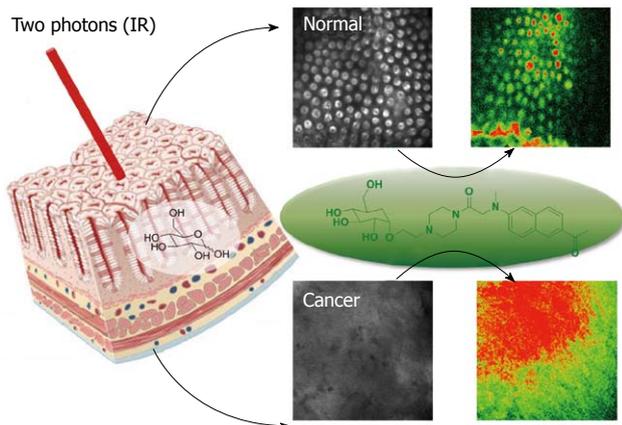


Figure 2 Images of normal tissue (above) and cancer tissue (below) treated with AG2. Normal tissues were incubated in artificial cerebrospinal fluid (ACSF) for 4 h, and cancer tissues were incubated in ACSF for 4 h, after which AG2 uptake was monitored. Right-side images are bright-field images, left-side images are pseudocolored two-photon microscopy (TPM) images obtained after incubation with AG2 for 4 h. The TPM images were obtained at a depth of 100 μm by collecting the two-photon excited fluorescence spectra in the range of 520-620 nm on excitation with fs pulses at 780 nm. IR: Infrared.

laborators have developed many new two-photon tracers. One tracer, a hydrogen probe, AH2, which emits fluorescence at $\text{pH} < 4$ can be used to obtain images of live esophageal tissue from the mucosal surface to 100 μm in depth. Emitted fluorescence of the hydrogen probe in reflux esophagitis tissue was stronger than that in control tissue. Multiphoton-emitted fluorescence of low esophageal tissue of the reflux model was similar to that of stomach^[26]. Visible images of pH changes in reflux esophageal tissue can be obtained by use of the multiphoton hydrogen probe.

Another new probe, AG2, that can be easily taken up by cancer cells and tissues through glucose-specific translocation has been developed. AG2 shows negligible cytotoxicity and high photostability. It can monitor glucose uptake in colon cancer tissues and visualize at depth of 75-150 μm by two-photon microscopy (Figure 2). This compound may be useful in diagnosing the early stages of cancer and make it possible to develop customized cancer therapy according to the uptake rates of AG2 in normal and cancerous tissues (Figure 3). In addition, this laboratory has used multiphoton laser scanning microscopy to study gastric and colon cancer with other probes. Multiphoton images of normal and cancer cell lines, as well as normal mucosa and dysplastic tissues, (adenoma, adenocarcinoma) labeled with the multiphoton microscopy probes AZn1 and ACu1, have been studied. The findings showed that the Cu1 content was higher, Zn1 content was lower, and the ratio of Cu1 to Zn1 was much higher in adenomas and adenocarcinoma than in the normal mucosa. These results suggest the possibility that multiphoton endomicroscopy might be developed further to use as a technique for performing virtual biopsies during the course of routine endoscopy.

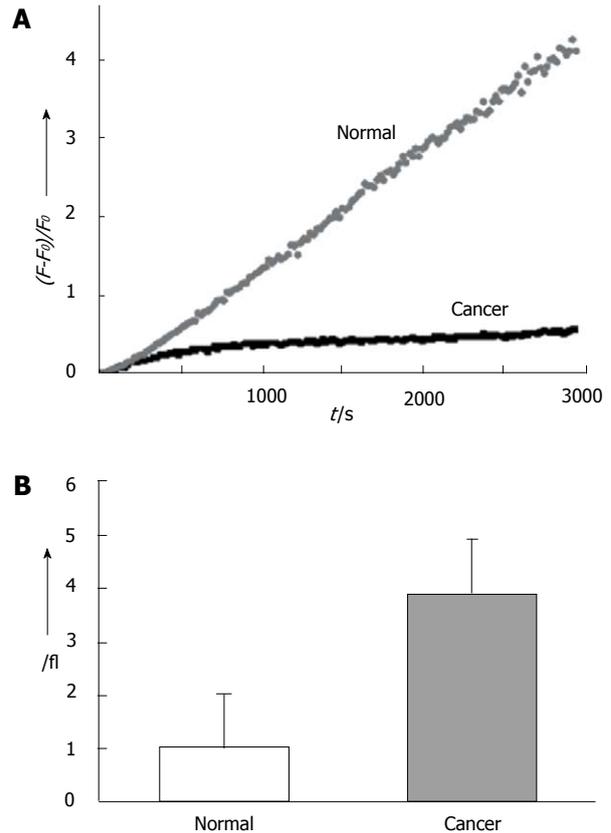


Figure 3 AG2 uptake in normal and cancerous tissues. A: Time course of AG2 uptake by normal tissue and cancer tissue at 100 μm depth as a function of time; B: Relative AG2 uptake by normal tissue and cancer tissue for 4000 s. The columns indicate the sum of the two-photon excited fluorescence intensities measured by photomultiplier tube at depths of 75, 100, 125 and 150 μm from the tissue surface, relative to that of normal tissue. The data are the average of three independent experiments.

CONCLUSION

Multiphoton microscopy has rapidly evolved and become a standard device for cell-based biological research in the fields of genomics, proteomics and tissue engineering. A major advantage of multiphoton microscopy is the ability to observe deep within intact organs and cells. Its applications are being extended beyond basic research to the clinical setting, such as detection of skin cancers, mucosal dysplasia of the intestinal tract, Alzheimer's disease, and metabolic disorders just by visualizing patient's tissue at the cellular level of resolution^[27].

Although multiphoton microscopy has already been used by many biologists for research and some clinicians, as mentioned briefly above, its advantages are partly limited by the bulkiness of the system including lasers, objective lenses, and scanning devices. Therefore, several groups currently are trying to develop smaller fluorescence microscopes, either by using a gradient index lens as a thin, rodlike probe to extend the working distance of a conventional objective^[15] or by using fiber optics to construct multiphoton endoscopes^[28]. Imaging of goblet cells as a marker for intestinal metaplasia of the stomach

by two-photon endomicroscopy has been reported^[29]. Its techniques can three-dimensionally observe goblet cells in mouse large intestine, and it provides the possibility that two photon endomicroscopy is advantageous in diagnoses.

The development of miniature laser scanning multiphoton endoscopes will provide advantages over currently available endomicroscopy technologies and be of great utility to gastroenterologists. Moreover, miniature multiphoton endoscopy may be used for minimally invasive endoscopic procedures and has enormous potential for histological evaluation of organs outside the gastrointestinal tract, namely, the liver, pancreas, and ovaries by transluminal endoscopic approaches^[30].

With the development of novel laser sources, new fluorophores and more specific probes, multiphoton microscopy and its applications will open up a wide range of possibilities. In addition, it can be combined with other imaging modalities such as ultrasound or magnetic resonance imaging, which provide complementary information.

The development of multiphoton microscopy marks a significant step in the advancement of imaging modalities and will likely aid in our understanding of the basis of disease as well as the management of the clinical manifestations of disease.

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S- Editor Tian L L- Editor O'Neill M E- Editor Li JY

Can intraluminal devices prevent or reduce colorectal anastomotic leakage: A review

Annelien N Morks, Klaas Havenga, Rutger J Ploeg

Annelien N Morks, Klaas Havenga, Rutger J Ploeg, Division of Abdominal Surgery, Department of Surgery, University Medical Center Groningen, 9700 RB Groningen, The Netherlands

Author contributions: Morks AN wrote the paper; Havenga K and Ploeg RJ both substantially contributed to the design of the review, revised the review, and approved the final version.

Correspondence to: Klaas Havenga, MD, PhD, Division of Abdominal Surgery, Department of Surgery, University Medical Center Groningen, BA 11, Hanzeplein 1, 9700 RB Groningen, The Netherlands. k.havenga@umcg.nl

Telephone: +31-50-3612283 Fax: +31-50-3614873

Received: January 3, 2011 Revised: February 18, 2011

Accepted: February 25, 2011

Published online: October 28, 2011

Key words: Anastomotic leakage; Colorectal surgery; Complication; Rectum; Device

Peer reviewer: Omar Vergara-Fernandez, MD, Departments of Surgery, National Institute for Medical Sciences and Nutrition Salvador Zubirán, Vasco de Quiroga No. 15, Col Seccion XVI Deleg Tlalpan, CP 14000, México

Morks AN, Havenga K, Ploeg RJ. Can intraluminal devices prevent or reduce colorectal anastomotic leakage: A review. *World J Gastroenterol* 2011; 17(40): 4461-4469 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4461.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4461>

Abstract

Colorectal anastomotic leakage is a serious complication of colorectal surgery, leading to high morbidity and mortality rates. In recent decades, many strategies aimed at lowering the incidence of anastomotic leakage have been examined. The focus of this review will be on mechanical aids protecting the colonic anastomosis against leakage. A literature search was performed using MEDLINE, EMBASE, and The Cochrane Collaborative library for all papers related to prevention of anastomotic leakage by placement of a device in the colon. Devices were categorised as decompression devices, intracolonic devices, and biodegradable devices. A decompression device functions by keeping the anal sphincter open, thereby lowering the intraluminal pressure and lowering the pressure on the anastomosis. Intracolonic devices do not prevent the formation of dehiscence. However, they prevent the faecal load from contacting the anastomotic site, thereby preventing leakage of faeces into the peritoneal cavity. Many attempts have been made to find a device that decreases the incidence of AL; however, to date, none of the devices have been widely accepted.

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INTRODUCTION

Colorectal anastomotic leakage (AL) is a serious complication after colorectal surgery and may lead to high morbidity and mortality rates. The incidence of AL varies between 2.5% and 20% and the aetiology is multifactorial^[1-5].

In recent decades, many strategies aimed at lowering the incidence of anastomotic leakage have been examined. A protective stoma reduces the consequence of anastomotic failure, thereby preventing the number of clinical leaks^[6,7]. The Dutch TME trial demonstrated a lower rate of surgical re-intervention in patients with a diverting stoma ($P < 0.001$)^[2]. However, a protective stoma can also result in stoma-related complications, and the obligatory operation to take down the stoma is associated with additional mortality, morbidity, and cost^[8]. In addition, so-called temporary protective stomas tend to be left *in situ* for much longer than initially anticipated, sometimes even lifelong^[9].

Many definitions are used to describe anastomotic leakage. The Surgical Infection Study Group (1991) categorised AL into clinical and subclinical leakage^[10]. In 2001, Bruce *et al*^[11] recommended the subdivision of anastomotic leakage into three groups: radiological (no

clinical signs), clinical minor (no intervention needed), and clinical major (intervention required) leakage. This grading of AL resembles the grading of AL proposed by the International Study Group of Rectal Cancer (IS-REC)^[12]. The ISREC defines AL as a communication between the intra- and extraluminal compartments due to a defect of the integrity of the intestinal wall at the anastomosis between the colon and rectum or the colon and anus. The extent or severity of AL should be graded according to the impact on clinical management. Grade A does not require active therapeutic intervention; grade B requires active therapeutic intervention, but is manageable without relaparotomy; and grade C requires relaparotomy.

In this review, we focus on the use and potential success of mechanical intraluminal devices that may protect a colonic anastomosis against leakage. Different strategies have been adopted to lower the incidence of AL. In this respect we will differentiate between transanal decompression, intracolonic, and biodegradable devices. In addition, devices encircling the bowel have been tested. The use of some of these devices showed promising results in lowering the incidence of AL (Table 1).

Considering the persisting associated morbidity and mortality of AL, and availability of intraluminal colonic devices today, a revival of the discussion of their effectiveness in lowering the incidence of AL is worthwhile.

LITERATURE SEARCH

This is a retrospective review describing the literature on devices protecting colonic anastomoses. In March 2010, an extensive literature search was performed using MEDLINE, EMBASE, and The Cochrane Collaborative Library for all papers related to prevention of anastomotic leakage by placement of an intraluminal device in the colon. Our search comprised the following: (tube OR tubes OR bypass* OR by-pass OR stent* OR device* OR coloshield) AND (anastomosis OR anastomo*) AND (leak* OR dehiscen*) AND (colon OR rectum OR colonic OR intracolonic OR colorectal) AND (prevent* OR protect*). Articles were marked as relevant if an intraluminal device was studied that protected a colonic anastomosis from leakage. Articles describing glues or fluids that protect the anastomosis are not included in this review, neither are studies on techniques of anastomosing the bowel. The reference list of each relevant article was checked for further relevant papers. All first authors of relevant papers were checked for other relevant publications. All articles were selected by one reviewer and in case of doubt, a second reviewer was consulted. The Internet was also searched using www.scholar.google.com. The search yielded 337 articles of which 44 were related to an intraluminal device intended to protect a colonic anastomosis. These 44 articles include experimental animal studies, as well as retrospective and prospective clinical studies.

TRANSANAL DECOMPRESSION DEVICES

A decompression device functions by keeping the anal sphincter open, thereby decreasing the intraluminal pressure, as well as the pressure on the anastomosis. In this way, the device serves as a protective vent. In addition, a number of authors have hypothesized that some tubes permit reinforcement and prevent angulation of the bowel and anastomosis. As early as in the 13th century, Lanfrank reported the placement of a reed pipe as an intraluminal stent in the colon^[13]. More recently, Gurjar^[14] assessed the current practice of rectal tubes in the United Kingdom and Ireland. A questionnaire was sent to all members of the Association of Coloproctology (ACP-GBI). The response rate was 58%, and 35% of those reported to use a rectal tube, in the majority of cases after ileo-anal or colonic pouch surgery. Sixteen percent used the tube after low anterior resection (LAR). Predominantly, a Foley catheter was positioned above the anastomosis (80%). The catheter was left *in situ* for a median of five days. Most respondents used the tube with the intention to decompress the rectum and/or pouch.

Animal studies

In 1988, Goldman *et al*^[15] tested an intrarectal, conically shaped flexible silastic tube in a dog model of LAR. The tube was fixed to the submucosa 5 cm proximal to the anastomosis. Twenty-five dogs underwent an LAR; 15 with a tube and 10 controls. In some animals, the anastomosis was deliberately made incomplete, leaving gaps. Mortality occurred only in the control group. Morbidity in the control group was six times higher (three colocolic fistulae and three anastomotic abscesses). Only one dog with a tube and an incomplete anastomosis was diagnosed with a pelvic abscess. The tubal fixation sites showed oedema and minor inflammatory reaction on microscopic examination. The authors concluded that their procedure presented effective practical implications, such as omitting the need for a proximal protective colostomy.

Human studies

Indwelling rectal tubes: Stewart^[16] used an indwelling rectal tube in 153 patients who underwent a left hemicolectomy or sigmoid resection. After completion of the anastomosis, a No. 32-34 French latex tube was introduced through the anal canal and directed through the anastomosis to a distance of approximately 15 centimeters above the anastomosis. The rectal tube was sutured to the perianal skin for fixation. Twice daily, the tube was irrigated with neomycin solution and after five or six days, the tube was removed. Suture-line complications occurred in seven patients (4.6%), with three patients being graded as C according to the ISREC classification. In the other four patients, the anastomotic complications were grade A/B (haematoma, stricture, and abscess noted only on sigmoidoscopy). Adverse effects of the tube (e.g., ulceration of the colon) were not observed.

Table 1 Studies on intracolonic devices aimed at preventing anastomotic leakage

Study ^[Ref.]	Yr	n	Site	Device	Anastomotic complications
Rack ^[17]	1966	32	Sigmoid or rectum resection	Rectal tube	0 AL
Stewart <i>et al</i> ^[16]	1968	153	Left colon or colorectal resection	Rectal tube	4 Grade A/B AL (3%) 3 Grade C AL (2%)
Balz <i>et al</i> ^[18]	1978	392 (including 153 patients from study Stewart)	Left colon or colorectal resection	Rectal tube	3 Grade A/B AL (1%) 6 Grade C AL (2%)
Castrini <i>et al</i> ^[33]	1984	19	Left colon or rectal resection	Intracolonic bypass	0 AL
Ravo <i>et al</i> ^[35]	1987	28	Sigmoid resection	Intracolonic bypass	0 AL
Cuilleret <i>et al</i> ^[53]	1991	14	Left colon resection	Intracolonic bypass	0 AL
Ravo <i>et al</i> ^[34]	1985	29	Left colon or rectal resection	Intracolonic bypass	0 AL
Ravo ^[29]	1988	Case report	Sigmoid resection	Intracolonic bypass	0 AL
Keane <i>et al</i> ^[37]	1988	6	Sigmoid or rectal resection	Intracolonic bypass	0 AL
Rosati <i>et al</i> ^[36]	1992	29	Left colon or rectal resection	Intracolonic bypass	2 AL (7%)
Egozi <i>et al</i> ^[38]	1993	Case report	Sigmoid	Intracolonic bypass	Colon necrosis at site of tube
Yoon <i>et al</i> ^[44]	1994	10	LAR	Condom	0 AL
Sterk <i>et al</i> ^[19]	2001	50	LAR	Transanal tube	3 Grade A AL (6%) 2 Grade C AL (4%)
Amin ¹ <i>et al</i> ^[20]	2003	76	LAR	41 transanal stent 35 loop stoma	Stent: 3 Grade C AL (7%) Stoma: 1 Grade A AL (3%) 1 Grade C AL (3%)
Bülow ² <i>et al</i> ^[21]	2006	194	LAR	98 Transanal stent 96 controls	Stent: 17 AL (17%) Control: 8 AL (8%)
Ye ³ <i>et al</i> ^[47]	2008	83	LAR	44 VIB 39 Loop ileostomy	VIB: 2 Grade A AL (5%) Stoma: 2 Grade A AL (5%)
Kolkert <i>et al</i> ^[48]	2010	15	Sigmoid or rectal resection	C-seal	0 AL

¹Randomized trial; ²Randomized trial, with/without stent and with/without ostomy; ³Patient could choose between VIB and LI. AL: Anastomotic leakage, according to the ISREC classification^[12]; LAR: Low anterior resection; VIB: Valtrac-secured intracolonic bypass; LI: Loop ileostomy; ISREC: International Study Group of Rectal Cancer.



Figure 1 Transanal stent.

In 1978, Balz *et al*^[18] reviewed a series of 392 patients undergoing anterior resection with placement of an indwelling rectal tube. Anastomotic complications occurred in 3.8%. In addition to decompression, the rectal tube facilitated intraluminal antibiotic irrigation of the anastomosis.

Transanal rubber drain: In 2001, Sterk *et al*^[19] used a transanal rubber drain to protect the anastomosis after low anterior resection in 50 patients. The maximal distance between the anastomosis and the anal skin was 7 cm. The transanal rubber drain had openings on the side, a length of 40 cm, and a diameter of 12-15 mm. The tip of the drain was positioned about 10 cm proximal to the anastomosis; the other end was fixed to the

perineal skin. Two patients (4%) developed a grade C AL and three patients (6%) a grade A AL. The authors concluded that the transanal drain was at least equivalent to a conventional colostomy to reduce symptomatic AL.

Human trials

Transanal stent: The transanal stent (TAS) is a radio-opaque soft silicone tube, 4 cm in length with funnel-shaped flanges. It is inserted into the anal canal at the end of the procedure, and is left *in situ* for 5-7 d (Figure 1). Amin *et al*^[20] performed a randomised trial with the TAS in LAR for rectal cancer. Forty-two of 118 patients were not randomised because of high dose pre-operative radiotherapy, concern about the anastomosis, or obstructing tumours. Seventy-six patients were randomised to TAS or a proximal defunctioning loop stoma (LS). No significant difference in AL rate was demonstrated between the two groups (TAS: three AL's, all grade C; LS: two AL's grade A and C). Patients with a TAS had fewer general infectious complications (17% *vs* 35%) and a shorter hospital stay (13 *vs* 23 d; $P < 0.001$). This study is one of the few trials that actually tested an intracolonic device. Unfortunately, the randomisation strategy is not very clear and no control group with patients without an LS or TAS were described.

In 2006, Bülow *et al*^[21] performed a prospective randomised trial to evaluate TAS in patients undergoing anterior resection for a mobile rectal tumour. The use of a protective ileostomy was left to the discretion of the operating surgeon. After completion of the opera-

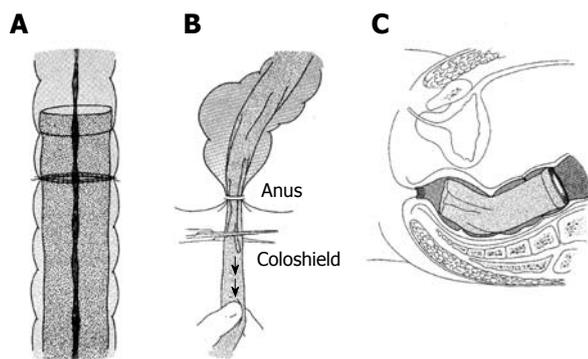


Figure 2 The Coloshield. A: The coloshield is sutured to the submucosa of the bowel proximal of the anastomosis; B and C: Slight traction is placed on the coloshield and it is cut so that it lies in the rectal ampulla.

tion, patients were randomised to the TAS group or the control group. After inclusion of 194 patients (of the planned 448 patients) an interim analysis was performed because of the occurrence of several leaks over a short time. Clinically significant leakage was diagnosed in 25 patients (13%), of whom seven were treated with drainage only and 18 with relaparotomy. AL occurred more frequently in the TAS groups (17%) than in the control group (8%). Although the difference in leak rate appears to be clinically relevant, the difference was not statistically significant because of the small sample size ($P = 0.09$). The study was stopped prematurely for ethical reasons because of this trend. Although this study was unable to demonstrate a statistical difference in AL between the groups, it seems unlikely that inserting the TAS reduces the incidence of AL (Figure 1).

INTRALUMINAL DEVICES

Intracolonic devices do not aim at preventing anastomotic dehiscence. However, they may prevent the faecal load from contacting the anastomotic site, thereby preventing leakage of faeces into the peritoneal cavity when the walls of the anastomosis have become dehiscent. When the faecal stream is bypassed from contacting the bowel mucosa, a gap in the anastomosis will not lead to extravasation of intraluminal content. Shielding the anastomosis from contact with faeces might also reduce the incidence of AL^[22].

Animal studies

Coloshield: In the 1980's, Ravo and Ger developed an intraluminal colonic tube to prevent anastomotic leakage. The application procedure is illustrated in Figure 2. After the bowel resection, the proximal loop is inverted for 4–6 centimetres. The proximal end of the tube, reinforced with a cloth strip, is fixed to the proximal bowel loop using polyglycolic acid sutures. The inverted intestinal portion is overturned to its normal anatomic position and the posterior half of the anastomosis is performed. Then, a rectal probe is introduced through the anus and

is connected to the tube by a built-in connector. The probe is drawn outside the intestinal lumen through the distal bowel and the anastomosis is completed by suturing the anterior part. The tube is then cut at the level of the anal orifice after a light traction, spontaneously returning inside. In cases where the mid- or lower rectum is resected, the tube is left protruding from the anus and an incontinence bag is attached to the perineum^[23]. Studies on dogs were performed using different tubes varying in width and length, material (latex, silicone, rubber), and suture technique. The colon tube placement was found to be a safe, uncomplicated procedure and none of the dogs (three studies, 14 dogs per study) developed AL (evaluated by laparotomy and barium studies). All tubes were expelled naturally together with the faecal stream^[24–26]. Even when an intentionally incomplete anastomosis was made after inserting the tube, no AL occurred. In 1985, the bypass tube was successfully tested on five dogs using a (circular) stapler^[27].

Silicone prosthesis: In 1992, Serra *et al.*^[28] studied the efficacy of intracolonic silicone prosthesis in 42 dogs. The use of the prosthesis is similar to the technique described by Ravo and Ger^[23,26,29]. The primary objective of the study was to evaluate the efficacy of the intracolonic silicone prosthesis in protecting the anastomosis. Three groups of 14 dogs each (colonic occlusion, diverticulitis, and control) were randomized to undergo resection and anastomosis with or without the silicone prosthesis. A significant difference in mortality was found: six dogs without prosthesis developed anastomotic failure, of which three died. No deaths or AL occurred in the prosthesis groups.

Soft latex tube: Intraluminal colonic tubes were studied by Ross^[30] in a rat model. The rats were divided in four groups; all underwent colon diversion with creation of an incomplete anastomosis. The first group consisted of rats treated with an intracolonic tube made from rat duodenum. In the second group, an intracolonic soft latex tube was introduced. The third group had a tube placed outside the colon lumen, and the last group was a control group with an incomplete anastomosis. The tubes were attached 1.5 cm above the incomplete anastomosis. The tubes remained inside the rectum, barely reaching the dentate line and were removed after five or six days. Rats treated with latex and rat duodenum tubes showed a better survival compared to controls (52% and 71%, respectively *vs* 25% in controls). Rats treated with rat duodenum showed a significant better survival compared to the control group ($P < 0.02$). A mortality rate of 100% was found in rats with a tube placed outside the lumen of the colon. The results suggest that only intraluminal tubes have a survival advantage compared to controls. This finding may be explained by the fact that tubes will prevent faecal contamination of the anastomotic site and allow time for secondary healing of the anastomosis.



Figure 3 Polyflex stents with a proximal flare.

Polyflex self-expandable covered plastic stent: As a result of achievements in biomedical technology, in 2008 Tsereteli *et al*^[31] performed a randomized controlled trial in 16 pigs comparing the incidence of AL after open rectosigmoid resection with or without a 21 mm Polyflex self-expandable covered plastic stent (Figure 3). The stent was placed over a guidewire with use of a flexible colonoscope and deployed under fluorescence control. A 2-cm anastomotic gap was created. After 6-9 d, stents were spontaneous expelled. At autopsy, none of the animals in the study group ($n = 8$) showed leak-related complications, although two pigs developed an unrelated postoperative complication (evisceration and bladder necrosis) and died. Five out of eight control animals (63%) showed intra-abdominal infection around the anastomosis at autopsy, with four abscesses and one fistula. This demonstrated a significant beneficial effect of the stent group *vs* controls ($P = 0.002$). The authors stated that the stent could be a breakthrough solution for the complicated colorectal anastomosis, avoiding the necessity of a stoma during the healing process. A potential new indication for this stent was also to seal an acute anastomotic leak, which is supported by one case report describing the successful use of a coated stent in healing a 1-cm fistula from a rectosigmoid anastomosis two weeks after surgery^[32]

Human studies

Coloshield: The development of the intraluminal tube led to the final version of the Coloshield: a soft, pliable tube like a surgical glove. This intraluminal protective device, developed by Ravo, was first used in humans in 1984^[25,33]. Indications for use include perforated diverticulitis, colonic obstruction, volvulus, carcinoma, and fistula. Several non-randomized studies were performed in patients (ranging from $n = 6$ to $n = 98$) undergoing colon surgery with the Coloshield. The reported anastomosis-related complication rates varied between 0% and 8.7%^[23,25,34-37]. The Coloshield related complications included two anastomotic dehiscences (from a group of 98 patients) following low anterior resection, both attributed to technical errors^[23]. Egozi *et al*^[38] described a case with a complicated course after insertion of a rigid

intracolonic bypass. On the 8th postoperative day, the Coloshield was found to have eroded through the colon. Castrini *et al*^[33] tested an intracolonic latex bypass in 19 patients undergoing left colon or rectal resection. None of these patients developed anastomotic complications. Regrettably, no detailed information concerning procedures, patients, and complications was reported.

The last article concerning the Coloshield was published by Ravo in 1991. Ravo described a method of inserting the Coloshield in the proximal colon after completion of the anastomosis by performing a longitudinal colostomy on the antimesenteric border of the afferent loop, proximal to the anastomosis^[39]. Ravo and Ger pioneered the use of intracolonic stents, testing different materials (silicone, rubber, and latex) before developing and, finally, filing the patent of the latex Coloshield^[40]. They concluded that the one-stage intracolonic bypass procedure is a viable alternative to the two- or three-stage procedure because it reduces the length of hospital stay and the length of disability. Despite its promise, the Coloshield has not been widely accepted. Ravo still uses the Coloshield (personal communication).

Condom: In 1994, Yoon *et al*^[41] used a condom instead of a Coloshield to protect the colo-anal anastomosis. Ten patients with rectal carcinoma undergoing LAR received this condom. The ring of the sterilised condom was sutured to the mucosal and submucosal layer of the proximal colon before completing the anastomosis. The condom was brought to the exterior and transected with scissors. The device is expelled naturally from the anus between the 10th and 14th postoperative day. No anastomotic dehiscence, leakage, or colonic necrosis occurred. In 1995, Ruiz *et al*^[42] described the same method using a condom (termed a skinless skin) as a protective device in a colonic anastomosis. When using a stapler, the distal end of the condom is attached to the anvil of the EEA stapler with two stay sutures, permitting it to be pulled through the anastomosis. Ruiz *et al* hypothesized that a latex condom is a cheap and safe device that decreases the risk of dehiscence and permits the performance of a large number of primary anastomoses. Unfortunately, for both studies, no detailed information on the procedures and patients are available.

BIODEGRADABLE DEVICES

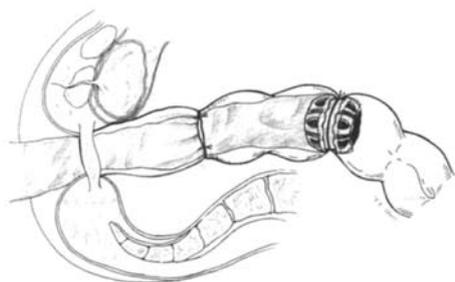
Animal studies

In 1993, Winkeltau tested the protective effect of biodegradable bipolymer intraluminal stents in 90 rats under the adverse condition of induced general peritonitis (verified by inspection, microbiology, and histology)^[43]. Peritonitis was induced using the cecal ligation and puncture model^[44]. Stents of various shapes and biodegradable materials were compared to controls with no stents, in rats undergoing jejunostomy. The survival rate in the control group was 25% and rats receiving a tube had a significantly better survival, varying between



Figure 4 Murphy's button. John Benjamin Murphy developed his device in 1892 as a quick and safe method of intestinal anastomosis. The steel Murphy button had two rounded heads mounted on hollow shafts. After the intestinal ends were tied on the shafts, the heads were screwed together to compress the tissue.

A



B

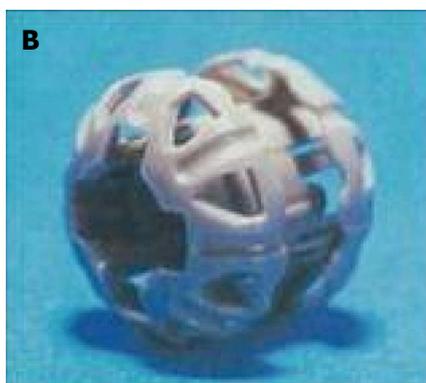


Figure 5 Valtrac-secured intracolonic bypass device. A: Rough colorectal anastomosis with large gaps between sutures protected by the intracolonic bypass; B: Biofragmentable anastomosis ring.

65% and 90%. The best results were obtained in rats with a funnel shaped BCL-004 tube, mainly composed of polyhydroxybutyric acid (PHB). The use of degradable materials is not restricted to the distal parts of the gastrointestinal tract, since it does not carry the risk of causing an obstruction.

Valtrac secured intracolonic bypass (VIB): Chen^[45] introduced the VIB, which consists of a soft vinyl tube attached to a biofragmentable anastomosis ring (BAR). The

BAR was introduced by Hardy *et al*^[46] in 1985 (inspired by Murphy's button). The BAR realises a sutureless intestinal anastomosis composed of two identical segments. The two components interdigitate and can be approximated to a semi-closed position with a 6 mm gap between the two edges of the ring (Figures 4 and 5).

Chen attached the BAR to the lumen of a pig's colon, 5-10 cm proximal from the anastomotic site, by putting a simple suture encircling the colon at the site of the BAR gap. The tube attached to the BAR passed through the anastomosis to the anus, thereby preventing contact between the anastomotic site and the faecal stream. Eighteen pigs underwent colonic resection with the deliberate creation of an incomplete anastomosis. Six pigs received the bypass, six received the bypass under the condition of a colonic outlet obstruction (created by tying a purse string suture at the level of the anus) and six pigs were controls. All pigs with the bypass had no anastomotic leakage (checked by a barium enema) and survived. Temporary anorexia and abdominal distension were noted in pigs with a colonic outlet obstruction. Four of six controls developed anastomotic leakage, of which three died.

Human studies

Valtrac secured intracolonic bypass: In 2002, the VIB was tested on 83 patients undergoing LAR for rectal cancer^[47]. After inclusion, the patient decided whether he/she wanted to be treated with the VIB or with a loop ileostomy (LI) to protect the anastomosis. The VIB was attached to the colon 5-7 cm proximal of the anastomosis by the same method Chen *et al* used in their experimental study. The fragmentation and excretion of the BAR occurred 12-22 d postoperatively. Fifty-three percent of patients chose the VIB and 47% chose the LI as treatment. Four subclinical anastomotic dehiscences were diagnosed, two in each group. Total hospital stay and costs were significant lower in the VIB group ($P = 0.001$); no readmission for a take down of the stoma was indicated. In two patients, the BAR detached en-bloc, which led to a difficult expulsion. In these cases the BAR was manually crushed and excreted through the anus. The authors concluded that the VIB is a safe and effective diverting technique to protect an elective low colorectal anastomosis; it avoids stoma-related complications and lowers the cost. This study can be criticized because the lack of randomization and high probability of introduction of bias.

C-seal: A recent development from the Groningen group is the C-seal: a thin walled tube like a soft sheet or condom, with a diameter of 4 cm, a length of 25 cm and a wall thickness of 70 μm (Figure 6). The C-seal is a tubular device composed of a biodegradable synthetic material. Two flaps with adhesive tape are located at one end of the tube. These flaps are used to attach the C-seal to the stapler cap, to facilitate an easy pull-through of the C-seal after the anastomosis is made (<http://www.jove.com/index/details.stp?ID=2223>). The C-seal remains

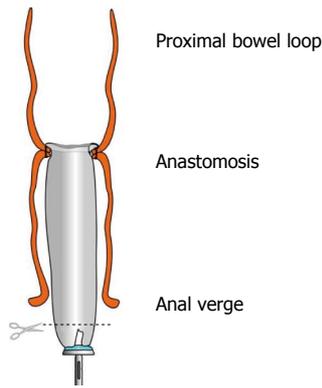


Figure 6 C-seal: A biodegradable drain protecting the anastomosis.

in place for about 10 d, according to the engineered composition of the biodegradable material. Thereafter, it loses strength, degrades, and is secreted from the body together with the gastrointestinal natural contents. In 2007, a pilot study was performed testing the C-seal in 15 patients diagnosed with colorectal carcinoma undergoing LAR with stapled anastomoses^[48]. No (sub) clinical AL was diagnosed in these 15 patients. Currently, the C-seal is being tested in a second phase study of 35 patients undergoing (colo-)rectal resection with stapled colorectal anastomosis.

CONCLUSION

The relative high incidence of anastomotic leakage after colorectal surgery, with its major consequences for morbidity and mortality, remain of great concern. Some authors concentrate on early detection of anastomotic dehiscence to reduce the consequences of AL^[49-52]. The ideal situation, however, would be prevention of anastomotic leakage. Many devices have been developed to prevent AL by protecting the anastomotic site. We categorised these devices as transanal decompression, intraluminal, and biodegradable protective devices. A number of studies concerning intraluminal tubes demonstrate low leakage rates^[18,33-35,48]. Despite these positive results, the use of protective devices has not been widely implemented. Clinicians are probably reluctant to use these devices in clinical practice for a number of reasons.

First, the use of intraluminal devices has only a small basis of evidence in the literature. Most papers are either animal studies or small, non-randomized human studies, often without a control group. Furthermore, most studies are heterogeneous and use different devices^[33,37,41,53]. Only two randomized, controlled studies are published, both on decompression devices. Amin *et al.*^[20] compared a defunctioning stoma with the transanal stent. This study does not show a benefit of the transanal stent. The study suffers from unclear eligibility criteria and a non-transparent randomisation process: one-third of the registered patients were not randomised. With 76 evaluable patients, the study is not sufficiently powered to detect significant differences in leakage rate between

the groups. A similar study by Bülow^[21] was prematurely stopped due to a high overall leakage rate, with a trend for a higher leakage rate in the TAS group.

Contrary to the transanal stent, a number of papers suggest that the Coloshield may help to reduce AL, though this beneficial effect has only been demonstrated in small studies with no control group^[33-35,39,41,53]. Unfortunately, no proper, randomised trial comparing the Coloshield to the standard of care has been performed until now.

Another aspect of the Coloshield is that it is considered time-consuming and tedious to apply, making it less attractive than the standard procedure. Tsereteli hypothesized that the Coloshield never found wide acceptance because of its technical difficulties and the requirement of a laparotomy for placement^[31]. Finally, medical devices are often only successfully introduced by companies who can organize an optimal marketing campaign and a widespread network of representatives. According to Ravo, the Coloshield was never widely accepted because it lacked these factors. Nevertheless, Ravo still uses the Coloshield in daily practice (personal communication).

We conclude that there is currently no high-level evidence demonstrating a benefit of intraluminal devices to reduce AL. Based on the literature, we think that the intraluminal device holds clinical promise to reduce or prevent early leakage of colo-rectal anastomoses and concomitant sequelae (Table 1). Although a number of very innovative approaches have been reported, not all devices have been appropriately studied in a randomized, controlled fashion with sufficient power to rule out chance or bias.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Zhang DN

Fascin promotes the motility and invasiveness of pancreatic cancer cells

Yan-Feng Xu, Shuang-Ni Yu, Zhao-Hui Lu, Jian-Ping Liu, Jie Chen

Yan-Feng Xu, Shuang-Ni Yu, Zhao-Hui Lu, Jian-Ping Liu, Jie Chen, Department of Pathology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tsinghua University, Beijing 100730, China

Author contributions: Xu YF and Yu SN contributed equally to this work; Xu YF performed the majority of experiments and drafted the manuscript; Yu SN edited and revised the manuscript and was involved in data interpretation; Lu ZH and Liu JP contributed to the literature review; Chen J designed and supervised the research and gave funding support.

Supported by Grants from the Doctoral Fund from the Ministry of Education of China, No. 20060023013; the National Nature Science Foundation of China, No. 30471970 and 30973470; the National Science and Technology Support Project (the 11th Five-Year Plan) of China, No. 2006BAI02A14; the Scientific Research Special Projects of Health Ministry of China, No. 200802011; the National Data Sharing Project in Human Health, No. 2005DKA32403; Roche Company

Correspondence to: Dr. Jie Chen, Department of Pathology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tsinghua University, Beijing 100730, China. xhblk@163.com

Telephone: +86-10-65295490 Fax: +86-10-65295490

Received: February 22, 2011 Revised: June 8, 2011

Accepted: June 15, 2011

Published online: October 28, 2011

Abstract

AIM: To explore the role of actin-bundling protein, fascin during the progression of pancreatic cancer.

METHODS: The plasmid expressing human fascin-1 was stably transfected into the pancreatic cancer cell line MIA PaCa-2. The proliferation, cell cycle, motility, scattering, invasiveness and organization of the actin filament system in fascin-transfected MIA PaCa-2 cells and control non-transfected cells were determined.

RESULTS: Heterogeneous overexpression of fascin markedly enhanced the motility, scattering, and inva-

siveness of MIA PaCa-2 cells. However, overexpression of fascin had minimal effect on MIA PaCa-2 cell proliferation and cell cycle. In addition, cell morphology and organization of the actin filament system were distinctly altered in fascin overexpressed cells. When transplanted into BALB/c-nu mice, fascin-transfected pancreatic cancer cells developed solid tumors at a slightly slower rate, but these tumors displayed more aggressive behavior in comparison with control tumors.

CONCLUSION: Fascin promotes pancreatic cancer cell migration, invasion and scattering, thus contributes to the aggressive behavior of pancreatic cancer cells.

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Key words: Fascin; Invasiveness; Motility; Pancreatic cancer

Peer reviewer: Hendrik-Tobias Arkenau, MD, Sarah Cannon Research United Kingdom, 93 Harley Street, London W1G 6AD, United Kingdom

Xu YF, Yu SN, Lu ZH, Liu JP, Chen J. Fascin promotes the motility and invasiveness of pancreatic cancer cells. *World J Gastroenterol* 2011; 17(40): 4470-4478 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4470.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4470>

INTRODUCTION

Pancreatic cancer is characterized by aggressiveness and early metastasis, and the survival rate for this cancer is among the lowest of all cancer types. In an effort to elucidate additional targets for the detection and therapy for this type of cancer, our lab completed a proteomic analysis of primary pancreatic cancer and normal pancreas samples^[1]. We identified 70 proteins that were expressed at least 2-fold higher in pancreatic cancers when compared with normal pancreas samples. Of these proteins,

18 were involved in cytoskeleton regulation, and fascin was one of the identified proteins that had the greatest change between pancreatic cancer and normal pancreas samples. Because cell motility is based on rearrangement of the actin cytoskeleton and this process of rearrangement is governed by multiple actin-binding proteins, we postulated that these proteins may play some role in the invasion and metastasis of pancreatic cancer. Several other studies have previously shown that the actin-bundling protein, fascin, which is specifically expressed in pancreatic cancer when compared with normal pancreas, is closely associated with the status of pancreatic cancer cell differentiation and plays an important role in pancreatic cancer progression^[2-5].

Fascin was identified in the 1970s to be a 55-kD globular protein that cross-links F-actin into well-ordered and tightly packed parallel bundles that are concentrated in cell protrusions during cell migration. Fascin is highly expressed in specialized cells that are rich in filopodia, such as neurons, glial cells, mature dendritic cells and actively migrating cells, such as the endothelial cells of microvessels^[6,7]. Fascin expression is often absent in normal epithelial cells, such as the epithelia of the bile duct^[8], urinary bladder^[9], breast^[10], colon^[11], ovary^[12], pancreas^[1] and stomach^[13]. Fascin expression is upregulated in several human neoplasms, such as breast^[10], lung^[14], kidney^[15], ovary^[12], prostate and pancreatic cancers^[3,5,16]. Fascin overexpression is often correlated with an invasive tumor phenotype, poor prognosis and decreased disease-free survival.

The role of fascin in the malignant behavior of pancreatic cancer remains unknown. To determine the functional consequences of fascin overexpression in pancreatic cancer cells, we stably transfected a human pancreatic cancer cell line, MIA PaCa-2, with a plasmid containing full-length human fascin cDNA. The proliferation, cell cycle, motility, scattering, invasiveness and organization of the actin filament system were evaluated in fascin-transfected MIA PaCa-2 cells and in non-transfected control cells.

MATERIALS AND METHODS

Cell culture

The human pancreatic cancer cell lines, BxPC-3, MIA PaCa-2 and AsPC-1 were obtained from American Type Culture Collection (Rockville, MD, United States), and the PC-1, PC-4 and PC-7 cell lines were established and maintained in our laboratory. The BxPC-3, AsPC-1, PC-1, PC-4 and PC-7 cell lines were cultured in RPMI 1640 (GIBCO, Paisley, United Kingdom) with 10% fetal bovine serum (FBS) (HyClone Laboratories, United States) and penicillin-streptomycin (100 IU/mL-0.1 mg/mL). The MIA PaCa-2 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Paisley, United Kingdom) supplemented with 10% FBS and penicillin-streptomycin (100 IU/mL-0.1 mg/mL). All cells were maintained in a humidified 5% CO₂ atmosphere at 37 °C.

Antibodies

The fascin antibody (M3567) was purchased from DAKO (Glostrup, Denmark); the β -actin antibody was purchased from Sigma (MO, United States); the EnVasion™ Detection Kit was purchased from DAKO (Glostrup, Denmark); and the FITC-conjugated secondary antibody was purchased from Boster (WuHan, China).

Transfection

A pcDNA3 vector containing the full-length human fascin cDNA (pcDNA3-Fascin) was kindly provided by Dr. Josephine C. Adams (Lerner Research Institute, Cleveland, Ohio, United States). The insert was cut out with the EcoR I restriction enzyme to acquire the pcDNA3 control vector (pcDNA3-Vector). The sequence was verified by DNA sequencing. MIA PaCa-2 cells were transfected with either pcDNA3-Fascin or pcDNA3-Vector. Approximately 5×10^4 MIA PaCa-2 cells per well were seeded in a 6-well culture plate and were subsequently transfected with 5 μ g of plasmid using 10 μ L of Lipofectamine 2000 (GIBCO, United States) in 250 μ L of Opti-MEM (GIBCO, United States). After 48 h, G418 (GIBCO, United States) was added to the cells for selection at a concentration of 800 μ g/mL. After 10 to 14 d, antibiotic-resistant colonies were picked, pooled and maintained in DMEM containing 10% FBS and 400 μ g/mL G418.

Western blotting analysis

Cells were rinsed twice with D-Hanks and solubilized with lysis buffer [50 mmol/L Tris (pH 8.0), 1% Nonidet p-40, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate, 0.5% deoxysodium cholate, 1 \times cocktail (Roche, Mannheim, Germany)] for 30 min on ice. The total extract was cleaned by centrifugation at 12 000 r/min for 30 min at 4 °C, and the supernatant was collected. The protein concentration was determined with the Bradford assay (BioRad, CA, United States). A total of 40 μ g of total cell extract was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were transferred onto an Immobilon-PVDF membrane (Millipore, Bedford, MA, United States) and were blocked and incubated with the primary antibody overnight at 4 °C. The EnVasion™ Detection Kit with DAB liquid substrate (DAKO, Glostrup, Denmark) was used for protein detection.

Immunocytochemistry and Immunofluorescence

Cells were cultured on sterile coverslips and were incubated for 24 h in a humidified 5% CO₂ atmosphere at 37 °C. The coverslips containing the cells were then fixed with 4% paraformaldehyde in phosphate buffered solution (PBS) for 10 min, washed with PBS, permeabilized in 0.2% Triton X-100 in PBS for 5 min, washed and then blocked with normal goat-serum for 30 min at room temperature. Cells were then incubated with an appropriate primary antibody for 1 h at 37 °C, and were rinsed 3 times with PBS. For protein detection by immunocytochemistry, an EnVasion™ Chem™ Detection Kit

(DAKO, Glostrup, Denmark) was used. The reaction color was developed by incubating sections with DAB liquid substrate. The slides were then washed with water and counterstained with hematoxylin. The slides were then dehydrated and mounted with mounting media. For immunofluorescence, a FITC-conjugated goat anti-mouse IgG secondary antibody was used. After washing, the slides were mounted with glycerol and imaged with an immunofluorescence microscope (Olympus BX51).

Proliferation assay

For the proliferation assay, 1×10^4 MIA PaCa-2 cells per well were seeded in a 24-well culture plate in DMEM supplemented with 10% FBS. Every 24 h, cells from 3 independent wells were collected by trypsinization and counted using a hemocytometer.

Wound healing/cell migration assay

Cell migration was evaluated by the wound healing assay^[17]. Fascin-transfected MIA PaCa-2 cells and non-transfected control cells were plated separately into 6-well culture plates and cultured to 70%-80% confluence in DMEM containing 10% FBS. After a 24-h serum starvation period, the monolayer of cells was wounded by manual scratching with a sterile plastic 200 μ L micropipette tip, washed with PBS 5 times to remove cell debris, photographed with an inverted tissue culture microscope (Leica LEITZDM IL) and then placed in complete medium in a humidified 5% CO₂ atmosphere at 37 °C. After 20 h of incubation, the wells were re-evaluated under the microscope, and the wounded area was re-imaged for comparison.

Aggregation assay

The ability of the cells to aggregate was tested by hanging drop suspension cultures^[18]. Cells were trypsinized with 0.25% trypsin in the presence of 0.01% ethylene diamine tetraacetic acid, washed twice in PBS, and resuspended at 2.5×10^5 cells/mL in DMEM containing 10% FBS. Drops of medium (20 μ L in each drop, containing 5000 cells) were pipetted onto the inner surface of a Petri dish lid. The lid was then placed on the Petri dish, and the drops with the cells suspended were left hanging from the lid. To compensate for evaporation, 8 mL of serum-free culture medium was added to the bottom of the Petri dish. After incubation at 37 °C for 12 h, the lid of the Petri dish was inverted and photographed under an inverted tissue culture microscope.

Invasion assay

For the invasion assay, the BioCoat Matrigel Invasion Chamber (Becton Dickinson Bioscience, United States) was used according to the manufacturer's instructions. Briefly, 2.5×10^4 MIA PaCa-2 cells suspended in 500 μ L serum-free medium were seeded onto Matrigel-coated filters, and 750 μ L of DMEM containing 10% FBS was added as a chemoattractant in the lower portion of the wells. After incubation at 37 °C with 5% CO₂ for 24 h, the inserts were removed, and the non-invading cells that

remained on the upper surface of the filter were scraped off with cotton swabs. Cells on the bottom surface of the membranes were fixed with ethanol and stained with 0.05% crystal violet. The number of cells invading through the Matrigel membrane was counted. Data are presented as the average of triplicate determinants.

Cell cycle analysis

For the cell cycle analysis, a minimum of 1×10^6 cells were harvested and fixed in 70% ethanol at 4 °C. After 12 h, cells were centrifuged (1000 g, 7 min, 4 °C), resuspended in PBS containing 0.05 mg/mL RNase A (Sigma, United States) and then incubated at room temperature for 30 min. After the cells were washed, they were stained with 10 μ g/mL propidium iodide, filtered through a 60 μ m mesh, and analyzed by flow cytometry (Elite Epics ESP, Coulter, United States). A total of 10 000 cells were analyzed with MODFIT software.

Xenograft tumor model

All procedures involving mice were approved by the College Committee on Use and Care of Animals at the Peking Union Medical College and conformed to the relevant regulatory standards. Four-week-old male athymic nude (BALB/c-nu) mice (Vitalriver, Beijing) were housed in specific pathogen-free conditions. To generate tumor xenografts, 5×10^6 tumor cells suspended in 0.1 mL of medium were inoculated subcutaneously in the right flank of the mice. Animals were inspected every 3 d. When the tumors from fascin-overexpressing MIA PaCa-2 cells (named MIA PaCa-2 Fascin) or vector control MIA PaCa-2 cells (named MIA PaCa-2-Vector) developed to a visible size, the mice were euthanized, the tumors were collected, cut into 1 mm³ pieces and then implanted subcutaneously in the right flank of BALB/c-nu mice. A total of 6 mice were used in each group. Animals were inspected and the tumors were measured every 3 d. Mice were humanely euthanized when they were overwhelmed by tumor burden. All tumors and major organs were fixed in formalin and embedded in paraffin. Histopathological analysis was performed following routine hematoxylin and eosin (HE) staining on tissue sections. The tumor volume was calculated on the basis of the following formula: volume = ($\pi/6$) LWH (L = length, W = width, H = height).

Statistical analysis

The Student *t*-test and the Fisher exact probability test were used for statistical analysis; a *P* value of less than 0.05 was considered significant.

RESULTS

Fascin expression in pancreatic cancer cell lines and the generation of fascin-overexpressing pancreatic cancer cells

Western blotting analysis was performed to investigate the expression of fascin in different human pancreatic cancer cell lines. Fascin protein was present at different

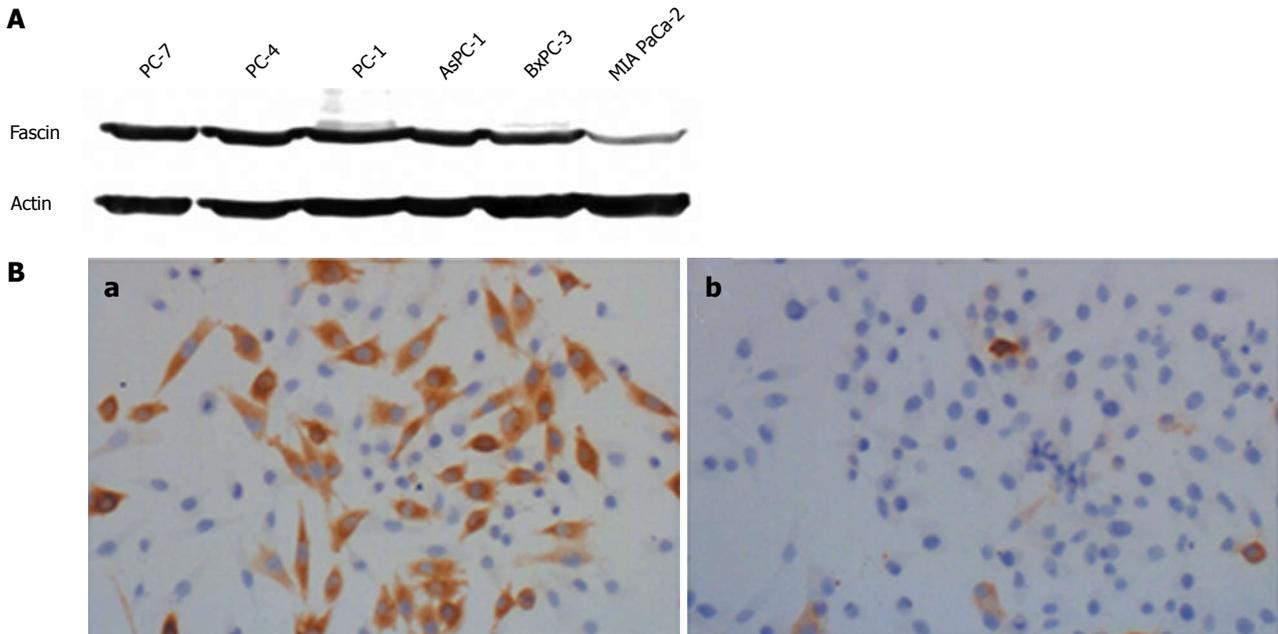


Figure 1 The selection and generation of fascin-overexpressing pancreatic cancer cells. A: Western blotting analysis of fascin expression in pancreatic cancer cell lines. Fascin protein was present in all pancreatic cancer cell lines at different expression levels. BxPC-3, AsPC-1, PC-1, PC-4 and PC-7 express fascin at a high level, whereas MIA PaCa-2 expresses fascin at a very low level. Actin served as a loading control; B: Immunohistochemical analysis of fascin expression in MIA PaCa-2 cells transfected with either pcDNA3-Fascin or pcDNA3-Vector. (a) MIA PaCa-2 Fascin represents the stable transfected fascin-expressing cell line; (b) MIA PaCa-2 Vector is the control cell line. Magnification is $\times 200$.

expression levels in all of the tested pancreatic cancer cell lines. BxPC-3, AsPC-1, PC-1, PC-4 and PC-7 expressed fascin at a high level, whereas MIA PaCa-2 expressed fascin at a very low level (Figure 1A). Because MIA PaCa-2 cells endogenously express fascin at low levels, we chose this cell line to examine the effect of heterogeneous fascin expression on the biological properties of pancreatic cancer cells.

MIA PaCa-2 cells were transfected with either pcDNA3-Fascin or the pcDNA3-Vector and stable clones were selected by G418 treatment. MIA PaCa-2 Fascin cells and MIA PaCa-2 Vector cells were used for further analysis (Figure 1B).

Fascin overexpression induces alteration of cell morphology and cytoskeleton

There was an increase in membrane protrusions in the MIA PaCa-2 Fascin cells when compared with the control MIA PaCa-2 Vector cells. Morphologically, the MIA PaCa-2 Vector cells were more rounded and had fewer projections, whereas MIA PaCa-2 Fascin cells were polarized with elongated membrane projections. In MIA PaCa-2 Fascin cells, actin filaments were distributed as bundles in the cytoplasm that protruded into membrane projections, whereas the actin filaments in MIA PaCa-2 Vector cells were distributed in a diffuse manner (Figure 2A). This result was also visualized *via* immunofluorescence as an accumulation of actin filaments in MIA PaCa-2 Fascin cells and as a diffuse distribution in MIA PaCa-2 Vector cells (Figure 2B).

Table 1 Cell cycle analysis of fascin-transfected MIA PaCa-2 cells (MIA PaCa-2 Fascin) and vector-transfected control MIA PaCa-2 cells (MIA PaCa-2-Vector) (mean \pm SD)

	G1 (%) ^a	S (%)	G2 (%)
MiaPaCa-2-Fascin	74.67 \pm 3.89	17.1 \pm 4.16	8.23 \pm 0.81
MiaPaCa-2-Vector	66 \pm 3.01	22.83 \pm 4.55	11.17 \pm 1.66

Percentages of the total cell population in different phases of the cell cycle were determined. The mean values of three experiments are shown. (^a*P* < 0.05).

Heterogeneous expression of fascin does not promote pancreatic cancer cell growth in vitro and in vivo

The growth curves of MIA PaCa-2 Fascin and MIA PaCa-2 Vector cells showed no significant difference between the two groups (Figure 3). Therefore, the heterogeneous expression of fascin does not seem to affect pancreatic cancer cell growth rate *in vitro*.

As shown in Table 1, fascin transfection induced an increase in G1 phase without a significant decrease in G2/M and S phases.

When transplanted into nude mice, both the MIA PaCa-2 Fascin and MIA PaCa-2 Vector cells developed solid tumor masses. The mean tumor volume from MIA PaCa-2 Fascin and MIA PaCa-2 Vector cells was $2.86 \pm 2.24 \text{ cm}^3$ and $3.08 \pm 1.16 \text{ cm}^3$, respectively. Tumors from fascin-transfected cells grew at a slightly slower rate in comparison with control tumors, but this difference was not significant (*P* = 0.8439). These results are in agreement with our *in vitro* experiments.

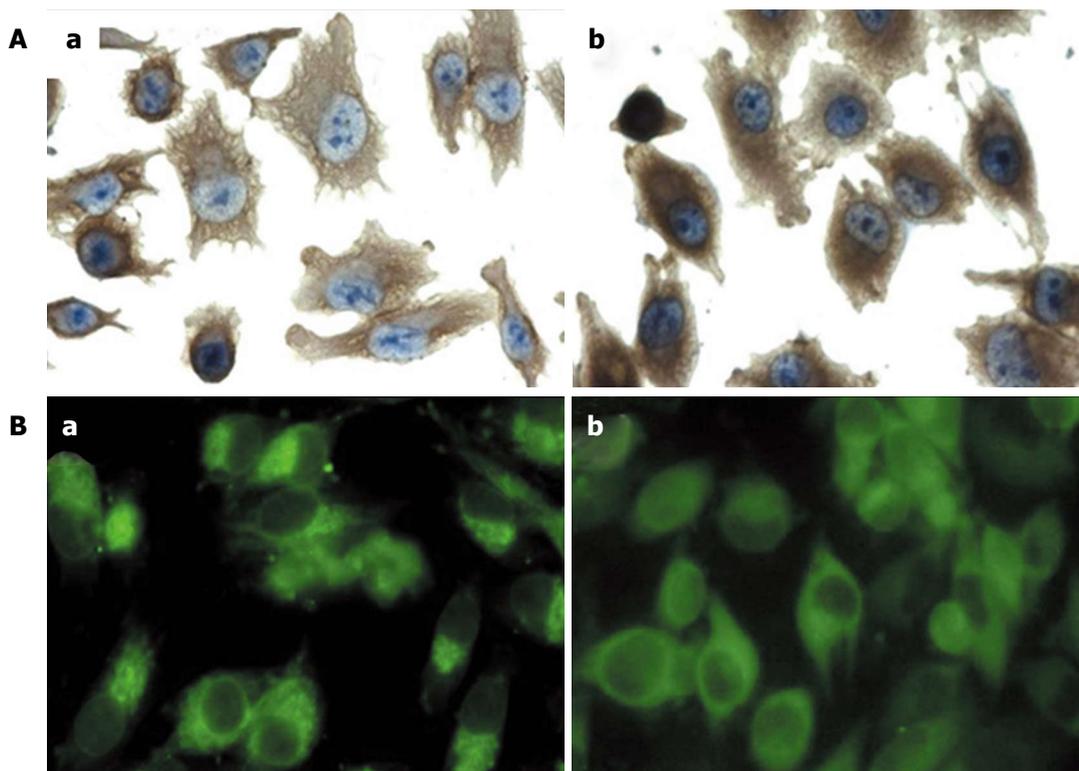


Figure 2 Fascin overexpression induces alteration of cell morphology and cytoskeleton. A: Immunohistochemical analysis of actin distribution in fascin-overexpressing cells and vector control cells ($\times 400$). (a) MIA PaCa-2 Fascin cells were more polarized with elongated membrane projections. Actin filaments were distributed as bundles in the cytoplasm which protruded into membrane projections in MIA PaCa-2 Fascin cells. (b) MIA PaCa-2 Vector cells showed a diffuse actin distribution; B: Immunofluorescence analysis of actin distribution in fascin-overexpressing cells and vector control cells ($\times 400$). (a) Actin accumulated in a polarized manner in MIA PaCa-2 Fascin cells, whereas (b) MIA PaCa-2 Vector cells demonstrated a diffuse actin distribution.

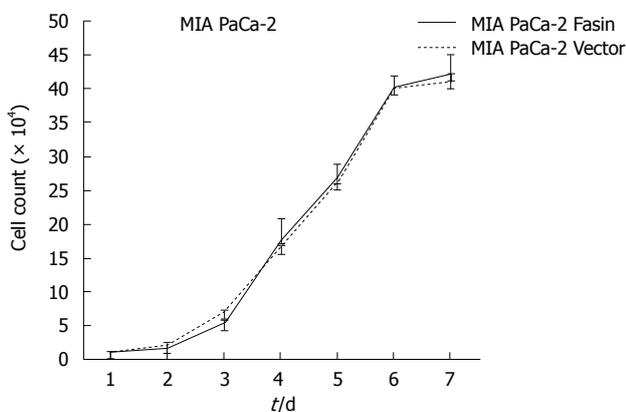


Figure 3 The growth curves of MIA PaCa-2 Fascin and MIA PaCa-2 Vector cells. There were no significant differences between the two groups.

Fascin promotes cell migration and inhibits cell aggregation

To investigate the effects of fascin on cell migration, *in vitro* wound healing assays were performed. After wounds were made for 20 h, the MIA PaCa-2 Fascin and MIA PaCa-2 Vector cells exhibited a cell reorientation response along the wounded edge margin and migrated into the wound area. MIA PaCa-2 Fascin cells repopulated the open space more efficiently than did MIA PaCa-2 Vector cells (Figure 4A).

Cell aggregation is an important factor that may critically affect tumor cell metastasis. We tested this using a hanging drop cell aggregation assay. Our results showed that the heterogeneous expression of fascin resulted in a reduction in aggregation when compared with vector control cells (Figure 4B).

Fascin promotes pancreatic cancer cell invasiveness in vitro and in vivo

To determine whether fascin promotes pancreatic cancer cell invasion, an *in vitro* invasion assay was performed using a Matrigel Invasion Chamber. Overexpression of fascin dramatically increased the cell invasive properties of the MIA PaCa-2 cells when compared with control MIA PaCa-2 cells (Figure 5A).

When transplanted into nude mice, the tumors developed from fascin-overexpressing MIA PaCa-2 Fascin cells grew in a more aggressive pattern, as 4 out of 6 of these tumors showed skin invasion, whereas only 1 of the control tumors exhibited skin invasion (Figure 5B and 5C).

DISCUSSION

We detected fascin expression in 6 pancreatic cancer cell lines (BxPC-3, AsPC-1, MIA PaCa-2 and 3 cell lines established by our laboratory: PC-1, PC-4 and PC-7). All of the cell lines expressed fascin at a relatively high

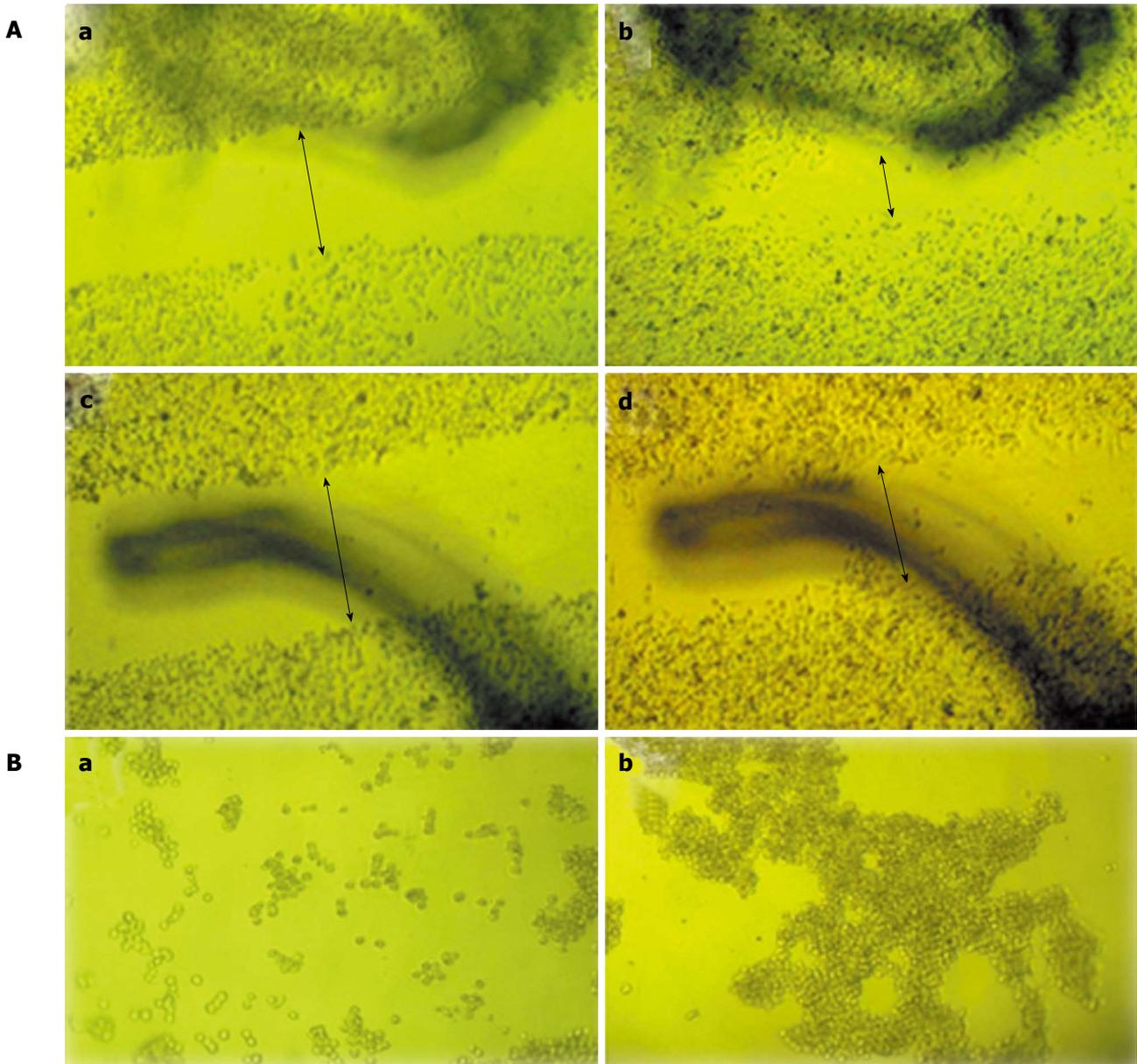


Figure 4 Fascin promotes cell migration and inhibits cell aggregation. A: The effect of fascin overexpression on the cell migration of MIA PaCa-2 cells. Images were taken at 0 h (a, c) and 20 h (b, d) ($\times 60$). MIA PaCa-2 Fascin cells (a, b) repopulated the open space more efficiently than did MIA PaCa-2 Vector cells (c, d). The arrows in figure 4a (MIA PaCa-2-Fascin cells group) and figure 4c (MIA PaCa-2-Vector cells group) showed initial distances between two side of cells (0 h), and the arrow in figure 4b (MIA PaCa-2-Fascin cells group) was short than the one in figure 4d (MIA PaCa-2-Vector cells group) in 20 h, which showed the ability of cell migration increased in the state of fascin overexpression; B: The effect of fascin overexpression on the aggregation of MIA PaCa-2 cells. Images were taken at 12 h ($\times 60$). The heterogeneous expression of fascin resulted in a reduction in aggregation compared with control cells. a: MIA PaCa-2 Fascin cells; b: MIA PaCa-2 Vector cells.

level, except MIA PaCa-2. This finding may indicate that fascin overexpression is a common event in pancreatic cancer, but the pathogenic effects of fascin are different among these cell lines. To elucidate the function of fascin in pancreatic cancer cells, we introduced a fascin-expression vector into MIA PaCa-2 cells and found that heterogeneous expression of fascin resulted in an increase in cell invasiveness and motility with a decrease in cell aggregation. The proliferation and cell cycle distribution of pancreatic cancer cells was not obviously affected by fascin overexpression. To our knowledge, this is the first study to ascertain the function of fascin by means of heterogeneous overexpression in pancreatic

cancer cells.

Pancreatic cancer progresses rapidly and demonstrates strong invasion and early metastatic properties with poor prognosis. The characteristics of tumor progression, cell motility and invasiveness, result from a rearrangement of the cytoskeletal microfilaments that is modulated by several types of actin cross-linking proteins^[19]. Among these molecules, fascin is implicated in the organization and persistence of filopodia, which plays an important role in cell-matrix adhesion, cell interactions and cell migration^[20,21]. Additional evidence has shown fascin-overexpressing tumors to have increased invasive properties. In breast cancer cells, overexpression

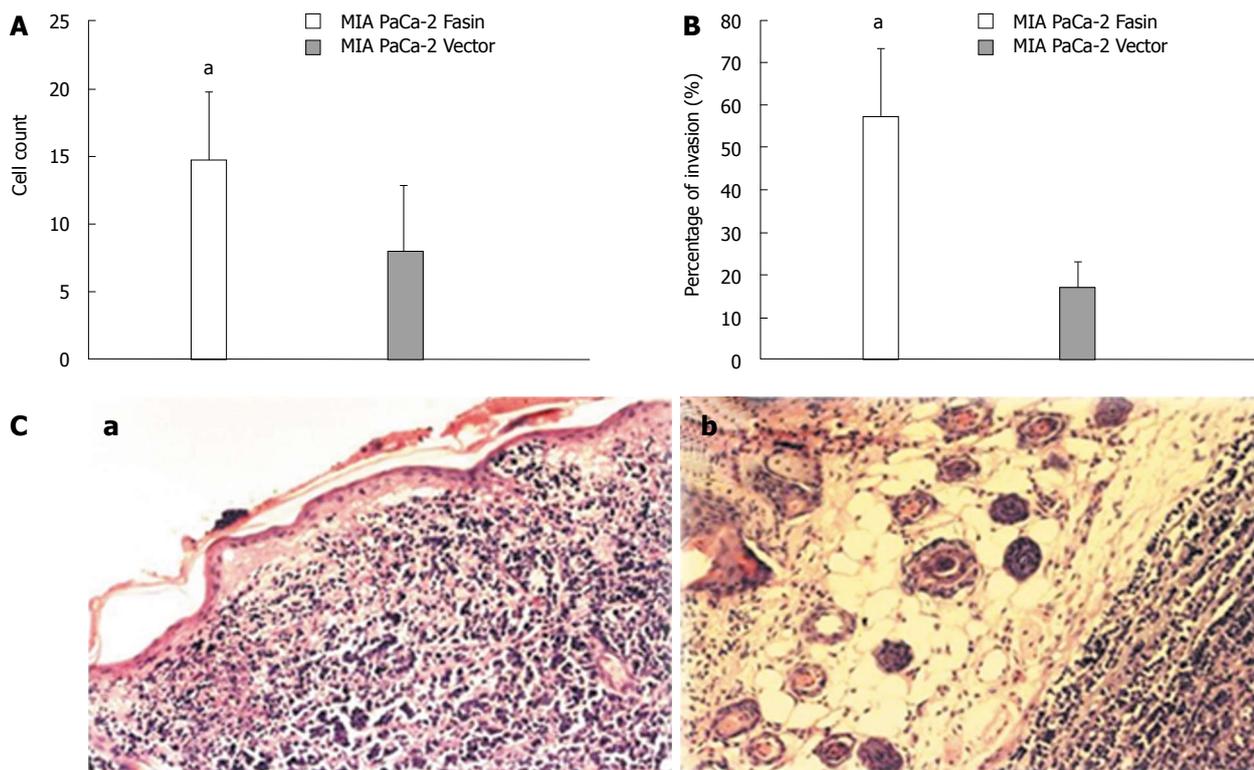


Figure 5 The effect of fascin overexpression on invasiveness of MIA PaCa-2 cells. A: Matrigel invasion assay showed that the overexpression of fascin dramatically increased cell invasive properties compared with control cells. ($^*P < 0.05$); B: *In vivo* invasion assay. Tumors from fascin-overexpressing MIA PaCa-2 Fascin cells showed an increase in skin invasion when compared with control MIA PaCa-2 Vector cells; C: Histological examination of skin invasion of tumors from MIA PaCa-2 Fascin cells and control MIA PaCa-2 cells. (a) Tumor from MIA PaCa-2 Fascin cells had skin invasion and (b) tumor from MIA PaCa-2 Vector cells showed no skin invasion (HE, $\times 150$). HE: Hematoxylin and eosin.

of c-erbB2 resulted in an increase in fascin expression and tumor cell motility^[22]. Also in gastric carcinoma, the outer edges of the tumors tended to have the most intense fascin staining in an immunohistochemical assay^[13]. Jawhari *et al*^[23] found that de novo expression of fascin in well-differentiated colon cancer cells increased cell migration through collagen type I- or IV-coated filters, and the cells showed a significant increase in dynamic membrane activity. In addition, in esophageal squamous cell carcinoma the down-regulation of endogenous fascin by RNA interference resulted in a dramatic decrease in cell invasiveness^[24]. In this study, we found that heterogeneous overexpression of fascin in pancreatic cancer cells resulted in an increase in cell motility and invasiveness. In fascin-overexpressing cells, there were more membrane protrusions, and the actin filaments were arranged as bundles in the cytoplasm which protruded into the membrane projections. In contrast, the control cells were rounded with diffusely distributed actin filaments and fewer projections. Thus, it seems likely that the rearrangement of the actin cytoskeleton induced by fascin overexpression in pancreatic cancer cells promoted their motility and invasion, which resulted in a more aggressive phenotype.

Cancer cell adhesion and migration are distinct but related events in the process of cancer progression, and cell dissociation is one of the limiting steps during the course of cancer cell migration. Heterogeneous

overexpression of fascin in pancreatic cancer cells resulted in an obvious decrease in cell-cell adhesion, as shown in the aggregation assay. To date, there are few reports on the role of fascin in cell-cell adhesion. Ectopic expression of fascin in rat Con8 cells disrupted the dexamethasone-induced formation of tight junctions and adherent junctions by preventing the recruitment of occludin and β -catenin to the site of cell-cell contact, which suggested that fascin was a negative regulator of cell-cell interactions^[25]. Another study demonstrated that fascin competed with E-cadherin for an association with β -catenin *in vitro*^[26], and it is conceivable that fascin plays a role in modulating cell adhesion. In contrast, a study in colon cancer cells did not find an effect of fascin on the E-cadherin- β -catenin association and distribution^[27]. Thus, the molecular mechanism of fascin involved in cell-cell adhesion still needs to be further explored.

In this study, the overexpression of fascin had no obvious effect on pancreatic cancer cell proliferation, which is in contrast to Jawhari *et al*^[23], who reported that fascin promoted the proliferation of colon cancer cells. In lung carcinomas, highly fascin-positive tumors had a high Ki-67 index^[14]. However, in colorectal adenoma, fascin and ki-67 were inversely correlated^[11]. The reasons for these divergent findings are currently unknown and may be related to the differences in growth-regulating signaling pathways. As a poorly differentiated pancreatic cancer cell line with the shortest doubling time compared

to the other 11 pancreatic cancer cell lines^[28], the MIA PaCa-2 cell line may have growth-regulating signaling pathways which are less dependent on fascin expression.

In summary, our study showed that overexpression of fascin promoted pancreatic cancer cell dissociation, migration and invasion, indicating its usefulness as a pancreatic cancer gene therapy target.

ACKNOWLEDGMENTS

We thank Dr. Josephine C Adams for kindly providing the fascin expression plasmid and thank Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences for its support.

COMMENTS

Background

Pancreatic cancer is one of the most devastating human malignancies, with an overall 5 year survival rate of 5% and a median survival time of 6 mo, and the major biological hallmarks of this disease are its early and aggressive local invasion and metastasis. Fascin is associated with cell movement and was identified to show the greatest change between pancreatic cancer and normal pancreas samples.

Research frontiers

Fascin expression is often absent in normal epithelial cells, and its expression is upregulated in several human neoplasms. Fascin overexpression is often correlated with an invasive tumor phenotype, poor prognosis and decreased disease-free survival. The role of fascin in the malignant behavior of pancreatic cancer remains unknown. In this study, the authors demonstrate that the overexpression of fascin could be a potential mechanism for migration and invasion in pancreatic cancer.

Innovations and breakthroughs

Recent reports have highlighted the importance of fascin in many types of cancer. This is the first study to verify that fascin is over-expressed in pancreatic cancer cells and that it promotes tumor migration and invasion. Furthermore, our *in vitro* and *in vivo* studies would suggest that this protein may be a positive factor of invasion and metastasis in this cancer.

Applications

By understanding fascin's overexpression and whether it induces migration and invasion, the findings of this study may represent a future strategy for therapeutic intervention in the treatment of patients with pancreatic cancer.

Terminology

The cytoskeletal protein, fascin, is an actin-bundling protein that plays a role in cell matrix adhesion, cell interaction and migration. Its overexpression has been reported in many types of tumors, but its function in pancreatic cancer is still unknown.

Peer review

The authors explored the role of Fascin during the progression of pancreatic cancer in pancreatic cancer cell lines and a mouse model. The key findings were that fascin promotes cancer cell migration, invasion and scattering leading to a more aggressive phenotype. Fascin overexpression did not result in increased cell cycle and proliferation which is in contrast to other tumor types, i.e., colon cancer. In general, the experiments are well explained and executed.

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S- Editor Tian L L- Editor Webster JR E- Editor Zhang DN

Expression and localization of paxillin in rat pancreas during development

Jing Guo, Li-Jie Liu, Li Yuan, Ning Wang, Wei De

Jing Guo, Li Yuan, Ning Wang, Wei De, Department of Biochemistry and Molecular Biology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Li-Jie Liu, Department of Physiology, Southeast University, Nanjing 210009, Jiangsu Province, China

Author contributions: Guo J and Liu LJ performed the majority of the experiments; Yuan L and Wang N provided vital reagents and analytical tools and were also involved in editing the manuscript; De W designed the study and wrote the manuscript; all authors read and approved the final manuscript.

Supported by The National Natural Science Foundation of China, Grant No. 81070620; International Cooperation Project of Jiangsu Province, Grant No. BK2007117 and BZ2008062

Correspondence to: Wei De, MD, PhD, Professor of Molecular Biology, Department of Biochemistry and Molecular Biology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. dewei@njmu.edu.cn

Telephone: +86-25-86862728 Fax: +86-25-86862728

Received: December 21, 2010 Revised: February 18, 2011

Accepted: February 25, 2011

Published online: October 28, 2011

Abstract

AIM: To investigate the expression and localization of paxillin in rat pancreas during development.

METHODS: Pancreata from Sprague Dawley rat fetuses, embryos, young animals, and adult animals were used in this study. Expression levels of paxillin in pancreata of different development stages were detected by reverse transcription polymerase chain reaction and Western blotting. To identify the cell location of paxillin in the developing rat pancreas, immunohistochemistry and double-immunofluorescent staining were performed using antibodies for specific cell markers and paxillin, respectively.

RESULTS: The highest paxillin mRNA level was detected at E15.5 (embryo day 15.5) following a decrease in the later developmental periods ($P < 0.05$ vs E18.5, P0

and adult, respectively), and a progressively increased paxillin protein expression through the transition from E15.5 to adult was detected. The paxillin positive staining was mainly localized in rat islets of Langerhans at each stage tested during pancreas development.

CONCLUSION: The dynamic expression of paxillin in rat pancreas from different stages indicates that paxillin might be involved in some aspects of pancreatic development.

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Key words: Pancreas development; Islet remodeling; Paxillin; Cell adhesion; Migration

Peer reviewers: Parviz M Pour, Professor of Pathology, Member of UNMC/Eppley Cancer Center, 98168 Nebraska Med Center, Omaha, NE 68198-680, United States; Raffaele Pezzilli, MD, Department of Internal Medicine and Gastroenterology, Sant'Orsola-Malpighi Hospital, Via Massarenti, 9, Bologna 40138, Italy; Mukaddes Esrefoglu, Dr., Professor, Department of Histology and Embryology, Inonu University, 44280 Malatya, Turkey

Guo J, Liu LJ, Yuan L, Wang N, De W. Expression and localization of paxillin in rat pancreas during development. *World J Gastroenterol* 2011; 17(40): 4479-4487 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4479.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4479>

INTRODUCTION

Development of the endocrine and exocrine pancreas involves a complex process of cell differentiation that ultimately gives rise to four distinct hormone producing cell types (α , β , δ , PP) and two kinds of enzyme secreting cells (acinar and ductal cells)^[1]. Organogenesis of the pancreas is a highly coordinated process. Morphologic development of the mouse pancreas first appears at E9.5. At approximately E16 the islet progenitor cells leave the

contiguous epithelium, migrate through the adjacent extracellular matrix (ECM) into the surrounding mesenchyme, and aggregate to form the islets of Langerhans^[2,3]. The islets are not fully formed until shortly before birth in E18-E19, and undergo further remodeling and maturation for 2-3 wk after birth^[4]. Thus, the developing pancreas presents a challenge for developmental biologists because of the complex morphogenetic processes underlying the development of this organ.

The factors that control pancreatic organogenesis and tissue maintenance remain unclear. Of particular interest are the ECM and their receptors, integrins, which exert a profound role during development controlling morphogenetic decisions and maintaining homeostasis during adulthood. Progression in islet cell development is accompanied by, and dependent upon, cell adhesion *via* β 1 integrin and its respective α -subunits. The β 1 family of integrins play critical roles in islet cell architecture, development, integrity and function^[5].

Paxillin interacts directly with several focal adhesion proteins including vinculin, talin, and integrin β 1^[6,7]. A principal function for paxillin is in the integration and dissemination of signals from integrins and growth factor receptors to provide efficient cellular migration^[8]. Paxillin is an important mediator of signal cross-talk in the complex multistep process of net cellular movement through its phosphorylation and multipotent associations^[9-12], and functions as an adaptor protein coordinating the activities of many focal adhesion proteins. Thus, paxillin is in a position to play a role in the integration and regulation of adhesion and signaling, yet little is known regarding its function during embryogenesis^[13]. Given that it mediates integrin signal transduction, it might be expected that paxillin may be involved in numerous aspects of cell behavior and development in the pancreas. To the best of our knowledge, no study has investigated the relationship between paxillin expression and pancreas development, and the expression of paxillin during pancreatic development in rats is poorly understood. Knowledge of the regional and temporal expression of paxillin will be useful in understanding its potential role in pancreatic development. Therefore, we examined the expression of paxillin in rat pancreas during development.

MATERIALS AND METHODS

Animals and preparation of rat pancreatic tissue

Sprague-Dawley (SD) rats were purchased from the Animal Center of Nanjing Medical University (Nanjing, China). SD rats (2:1, male:female) were mated overnight. At noon the next day, if a vaginal plug was discovered, it was considered as Day 0.5 of gestation (E0.5). Embryos were removed at E12.5, E15.5 and E18.5 from the uterus of pregnant rats, which were sacrificed by cervical dislocation. Pancreata from E15.5 and E18.5 rat embryos were isolated according to their specific vacuolated morphology, as previously described^[14], under a stereomicroscope. Rat pancreata at postnatal (P) days 0, 7, 14, 21 and from adults, were directly isolated by the unaided eye.

All experiments were conducted in accordance with the Chinese Law for Animal Protection and were approved by the local animal care committee. Five rats were used at each age stage. Dissected tissues were immediately rinsed 3 times with phosphate buffered saline (PBS) to remove serum proteins, and fixed with 4% paraformaldehyde in PBS overnight for histology, or frozen in liquid nitrogen for RNA and protein isolation.

Immunohistochemistry

Pancreata from E15.5, E18.5, P0, P14, P21, and nonpregnant adult rats were fixed with 4% paraformaldehyde in PBS overnight and embedded in paraffin. Pancreata were cut into 5- μ m sections and mounted on gelatin/chrome alum-coated glass slides. Following deparaffinization, the presence of paxillin and insulin was determined immunohistochemically. To expose antigenic sites for paxillin/insulin, dewaxed sections were heated four times to 95 °C in a 600 W microwave oven maintained for 5 min and allowed to cool for 20 min. Endogenous peroxidase activity was then eliminated by incubation with 0.5% (v/v) hydrogen peroxide solution in absolute methanol for 15 min at 20 °C. Non-specific protein binding was eliminated by incubation with 10% non-fat dry milk in PBS for 1 h at 20 °C. Sections were then incubated with a polyclonal antibody (sc-7336; Santa Cruz Biotechnology) against paxillin or insulin (sc-9168; Santa Cruz Biotechnology) at a dilution of 1:200 and 1:500, respectively, for 18 h at 4 °C. Incubation for 1 h with horseradish peroxidase conjugated secondary antibody (1:500 dilutions) at room temperature followed. The antigen-antibody complex was then visualized by incubating the sections with 3, 3'-diaminobenzidine solution in the dark for 3 min. Sections counterstained with hematoxylin were dehydrated, and coverslipped. Images were taken at a magnification of \times 400. Controls were processed by omitting the primary antibody in the immunolabeling procedure.

Double fluorescence immunohistochemistry

The paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol and distilled water. The non-specific binding sites were blocked in 1% bovine serum albumin for 30 min. For paxillin and amylase or glucagon double immunofluorescence, the goat anti-paxillin primary polyclonal antibody was applied and revealed using fluorescein isothiocyanate-labeled rabbit anti-goat IgG (1:400, sc-2777, Santa Cruz Biotechnology). Mouse anti-amylase primary polyclonal antibody (1:500, sc-45667; Santa Cruz Biotechnology) or mouse anti-glucagon primary polyclonal antibody (1:1000, G2654; Sigma Aldrich) was then applied and revealed by cy3-labeled anti-mouse IgG (1:400, AP192C; Chemicon International, Inc. Temecula, CA, United States). Sections were placed in gel mounted aqueous mounting medium (G0918; Sigma, St. Louis, United States) with a cover glass, and examined under an Olympus BX51 Research Microscope (Olympus Optical, Tokyo, Japan). To rule out cross-reactivity in this staining system, the controls used were: first, single stain-

Table 1 mRNA expression of genes related to paxillin in the developing rat pancreas

Gene symbol	Probeset ID	E12.5		E15.5		E18.5		P0		Adult	
Integrin β 1	1368819_at	748.3	P	3277.2	P	3847.5	P	1055.6	P	427.5	P
Integrin β 4	1368612_at	58.2	A	295.3	P	542.6	P	149.4	P	115.5	P
Integrin β 5	1370801_at	25.4	A	49.5	P	56.3	P	5.2	A	2.8	A
Vinculin	1375538_at	168.7	P	1532.5	P	1790.7	P	141.9	P	23.1	P
ILK	1387777_at	634.5	P	1388.5	P	1449.5	P	583.3	P	122.8	P
Clathrin	1398842_at	1040.0	P	2918.2	P	3682.7	P	1306.9	P	429.0	P
Paxillin	1371664_at	298.8	P	997.5	P	847.0	P	285.3	P	62.4	P
Actopaxin	1370266_at	57.7	A	425.6	P	212.5	P	42.5	P	10.2	A

The Probeset IDs were the reference number for the probes of the Affymetrix oligonucleotide microarray (RAE230A). P: Present; A: Absent.

ing with the alternative secondary antibody and second, staining in the absence of primary antibody. In neither case was staining detectable. Images were taken at a magnification of $\times 400$.

Preparation of protein samples

The pancreata was homogenized in a detergent lysis buffer containing 8 mol/L urea, 2% CHAPS, 40 mmol/L Tris, 65 mmol/L DTT and 2% IPG buffer. The lysate was then centrifuged at 15000 *g* for 1 h at 4 °C. The total protein concentration of each sample was analyzed using a modified Bradford assay. All samples were stored at -80 °C prior to the electrophoresis.

Western blotting analysis

An equal amount of protein sample (40 μ g) from each time point was resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). After transfer, the membrane was blocked with 5% fat-free milk in Tris-buffered saline and 0.05% Tween 20 overnight at 4 °C. Primary antibodies were incubated with the membrane as described above and detected by peroxidase-linked rabbit anti-goat conjugates (Santa Cruz Biotechnology). Densitometric quantification of bands at subsaturating levels was performed using the Syngene-tool gel analysis software (Syngene, Cambridge, United Kingdom). Loading controls of presumably constantly expressed proteins such as β -tubulin were used; however, their variability and increase in development precluded their use^[5]. For negative controls, the primary antibody was omitted.

RNA extraction, reverse transcription-polymerase chain reaction

Total RNA was extracted from the pancreata at each time point with TRIZOL reagent (Invitrogen Life Technologies, Burlington, Ontario, Canada), according to the manufacturer's instructions. The quality of the RNA was verified by agarose gel electrophoresis using ethidium bromide staining. For each polymerase chain reaction (PCR), 2 μ g DNA-free total RNA with oligo (deoxythymidine) primers and reverse transcriptase were used. PCR was performed in 25- μ L reactions

containing 25 ng of cDNA, 0.2 nmol of each primer pair, and 0.3 μ L of *Taq* DNA polymerase. PCR was carried out in a T-gradient Biometra PCR thermal cycler (Montreal Biotech Inc., Kirkland, Quebec, Canada) to determine the annealing temperature for paxillin primers. The primer pairs used were as follows: paxillin, forward: 5'-GGAGCAGAACGACAAGCC-3', reverse: 5'-GCACAGAGCCCAGGAGA-3' (256 bp); 18S rRNA, forward: 5'-ACGAACCAGAGCGAAAGC-3', reverse: 5'-GGACATCTAAGGGCATCACAG-3' (514 bp). PCR conditions were as follows: 2 min at 94 °C for hot start, followed by up to 35 cycles of 94 °C for 30 s, 53.4 °C for 30 s, and 72 °C for 45 s, with a final extension of 5 min at 72 °C. To estimate the linear range of the nested reactions, we analyzed the PCR products at 10, 15, 20, 25, 30, and 35 cycles. The amplified products were analyzed on 1% agarose gels and visualized by ethidium bromide staining. The data were normalized by 18S rRNA.

cRNA probe generation and hybridization to Affymetrix microarray chips

Total RNA samples were used to generate cRNA probes by two rounds of transcription. A poly (dT) primer (with its 5' end carrying T7 promoter sequence) was used to synthesize cDNA from total RNA. The cDNA were used to amplify cRNA using T7 polymerase. The cRNA product from this first round amplification was then used to generate more cDNA by random priming, with the 3' end carrying a T7 promoter sequence. This cDNA was used to transcribe biotinylated cRNA, which was used to hybridize to the RAE 230A microarrays produced by Affymetrix.

Statistical analysis

Analysis of the experimental data was performed using PD Quest 7.0 software and the paired Student *t*-test. *P* < 0.05 was considered statistically significant. Data are presented as the mean \pm SD.

RESULTS

Paxillin mRNA expression in the process of pancreatic development

The mRNA expression levels of paxillin in the whole

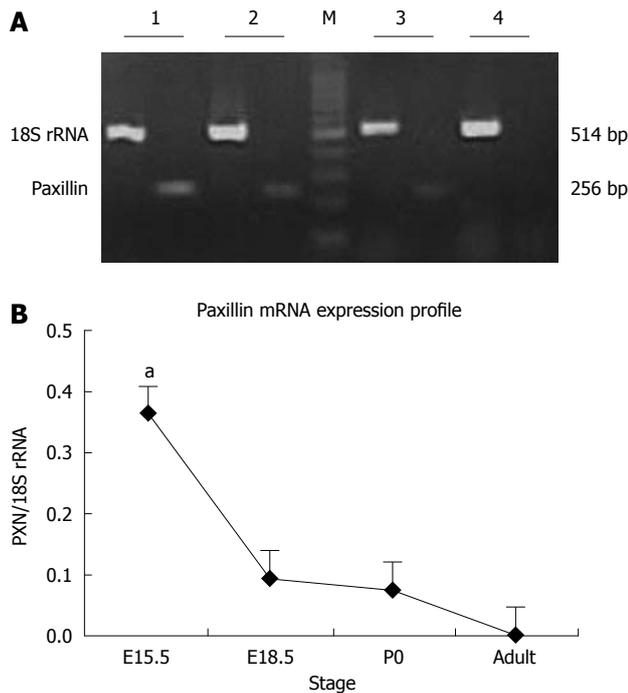


Figure 1 Paxillin mRNA expression level in the developing rat pancreas measured by reverse transcriptional polymerase chain reaction. A: From embryonic day (E) 15.5 to adulthood, paxillin mRNA expression in pancreas was highest at E15.5, following a dramatic decrease. Paxillin mRNA was almost undetectable in the adult pancreas. 1: E15.5; 2: E18.5; 3: P0; 4: Adult; M: Marker; B: Paxillin mRNA expression was analyzed and normalized to 18S rRNA. Results are indicated in percentages above the 18S rRNA value and are representative of three independent experiments. Compared to E15.5, the level of paxillin mRNA was lower at E18.5, P0 and adult ($^*P < 0.05$ vs E18.5, P0 and adult, respectively).

pancreas of the developing rat were examined through Affymetrix oligonucleotide microarray (RAE230A) (Table 1) and reverse transcription-PCR (Figure 1). As shown in Figure 1, the highest paxillin mRNA expression level was observed at E15.5, followed by decreased mRNA level of paxillin from E18.5 to adult. At the same time, mRNA expression of genes related to paxillin was also detected by Affymetrix oligonucleotide microarray (RAE230A), such as actopaxin, ILK, clathrin, vinculin, and Integrin β 1, as shown in Table 1. Absolute signal values of a selection of genes expressed in E12.5, E15.5, E18.5, P0 and adult rat pancreata were given. The highest expression level of these genes was almost detected at E15.5 (paxillin and actopaxin) or E18.5 (Integrin β 1, Integrin β 4, Integrin β 5, vinculin, ILK and clathrin). Expression profile of these mRNAs was similar to paxillin.

Regional localization of paxillin protein in rat pancreas at different developmental stages

To investigate the spatiotemporal expression of paxillin within the rat pancreas through fetal to postnatal life, we examined paxillin expression through immunohistochemistry (Figure 2). We found that paxillin maintained expression in the pancreas from E18.5 to adult, and at P14 and P21 paxillin was mainly localized in rat islets of Langerhans. Furthermore, double immunofluorescence

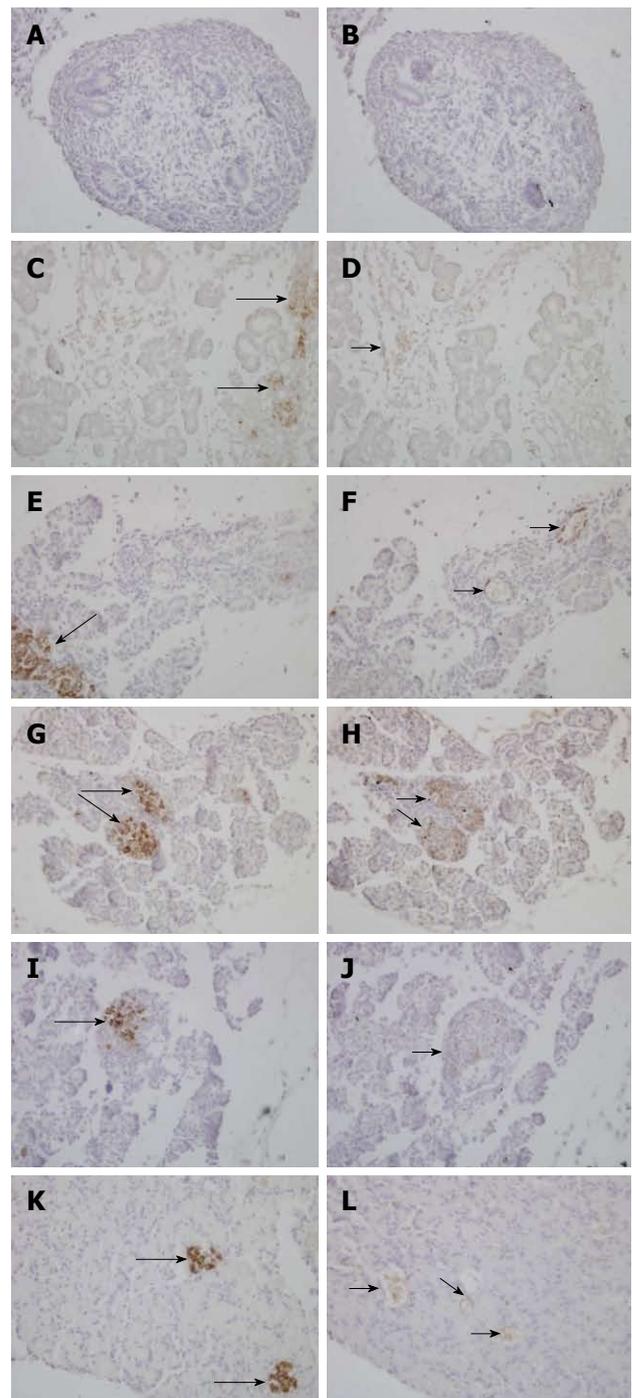


Figure 2 Immunohistochemical analysis of insulin and paxillin in the serial sections of the rat pancreas at E15.5 (A, B), E18.5 (C, D), P0 (E, F), P14 (G, H), P21 (I, J) and adult (K, L). Adjacent pancreatic sections from six developmental stages were stained with antibodies against insulin (left lane) and paxillin (right lane), respectively. We acquired images using an OLYMPUS DP70 digital camera. Strong cytoplasmic staining was observed for insulin (long arrows) for five stages except E15.5. Immunolocalization for the paxillin revealed a sporadic positive staining (short arrows) in the pancreas. In E18.5 and P0 rats, some cells in the pancreas, but not in islets, were stained. As shown in G-J, at P14 and P21 paxillin was mainly localized in islets. All magnifications are $\times 400$.

was used to detect expression of paxillin and amylase (Figure 3) or glucagon (Figure 4). As shown in Figure 3, although little co-expression of paxillin and amylase was

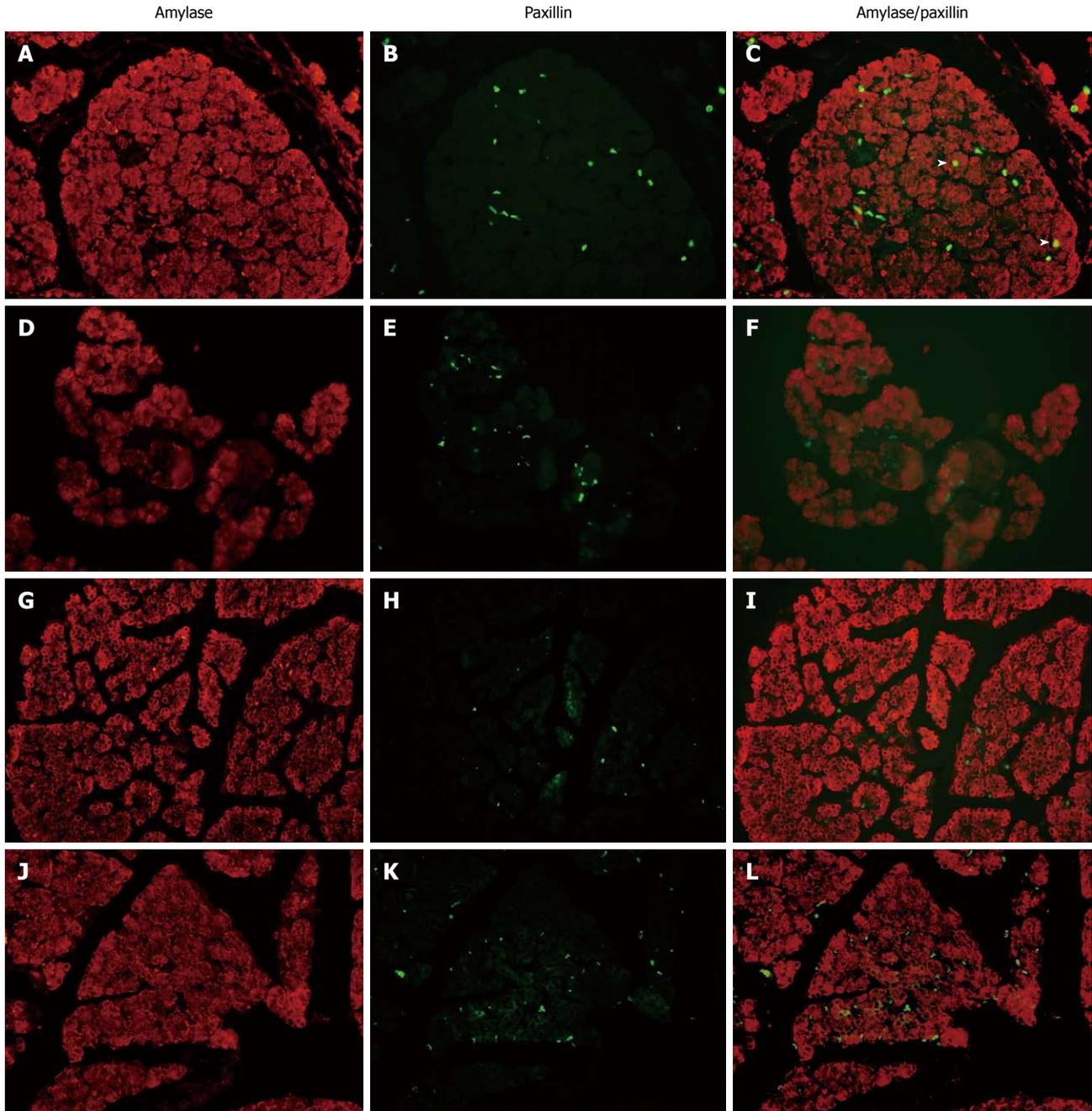


Figure 3 Immunofluorescent localization of paxillin and amylase in the pancreas of P0, P7, P21 and adult rats. The paxillin antibody was detected with an fluorescein isothiocyanate (green)-labeled secondary antibody and the amylase antibody was detected with a Cy3 (red)-labeled secondary antibody. Overlap between paxillin (green) and amylase (red) labeling is indicated by arrowheads. Original magnification 400 ×. A-C: P0; D-F: P7; G-I: P21; J-L: Adult.

detected, paxillin could be detected in the exocrine portion during development as has been reported previously^[15]. As shown in Figure 4, although little co-expression of paxillin and glucagon was detected, paxillin positive cells could be found in the center of islets of Langerhans from E18.5. To the best of our knowledge, there have been no reports concerning the expression of paxillin in rat islets of Langerhans.

Paxillin protein expression in the process of pancreatic development

Examining the abundance of paxillin at the protein level

during rat pancreatic development, from embryonic to postnatal life in whole pancreata by Western blotting analysis (Figure 5), revealed that there exist two isoforms of paxillin protein at 68 KD in pancreas during the developmental process, which was in agreement with previous reports^[13]. During embryonic and neonatal development phases, only the smaller isoform was detected, while the larger isoform was detected only after birth. Interestingly, in the adult pancreas, the two isoforms could both be identified. Paxillin began to be expressed at E15.5, and significantly increased after birth, with a 10 fold increase at birth. The differential expression pattern of two iso-

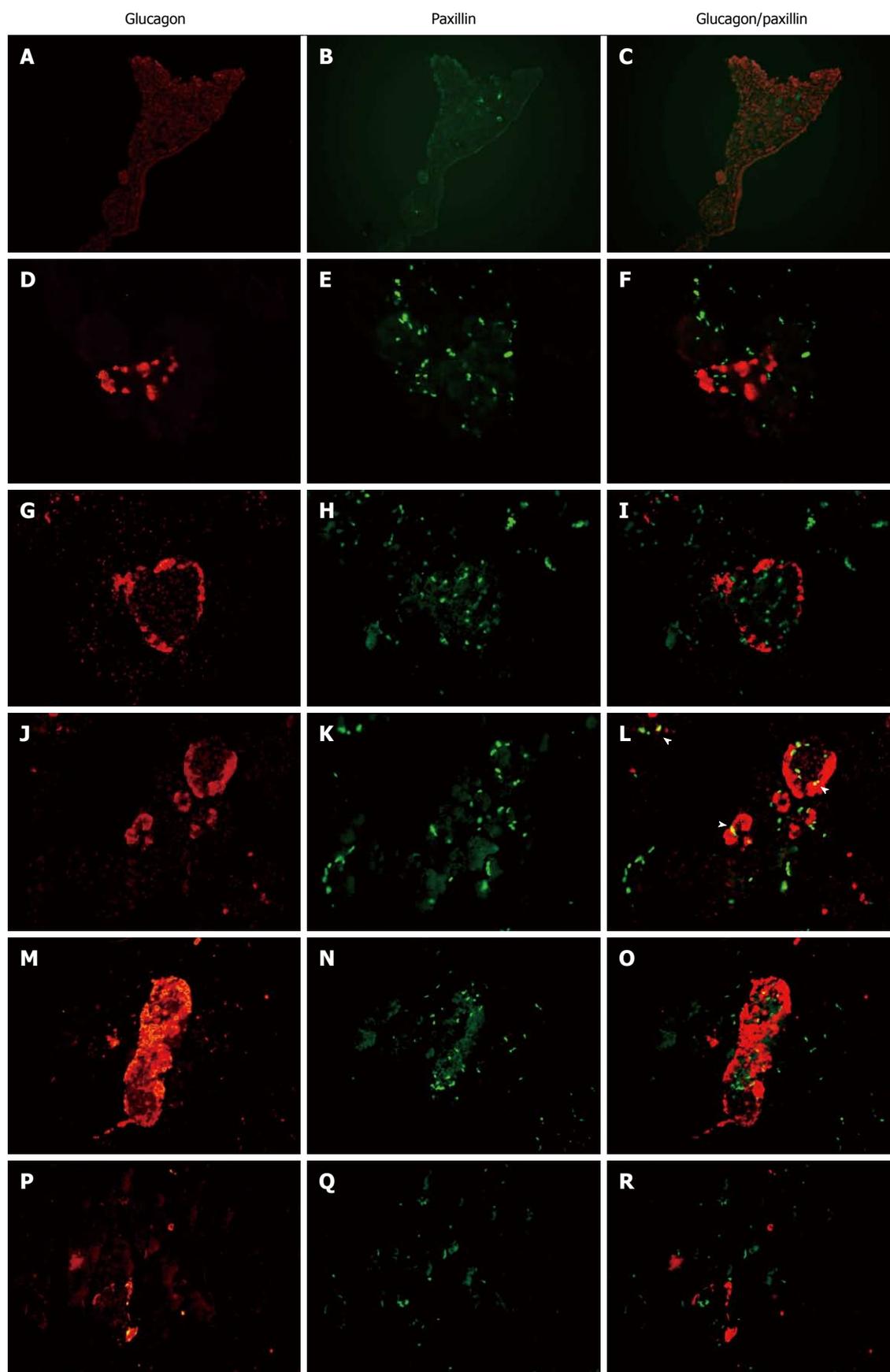


Figure 4 Immunofluorescent localization of paxillin and glucagon in the pancreas of E15.5, E18.5, P0, P7, P21 and adult rats. The paxillin antibody was detected with an fluorescein isothiocyanate (green)-labeled secondary antibody and the glucagon antibody was detected with a Cy3 (red)-labeled secondary antibody. Overlap between paxillin (green) and glucagon (red) labeling is indicated by arrowheads. Original magnification 400 ×. A-C: E15.5; D-F: E18.5; G-I: P0; J-L: P7; M-O: P21; P-R: Adult.

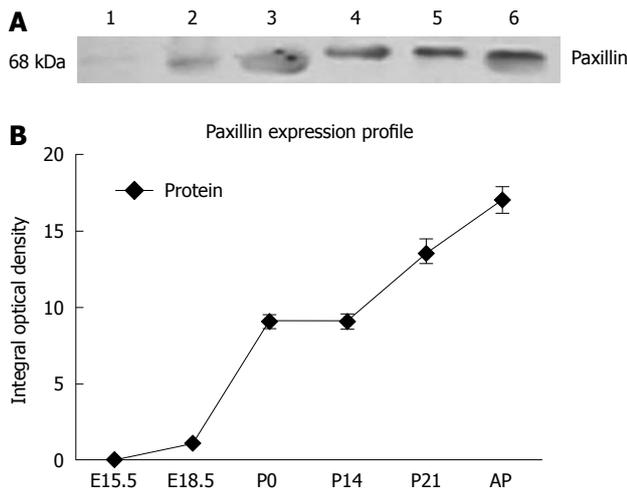


Figure 5 Paxillin protein expression level in the developing rat pancreas measured by Western blotting. A: From E15.5 to adulthood, paxillin protein (68 KD) expression in pancreas was highest at adult. 1: E15.5; 2: E18.5; 3: P0; 4: P14; 5: P21; 6: Adult; B: Progressively increased level of paxillin protein through the transition from E15.5 to adult is observed. Results are representative of three independent experiments.

forms of paxillin in the developmental process suggested their distinct physiological function^[7].

DISCUSSION

During organogenesis, specialized cell types are generated from progenitor cell populations and are precisely organized into the elaborate structure of the adult organ^[16,17]. Biochemical and ultrastructural studies delineated three distinct phases of pancreatic development in rats and mice. The first is during early organogenesis and is known as the “primary transitional phase” in which cytodifferentiation into exocrine or endocrine cells is minimal (approximately from E10 to E12/12.5 in rat). This is followed by the “proto-differentiated state” (from E12.5 to E15.5), in which epithelial cell proliferation is high, leading to rapid growth and lobulation, while cytodifferentiation is poor. During late organogenesis, a third phase or “secondary transitional phase” (from E15.5 to E18.5) is detected in which a dramatic increase in the number of endocrine and exocrine cells with high levels of insulin and exocrine enzymes is observed. During the secondary transition, endocrine cell migration and adhesion, and primary islet formation are key events. At approximately E16 the islet progenitor cells leave the contiguous epithelium, migrate through the adjacent ECM into the surrounding mesenchyme, and aggregate to form the islets of Langerhans. The islets are not fully formed until shortly before birth in E18-E19 and undergo further remodeling and maturation for 2-3 wk after birth^[4]. Although some studies have addressed the function of single gene, such as integrins in islet formation, much less is known about the multifactor network that guides islet formation during embryogenesis^[18-20]. The present study demonstrates that pancreatic development is accompanied by a specific spatiotemporal pattern of protein and

mRNA expression of paxillin, which is a mediator in integrin signaling, suggesting a role for paxillin during pancreatic development.

The protein and mRNA expression of paxillin in the whole pancreas determines a specific temporal pattern of expression from embryonic development to postnatal development. High mRNA expression, along with low protein level of paxillin, was observed in the embryonic pancreas (Figures 1 and 5). Subsequently, into postnatal life, decreased paxillin mRNA expression was paralleled to increased protein levels in postnatal life. What is more, genes related to paxillin, such as ILK, clathrin, vinculin and integrin β 1, shared the same expression pattern with paxillin. The highest expression level of these genes was almost detected at E15.5 or E18.5. In the present study, a dramatic increase of paxillin protein expression in the neonatal pancreas, compared with embryonic period, was detected. From the 18th day of gestation until birth, active cellular reorganization takes place to form the classic cellular features of the islet. After birth, apoptosis of a great amount of islet cells take place. From P14 to P21, islet cell proliferation increased dramatically and islet structure is remodeled. Cell adhesion and migration are necessary to islet structure remodeling. Immunohistochemical staining of paxillin demonstrated its expression within rat islets at P14 and P21. To the best of our knowledge, there have been no reports concerning the expression of paxillin in islets of Langerhans. Given the pivotal role of paxillin in cell adhesion and migration, the specific high expression of paxillin protein after birth indicates a potential role in islet structure remodeling.

The expression of paxillin at both the mRNA and protein level indicates that paxillin may be critical in mediating the biological functions of the developing pancreas, especially in maintaining islet structure and function. Regardless, these studies demonstrate that paxillin is expressed at the protein and mRNA level throughout development, indicating potential molecular signals mediated by paxillin that may control various aspects of pancreatic function and development.

In higher eukaryotes, paxillin exists as multiple isoforms (α , β , γ and δ)^[6]. Paxillin α is the principal, ubiquitously expressed isoform, whereas the β - and γ -isoforms exhibit restricted expression. The β - and γ -isoforms contain a 34- and 48-amino acid insertion, respectively, between amino acids 277/278. However, there have been reports suggesting the lack of a murine γ -isoform^[21,22]. Our study revealed that there exist two isoforms at 68 KD of paxillin protein in the pancreas during development, with different expression profiles. During the embryonic and neonatal development phases, only the smaller isoform was detected, while the larger isoform was detected only after birth. Interestingly, in the adult pancreas, the two isoforms could both be identified. It has been documented in a previous study that different paxillin isoforms show a distinct expression profile in whole embryos during development in mice, which suggested distinct physiological roles for each isoform^[7]. Thus, the differential expression of paxillin isoforms in the pancreas during development may indicate their distinct roles in pancreas

development. Of course, further studies are needed to verify our hypothesis.

The morphogenesis of embryos depends both on interactions between cells and their surrounding ECM *via* integrin complexes, and direct cell-cell borders perceived as discrete adhesion systems that may interact with each other during morphogenesis^[5,23-26]. The integrin family of transmembrane receptors physically connects the actin cytoskeleton of the cells to the ECM at focal adhesions, and thus, mediates migration and adhesion in many cells. The importance of focal adhesion in early development is well illustrated by mouse embryos deficient in fibronectin or focal adhesion components, including focal adhesion kinase (FAK), paxillin, or integrins. These embryos die early in development (days 5-10) with mesodermal defects. Data from previous studies support roles for paxillin and FAK in the organization of actin around tight junctions or adherens junctions; functions that would help stabilize or remodel cell-cell borders during the migration of this epithelial sheet^[9,12].

In summary, our present study has, for the first time, demonstrated that paxillin is localized to the islets of Langerhans in developing rat pancreata *via* immunohistochemistry and immunofluorescence. Given the important roles of paxillin in intercellular adhesion and cell migration, it may play important roles in pancreas islet formation, which is a process of cell-cell adhesion and cell migration, especially in islet structure and functional maintenance. Further investigations are needed to verify our hypothesis.

ACKNOWLEDGMENTS

Special thanks to Dr. Ying-Bing Ge for his technical skills. Our gratitude also goes to Dr. Zheng-Xian Tao for his comments and criticisms.

COMMENTS

Background

Knowledge of the regional and temporal expression of paxillin will be useful in understanding its potential role in pancreatic development. However, no study has investigated the relationship between paxillin expression and pancreas development, and the expression of paxillin during pancreatic development in rats is poorly understood.

Research frontiers

The authors examined the expression of paxillin in rat pancreas during development.

Innovations and breakthroughs

The study, for the first time, demonstrated that paxillin is localized to the islets of Langerhans in developing rat pancreata *via* immunohistochemistry and immunofluorescence. Given the important roles of paxillin in intercellular adhesion and cell migration, it may play important roles in pancreas islet formation, which is a process of cell-cell adhesion and cell migration, especially in islet structure and functional maintenance.

Peer review

This is an interesting and fairly well performed study.

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α_v integrin: A new gastrin target in human pancreatic cancer cells

Celine Cayrol, Claudine Bertrand, Aline Kowalski-Chauvel, Laurence Daulhac, Elizabeth Cohen-Jonathan-Moyal, Audrey Ferrand, Catherine Seva

Celine Cayrol, Claudine Bertrand, Aline Kowalski-Chauvel, Elizabeth Cohen-Jonathan-Moyal, Audrey Ferrand, Catherine Seva, INSERM UMR1037-Cancer Research Center of Toulouse, Paul Sabatier University Toulouse III, 31432 Toulouse cedex 4, France

Laurence Daulhac, INSERM U766, Department of pharmacology, Clermont 1 University of Pharmacy, 63001 Clermont-Ferrand, France

Author contributions: Ferrand A and Seva C planned the study and wrote the paper; Daulhac L and Cohen-Jonathan-Moyal E contributed to the design of the study and critically revised the manuscript; Cayrol C, Bertrand C and Kowalski-Chauvel A were responsible for data acquisition and analysis.

Supported by Grants from INSERM

Correspondence to: Dr. Catherine Seva, INSERM UMR1037-Cancer Research Center of Toulouse, Paul Sabatier University Toulouse III, 1 avenue J Poulhes, BP 84225, 31432 Toulouse cedex 4, France. cathy.seva@inserm.fr

Telephone: +33-5-61322408 Fax: +33-5-61322403

Received: November 30, 2010 Revised: February 12, 2011

Accepted: February 19, 2011

Published online: October 28, 2011

Abstract

AIM: To analyse α_v integrin expression induced by gastrin in pancreatic cancer models.

METHODS: α_v integrin mRNA expression in human pancreatic cancer cells was analysed using a "cancer genes" array and confirmed by real-time reverse transcription-polymerase chain reaction (PCR). Western blotting and semi-quantitative immunohistochemistry were used to examine protein levels in human pancreatic cancer cell lines and pancreatic tissues, respectively. The role of α_v integrin on gastrin-induced cell adhesion was examined using blocking anti- α_v integrin monoclonal antibodies. Adherent cells were quantified by staining with crystal violet.

RESULTS: Using a "cancer genes" array we identified α_v integrin as a new gastrin target gene in human pancreatic cancer cells. A quantitative real-time PCR approach was used to confirm α_v integrin gene expression. We also demonstrate that Src family kinases and the PI 3-kinase, two signalling pathways specifically activated by the CCK-2 receptor (CCK2R), are involved in gastrin-mediated α_v integrin expression. In contrast, inhibition of the ERK pathway was without any effect on α_v integrin expression induced by gastrin. Our results also show that gastrin modulates cell adhesion *via* α_v integrins. Indeed, *in vitro* adhesion assays performed on fibronectin show that gastrin significantly increases adhesion of pancreatic cancer cells. The use of blocking anti- α_v integrin monoclonal antibodies completely reversed the increase in cell-substrate adhesion induced by gastrin. In addition, we showed *in vivo* that the targeted CCK2R expression in the pancreas of Elas-CCK2 mice, leads to the overexpression of α_v integrin. This process may contribute to pancreatic tumour development observed in these transgenic animals.

CONCLUSION: α_v integrin is a new gastrin target in pancreatic cancer models and contributes to gastrin effects on cell adhesion.

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Key words: α_v integrin; Cell adhesion; CCK-2 receptor; Gastrin; Pancreatic cancer

Peer reviewers: Catherine Greene, PhD, Senior Lecturer, Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin 9, Ireland; Dae-Yeul Yu, PhD, Professor, Aging Research Center, Korea Research Institute of Bioscience and Biotechnology, 111 Gwahangno, Yuseong-gu, 305-806 Daejeon, South Korea

Cayrol C, Bertrand C, Kowalski-Chauvel A, Daulhac L, Cohen-Jonathan-Moyal E, Ferrand A, Seva C. α_v integrin: A new gastrin

target in human pancreatic cancer cells. *World J Gastroenterol* 2011; 17(40): 4488-4495 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v17/i40/4488.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4488>

INTRODUCTION

Pancreatic cancer has a poor prognosis with a 5-year survival rate < 5%. Despite intensive efforts to improve therapy, treatment remains unsatisfactory and most patients die within months as a result of rapid local spread of tumour or metastatic dissemination^[1]. This poor prognosis is mainly due to the propensity of this tumour to invade the adjacent structures and metastasize to distant organs early in the course of this disease; however, the molecular basis for these characteristics of pancreatic cancer is incompletely understood. A better understanding of the genes involved in tumour growth and migration may allow development of novel treatment strategies to rapidly tackle this disease.

Several lines of evidence support the role of gastrin, a digestive peptide hormone and its G protein-coupled receptor (CCK2R) in pancreatic cancer development. Gastrin and its receptor are up-regulated in human pancreatic adenocarcinoma as well as in preneoplastic lesions^[2,3]. A splice variant of the CCK2R has recently been identified, which has constitutive activity and is exclusively expressed in certain human colon and pancreatic cancers^[4-6]. In addition, we have reported in *Elas-CCK2* transgenic mice, expressing functional human CCK2R in pancreatic exocrine cells, an increased pancreatic growth, an acinar to ductal trans-differentiation, postulated to be a preneoplastic step in pancreatic carcinogenesis and the development of tumours^[7,8].

Besides proliferation, gastrin has been shown to modulate cell adhesion and migration. We and others have recently demonstrated *in vitro* that prolonged activation of the CCK2R by gastrin induces stress fibre formation, alters cell morphology, increases loss of cell-cell adhesion, as well as motility of epithelial cells^[9-12]. We have also shown the loss of intercellular adhesion in acini of *Elas-CCK2* mice before tumour formation^[13].

Several signalling pathways activated by the CCK2R have been implicated in the proliferative effects or cell migration induced by gastrin. They include: MAP-kinases^[14,15], the phosphatidylinositol 3-kinase and the JAK2/STAT3 pathway^[16,17]. In addition, Src family tyrosine kinases and p125FAK have also been shown to play a crucial role in these biological effects of gastrin^[18].

In gastric epithelial cells, several target genes of the CCK2R have already been identified. They include genes involved in gastric acid secretion^[19], early response genes, c-Fos^[20], c-Jun and c-Myc^[21,22] and other growth-related genes such as cyclin D1^[23], Reg-1^[24], or the HB-EGF^[25]. In addition, in the same cellular model, gastrin also regulates the expression of genes associated with cell migration

and invasion such as the *MMP9* gene, a matrix metalloproteinase^[26]. In several cellular models such as gastric and colonic cancer cells, intestinal epithelial cells or fibroblasts transfected with the CCK2R, gastrin has also been shown to enhance *cyclooxygenase-2* gene expression, known to play an important role in inflammation processes and carcinogenesis^[27-29].

In contrast, to our knowledge, very few gastrin-regulated genes have been identified in pancreatic models expressing the CCK2R. Recently, we showed that Reg proteins are targets of CCK2R activation and are induced during the early steps of carcinogenesis in *Elas-CCK2* mouse pancreas^[30]. In addition, we also identified β_1 integrin as a gastrin-regulated gene in human pancreatic cancer cells and demonstrated its involvement in modulation of cell adhesion by the CCK2R^[31].

In this study, we identified αv integrin, another member of the large integrin family, as a new gastrin target in the human pancreatic cancer cell line, Panc-1. Integrins which mediate cell adhesion play an important role in cell migration, survival and differentiation. Here we show *in vitro* that αv integrin is involved in the modulation of cell adhesion by the CCK2R. In addition, we demonstrate *in vivo* that the targeted CCK2R expression in the pancreas of *Elas-CCK2* mice, which present preneoplastic lesions and develop pancreatic tumours, leads to αv integrin expression.

MATERIALS AND METHODS

Cell culture

The human pancreatic cancer cell line, Panc-1 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS at 37 °C in a humidified atmosphere containing 5% CO₂. In all experiments, cells were serum-starved for 18 h prior to gastrin stimulation. Human gastrin 2-17_{ds} (Bachem, Switzerland) was used in all experiments.

RNA extraction and reverse transcription

Total RNA was isolated from Panc-1 cells treated with or without gastrin as indicated using the RNeasy RNA Isolation Kit (Qiagen, Valencia, CA, United States). After pretreating RNA with 10 units DNase (Invitrogen, Carlsbad, CA, United States), cDNA was produced from 1 μ g of total RNA using the Superscript First-Strand Synthesis System for reverse transcription-polymerase chain reaction (PCR) (Invitrogen, Carlsbad, CA, United States).

"Cancer super array"

A specific "Cancer array" (96 genes) from SuperArray (Bioscience Corporation, Beverly, MA, United States) was used in this study. Total RNA was isolated from Panc-1 cells as described above. Reverse transcription of cellular RNA was carried out with the RT-Labeling kit (SuperArray, Bioscience Corporation, Beverly, MA,

United States) according to the manufacturer's instructions. The biotinylated probes from gastrin-stimulated cells and unstimulated cells were hybridized overnight to separate membranes at 60 °C, washed with SSC/SDS solutions, incubated with the avidin-alkaline phosphatase conjugate and exposed to a chemiluminescent substrate. Analysis of the images and quantitation of the spots in both membranes were performed by the ScanAlyze 2.5 software, and normalization of the values and comparison of the intensities was achieved by the GE ArrayAnalyzer 1.3 (SuperArray, Bioscience Corporation, Beverly, MA, United States) software.

Real-time PCR

α_v integrin expression was determined *via* real-time PCR, using fluorescent SYBR green dye (Applied Biosystems, Framingham, MA, United States) to allow semi quantitative analysis of gene expression levels. Amplification was conducted using ABI-Stepone + Detection System (Applied Biosystems, Framingham, MA, United States). Relative fold changes were determined using the $2^{-\Delta\Delta CT}$ method, in which *18S* gene was used for normalization.

Primers used (18S: forward-CGCAGCTAGGAATA-ATGGAATAGG, reverse-CATGGCCTCAGTTCC-GAAA; α_v integrin: forward-TGCCAGCGCTCTTC, reverse-TGGGTGGTGTGTTGCTTTGG).

Western blotting

Western blotting analyses were performed on lysates from Panc-1 cells stimulated or not with gastrin. Fractions, containing identical levels of proteins, were separated by SDS-PAGE and analyzed by Western blotting with the indicated antibodies. The immunoreactivity was visualized with an enhanced chemiluminescence system (Pierce, IL, United States). Anti- α_v integrin antibodies were from Chemicon (Temecula, CA, United States).

Cell adhesion assay

Cell adhesion assays were carried out in 96-well plates using 10^5 cells/cm² in a final volume of 100 μ L/well of serum-free medium. Wells were coated overnight at 4 °C with fibronectin diluted at 5 μ g/mL in phosphate buffered solution (PBS) then washed twice with 100 μ L of PBS and blocked with 1% bovine serum albumin (BSA)-PBS for 30 min at room temperature before addition of the cell suspension. The cells were incubated for 2 h at 37 °C with or without gastrin. Adherent cells were fixed with 50 μ L of 96% ethanol for 10 min, stained with 50 μ L of 0.1% crystal violet, rinsed extensively with water and dried at room temperature. Stained cells were solubilised with 50 μ L of 0.2% Triton X-100 and quantified by measuring the absorbance at 570 nm. For adhesion inhibition experiments, cells were pretreated for 30 min at 37 °C with or without 5 μ g/mL function-blocking antibodies directed against α_v integrin and treated or not with gastrin for 2 h.

Animals

Homozygous *Elas-CCK2* mice used in this study have

been described previously^[8]. Homozygous *Elas-CCK2* mice in a B6SJLF1 background 3 at least 6-mo old and 3 corresponding control littermates were used. Mice were reared in a routine animal facility of the I2MR and maintained on a 12:12 h light-dark cycle. All the experiments were performed during the daytime. All procedures were approved by the I2MR Animal Facility Care Committee.

Immunohistochemistry

Mice were killed by decapitation, the pancreas was excised, fixed and embedded in paraffin using standard techniques. Immunohistochemistry was performed as previously described^[16] using anti- α_v integrin antibodies (Chemicon, Temecula, United States). Sections were incubated with the appropriate secondary and tertiary peroxidase-labelled antisera (DAKO, Glostrup, Denmark) at room temperature, exposed to a solution of diaminobenzidine. All dilutions and washes were performed with phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin.

Statistical analysis

All results are presented as mean \pm SE. Statistical significance was calculated using unpaired Student's *t* test. Values of $P < 0.05$ were considered statistically significant. All analyses were performed using "GraphPad Prism" software.

RESULTS

Gastrin increases α_v integrin expression in Panc-1 cells

In order to identify new gastrin-regulated genes, a human cancer array of 96 genes was probed with samples from either control Panc-1 cells or cells treated with gastrin for 24 h.

Among the genes positively modulated by the CCK2R in these experiments, we observed a significant increase in the expression of α_v integrin (Figure 1A). A quantitative real-time PCR approach was used to confirm and quantify the α_v integrin gene expression. In response to gastrin, the increase in α_v integrin gene expression was time-dependent. A significant effect due to gastrin was detectable 3 h after treatment. At 24 h, we observed a 5-fold increase in the expression of α_v integrin in response to gastrin (Figure 1B).

In addition, we also confirmed the increase in protein levels of α_v integrin in gastrin-stimulated cells using Western blotting analysis (Figure 1C and D).

Signalling pathways involved in α_v integrin expression stimulated by gastrin

As mentioned in the Introduction, gastrin exerts its trophic effects and modulates cell adhesion through a variety of intracellular pathways depending on the cellular model. We previously identified the signalling pathways specifically activated by the CCK2R in Panc-1 cells^[51]. They include the ERK pathway, the PI3K/AKT pathway

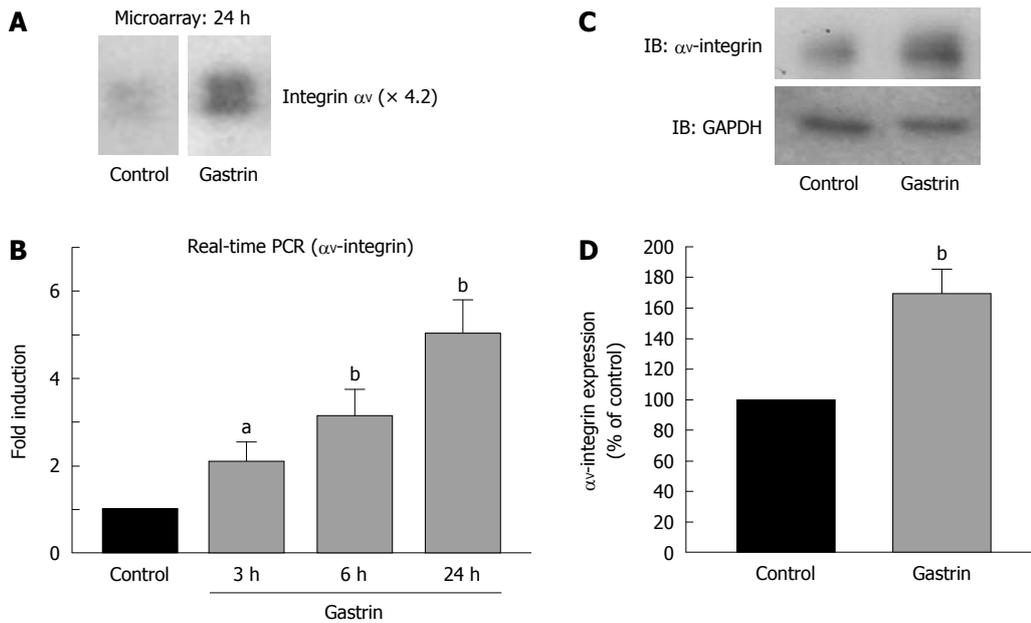


Figure 1 Increased expression of α_V integrin in response to gastrin in Panc-1 cells. A: Results of probing a 96 genes array with samples from unstimulated Panc-1 cells (control) or cells stimulated with 100 nmol/L of gastrin for 48 h; B: Real time polymerase chain reaction (PCR) analysis of α_V integrin mRNA expression in Panc-1 cells. Cells were treated or not with gastrin for the time indicated. Total RNA was isolated and α_V integrin mRNA expression was determined by real time PCR as described Materials and Methods; C, D: Expression of α_V integrin protein was examined by Western analysis following treatment of the cells with gastrin for 24 h. Blots were also probed with an antibody against GAPDH to ensure equal loading of proteins. Representative data from 3 experiments are shown. Quantifications of three experiments are presented as mean \pm SE. Significance was accepted at $P \leq 0.05$, ^a $P < 0.05$, ^b $P < 0.01$.

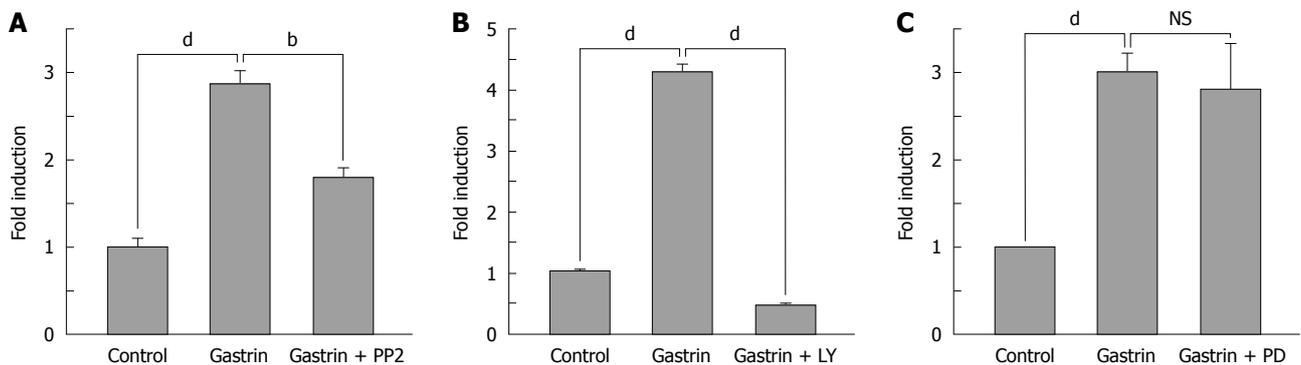


Figure 2 Signalling pathways involved in α_V integrin expression stimulated by gastrin. Cells were pretreated for 30 min with (B) a specific PI3K inhibitor (LY294002, 20 μ mol/L), (A) a Src-kinase inhibitor (PP2, 30 μ mol/L) or (C) a MEK inhibitor (PD PD098059, 20 μ mol/L) prior to gastrin stimulation. After 24 h, total RNA was isolated. Quantitative real-time polymerase chain reaction was performed as described in Materials and Methods. Quantifications of three experiments are presented as mean \pm SE. Significance was accepted at $P \leq 0.05$, ^b $P < 0.01$, ^d $P < 0.001$. NS: Not significant.

and the activation of Src-kinases.

To determine the cellular mechanism by which gastrin increased α_V integrin gene expression, we examined gastrin-regulated α_V integrin gene expression in Panc-1 by quantitative real-time PCR in the absence or presence of different specific inhibitors, LY294002, PP2, or PD098059 which block the PI 3-kinase pathway, Src family kinases and the ERK pathway, respectively. When cells were pre-incubated with PP2, the response to gastrin was decreased by 60% and totally blocked in cells pre-treated with LY294002 (Figure 2A and B), whereas the inhibitors alone did not significantly affect basal α_V integrin expression (PP2: 1.09 \pm 0.2 fold induction, LY204002: 0.93 \pm 0.35 fold induction). These results indicate that Src fam-

ily kinases and the PI 3-kinase pathway mediate gastrin-increased α_V integrin gene expression in Panc-1 cells. In contrast, the inhibitor of the ERK pathway was without any effect (Figure 2C).

Effect of gastrin on Panc-1 cell adhesion to fibronectin

In this study, we have identified α_V integrin as a new gastrin target in Panc-1 cells. Integrins act as adhesion receptors linking the extracellular matrix (ECM) to the cytoskeleton. For many cell types, integrin-mediated adhesion is required for cell growth and cell survival. In the second part of this study, we investigated whether gastrin had an effect on Panc-1 cell adhesion. In a cell adhesion assay using fibronectin-coated wells, we showed

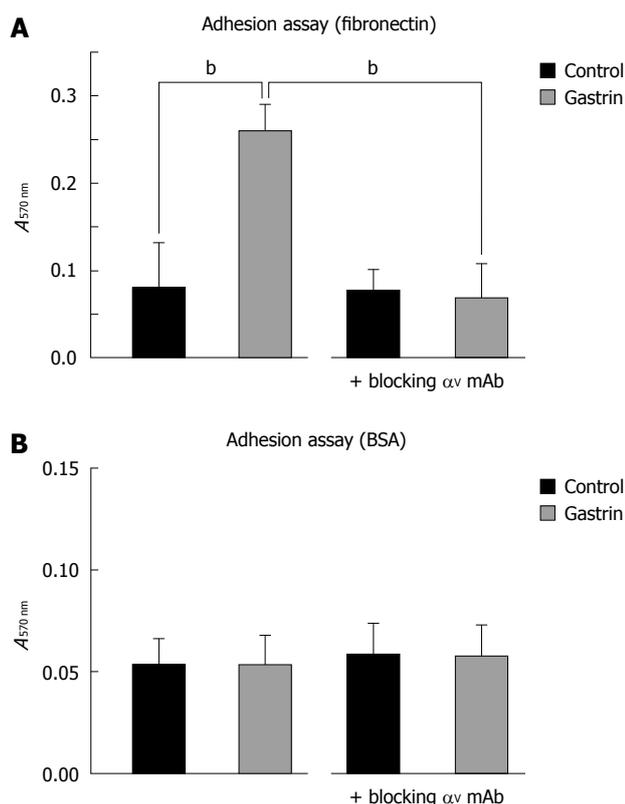


Figure 3 Effect of gastrin on Panc-1 cell adhesion. Cells were added to fibronectin-coated (A) or non-coated (BSA alone) (B) 96-wells for 2 h in the presence or absence of gastrin. Adherent cells were fixed and stained with crystal violet as described in Materials and Methods. After solubilisation, absorbance was measured at 570 nm. When indicated, Panc-1 cells were pre-incubated, with a blocking α_v mAb for 30 min prior to gastrin treatment for 2 h. Quantifications of three experiments are presented as mean \pm SE. Significance was accepted at $P \leq 0.05$. ^b $P < 0.01$. BSA: Bovine serum albumin.

that gastrin induced a significant increase in Panc-1 cell adhesion (Figure 3A). As expected in BSA only controls, we did not observe any effect of gastrin on cell adhesion (Figure 3B).

To determine the role of α_v integrin in gastrin-enhanced Panc-1 cell adhesion, we used blocking anti- α_v integrin monoclonal antibodies. When added 30 min prior to gastrin stimulation, the antibodies significantly decreased gastrin-stimulated Panc-1 cell adhesion (Figure 3A). This confirmed that α_v integrin plays an important role in Panc-1 cell adhesion stimulated by gastrin.

Immunohistochemical staining of α_v integrin in the pancreas of Elas-CCK2 mice

We recently described that Elas-CCK2 mice express human CCK2R in acini. These mice exhibited an increased pancreatic growth, an acinar to ductal trans-differentiation, postulated to be a preneoplastic step in pancreatic carcinogenesis, and developed tumors^[8].

Thus, to analyse *in vivo* the relevance of α_v integrin expression in correlation to CCK2R expression, we analysed α_v integrin overexpression in pancreatic tissue sections from Elas-CCK2 mice and control littermates using

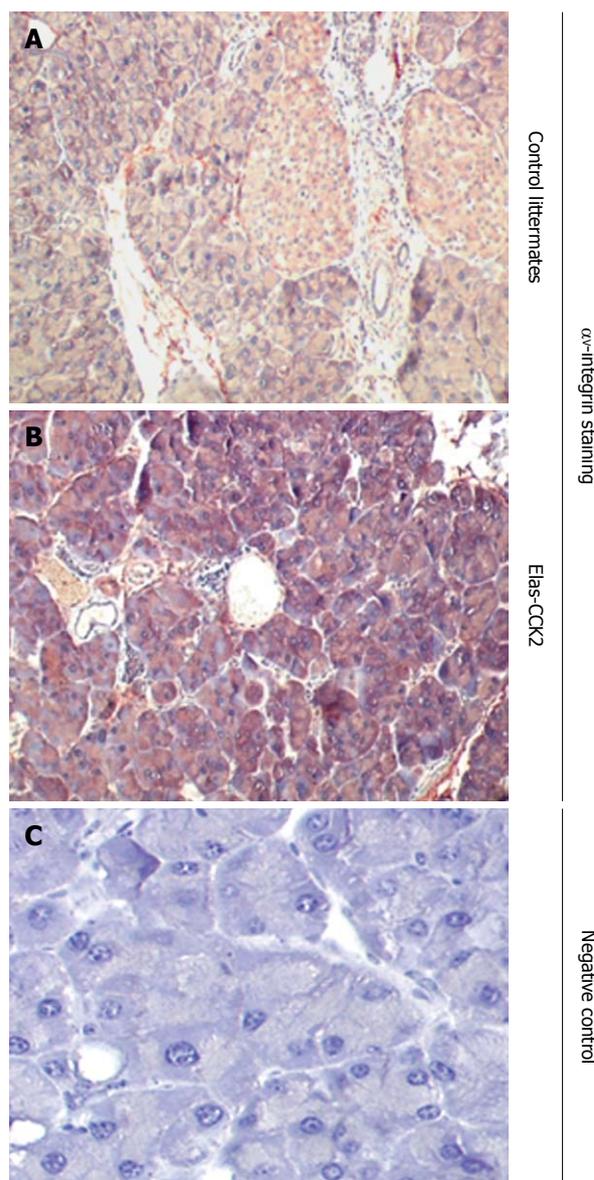


Figure 4 Overexpression of α_v integrin in the pancreas of Elas-CCK2 mice. Immunohistochemistry analysis of paraffin-embedded pancreatic tissues from Elas-CCK2 mice and control littermates were performed using antibodies specific for α_v integrin (A, B). Representative data from 3 experiments (3 different animals in each group) are shown. A negative control without secondary antibody was also included (C).

immunohistochemistry methods. As shown in Figure 4, tissues derived from Elas-CCK2 mice showed an upregulation of α_v integrin (Figure 4B) as compared to control mice (Figure 4A).

DISCUSSION

Several lines of evidence suggest that gastrin and CCK2R could contribute to pancreatic carcinogenesis by modulating processes such as proliferation, cell adhesion or migration. In the current study, we identified α_v integrin as a new gastrin-regulated gene in human pancreatic cancer cells and demonstrated its involvement in modulation of

cell adhesion by gastrin.

Integrins, a large family of cell-surface receptors, act as the bridge between ECM proteins and cytoskeletal proteins^[32]. They are crucial for cell migration but also modulate signal transduction cascades implicated in cell survival or proliferation. Several studies have demonstrated that integrins played a key role in the malignant behaviour of neoplastic cells and were important mediators of tumour invasion and metastasis formation through interactions with ECM proteins^[33-35]. Alterations in integrin expression have been correlated with aggressive growth and metastatic capacity of several tumours^[36-40]. In addition, several integrin subunits are upregulated in pancreatic carcinoma, in particular the fibronectin receptor β_1 and β_3 integrins, two subunits known to interact with α_v integrin^[41-43]. We previously identified β_1 integrin as a gastrin target in pancreatic cancer^[31]. Here, we show that gastrin increases the expression of another member of the integrin family, α_v integrin, at the mRNA and protein level in a human pancreatic tumour cell line. In addition, the use of blocking anti- α_v integrin monoclonal antibodies completely reversed the increase in cell-substrate adhesion induced by gastrin. Previously we showed an inhibitory effect of anti- β_1 integrin antibodies on gastrin-induced cell adhesion, suggesting that the heterodimer $\alpha_v\beta_1$ might be important in gastrin signalling. However, since the β_3 subunit is also overexpressed in pancreatic adenocarcinomas and can interact with α_v subunit, it might be important to analyse, using anti- β_3 integrin, whether it also contributes to gastrin-induced cell adhesion.

In gastric cells, the regulation by gastrin of numerous genes, including genes involved in gastric acid secretion^[19], early response genes^[20] or genes associated with cell migration^[26], involves the activation of the ERK1/2 pathway. In other cellular models such as colon cancer cells, the PI-3-kinase pathway is also involved in the regulation of gastrin target genes. To our knowledge, very little is known about gene regulation by gastrin in pancreatic tumour models. In this study, we demonstrated in pancreatic cancer cells that Src family kinases and the PI-3-kinase pathway play a crucial role in the expression of α_v integrin modulated by gastrin.

The present study and previously published studies by our group demonstrate that gastrin affects cell adhesion and migration by different complementary mechanisms. First, gastrin modulates cell-cell adhesion by inducing a dissociation of the E-cadherin-catenin-complex leading to cytoskeleton reorganization and cell invasion. Here, we show that gastrin also modulates cell-substrate adhesion *via* the α_v integrin.

Another important finding of this study is that the expression of a G protein-coupled receptor, namely the CCK2R, targeted in mouse pancreatic acinar tissue, leads to the over-expression of α_v integrin. These transgenic mice display an increased growth of the pancreas and develop preneoplastic lesions then pancreatic tumours presenting a ductal phenotype similar to that observed in

human pancreatic tumours.

ACKNOWLEDGMENTS

We thank Dr. André F (Marseille, France) for blocking anti- α_v integrin antibodies. We thank Dr. Dufresne M (Toulouse, France) for providing us with pancreatic tissue sections from Elas-CCK2 mice and control mice.

COMMENTS

Background

Pancreatic cancer has a poor prognosis with a 5-year survival rate < 5%. Despite intensive efforts to improve therapy, treatment remains unsatisfactory and most patients die within months as a result of rapid local spread of tumour or metastatic dissemination. A better understanding of the genes involved in tumour growth and migration may allow the development of novel treatment strategies to rapidly tackle this disease.

Research frontiers

Integrins play a key role in the malignant behaviour of neoplastic cells and are important mediators of tumour growth invasion and metastasis. Several publications support the role of gastrin, a peptide hormone, in pancreatic cancer development. However, the mechanism by which gastrin regulates integrin signalling in pancreatic cancer has not been addressed. In this study, the authors show that regulation of α_v integrin by gastrin may contribute to pancreatic tumour development.

Innovations and breakthroughs

This is the first study to report that α_v integrin is a gastrin target in human pancreatic cancer cells. Furthermore, we identified the signalling pathways involved in gastrin-mediated α_v integrin expression. Another important finding of this study is that the expression of a G protein-coupled receptor, namely the CCK2R, targeted in mouse pancreatic acinar tissue, leads to the over-expression of α_v integrin. These transgenic mice display an increased growth of the pancreas and develop preneoplastic lesions then pancreatic tumours presenting a ductal phenotype similar to that observed in human pancreatic tumours.

Applications

A better understanding of the genes involved in tumour growth and migration may allow the development of novel treatment strategies for patients with pancreatic cancer.

Terminology

Integrins, a large family of cell-surface receptors, act as the bridge between extracellular matrix proteins and cytoskeletal proteins. They are crucial for cell migration but also modulate signal transduction cascades implicated in cell survival or proliferation.

Peer review

This is a very well written and clearly laid out manuscript. The authors appear to have carried out the experiments to a high standard and the data are convincing. There are one or two experimental controls that are not included however if the authors can include these or comment on the fact that their inclusion would strengthen their observations.

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S- Editor Tian L L- Editor Webster JR E- Editor Zheng XM

Cell proliferation of esophageal squamous epithelium in erosive and non-erosive reflux disease

Carlo Calabrese, Lorenzo Montanaro, Giuseppina Liguori, Elisa Brighenti, Mauela Vici, Paolo Gionchetti, Fernando Rizzello, Massimo Campieri, Massimo Derenzini, Davide Trerè

Carlo Calabrese, Giuseppina Liguori, Paolo Gionchetti, Fernando Rizzello, Massimo Campieri, Department of Clinical Medicine, University of Bologna, 40138 Bologna, Italy
Lorenzo Montanaro, Elisa Brighenti, Mauela Vici, Davide Trerè, Department of Experimental Pathology, University of Bologna, 40138 Bologna, Italy

Massimo Derenzini, Clinical Department Radiological and Histocytopathological Sciences, University of Bologna, 40138 Bologna, Italy

Author contributions: Calabrese C, Montanaro L and Trerè D designed the research; Calabrese C, Montanaro L, Liguori G, Brighenti E, Vici M and Trerè D performed the research; Calabrese C, Montanaro L, Trerè D, Derenzini M, Gionchetti P, Rizzello F, Campieri M analyzed the data; Calabrese C, Montanaro L and Trerè D wrote the paper.

Correspondence to: Carlo Calabrese, MD, PhD, Department of Clinical Medicine, University of Bologna, 40138 Bologna, Italy. carlo.calabrese2@unibo.it

Telephone: +39-0516-364191 Fax: +39-0516-364191

Received: March 17, 2011 Revised: June 2, 2011

Accepted: June 9, 2011

Published online: October 28, 2011

Abstract

AIM: To elucidate cell proliferation in erosive reflux disease (ERD) and non-erosive reflux disease (NERD), we evaluated markers in squamous epithelial cells.

METHODS: Thirty-four consecutive patients with gastroesophageal-reflux-disease-related symptoms (21 NERD and 13 ERD) were evaluated for the enrolment into the study. All patients underwent 24-h pH monitoring, standard endoscopy, and biopsy for histological evaluation. The expression of cyclins D and A was evaluated by real-time reverse transcription polymerase chain reaction (RT-PCR) from isolated epithelial cells. In all samples, analysis of the isolated cell population revealed the presence of epithelial cells only.

RESULTS: Real-time RT-PCR showed that, in patients

with ERD, the relative expression of cyclin D1 mRNA in esophageal epithelium was strongly decreased in comparison with NERD patients. The mean value of relative expression of cyclin D1 mRNA in NERD patients was 3.44 ± 1.9 , whereas in ERD patients, it was 1.32 ± 0.87 ($P = 0.011$). Real-time RT-PCR showed that, in patients with ERD, relative expression of cyclin A mRNA in esophageal epithelium was decreased in comparison with that in NERD patients (2.31 ± 2.87 vs 0.66 ± 1.11). The mean bromodeoxyuridine labeling index in the NERD patients was $5.42\% \pm 1.68\%$, whereas in ERD patients, it was $4.3\% \pm 1.59\%$.

CONCLUSION: We confirmed reduced epithelial proliferation in ERD compared with NERD patients, and that individuals who develop ERD are characterized by weaker epithelial cell proliferation.

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Key words: Esophageal cell proliferation; Erosive reflux disease; Non-erosive reflux disease; Gastroesophageal reflux disease; Cyclin A; Cyclin D; Bromodeoxyuridine

Peer reviewer: Wojciech Blonski, MD, PhD, University of Pennsylvania, GI Research-Ground Centrex, 3400 Spruce St, Philadelphia, PA 19104, United States

Calabrese C, Montanaro L, Liguori G, Brighenti E, Vici M, Gionchetti P, Rizzello F, Campieri M, Derenzini M, Trerè D. Cell proliferation of esophageal squamous epithelium in erosive and non-erosive reflux disease. *World J Gastroenterol* 2011; 17(40): 4496-4502 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4496.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4496>

INTRODUCTION

Most of the patients with gastroesophageal reflux disease

(GERD) fall into one of two categories: non-erosive reflux disease (NERD) or erosive reflux disease (ERD). The two main phenotypes of GERD appear to have different pathophysiological and clinical characteristics. NERD is the most common phenotypic presentation of GERD. Although separation of ERD and NERD on a clinical level is difficult, there are clearly physiological, pathophysiological, anatomical, and even histological characteristics that are unique to NERD. Natural course studies have demonstrated that most NERD patients do not progress over time to ERD or even Barrett's esophagus. NERD patients compared to those with ERD demonstrate a highly variable and unpredictable symptomatic response rate to antireflux treatment^[1].

Cell replication of basal layers is hypothesized to be one of the causes implicated in the resistance of the mucosa and structural epithelial defense. In previous investigations, we have demonstrated that, in patients with GERD, the number of proliferating cells, evaluated by Ki-67 immunostaining, was reduced in esophageal mucosa exposed to chronic acid-peptic insult^[2,3]. Two reasonable hypotheses can be suggested to explain the reduced epithelial proliferation activity observed in GERD: (1) chronic cell damage induced by GER determines a reduction in the proliferation rate of esophageal epithelium; or (2) a constitutive lower capacity for cell proliferation brings a major susceptibility to damage induced by GER.

Our findings are in contrast to the results of a recent study^[4] on the cell proliferation of squamous epithelium in GERD. This study has shown a significantly higher number of proliferating cells in GERD patients compared with that in controls, as evaluated by Ki-67 immunostaining.

To elucidate the different proliferation in NERD and ERD patients, the present study evaluated squamous epithelial cell proliferation in patients with GERD, in comparison with NERD, by measuring the S-phase fraction using the bromodeoxyuridine labeling index (BrdU-LI), and by quantifying the expression of cyclins A and D, which are associated with cell cycle progression.

MATERIALS AND METHODS

Study design

Fifty consecutive patients with GERD-related symptoms were evaluated for enrolment into the study. Inclusion criteria were the presence of typical symptoms (heartburn and/or regurgitation) for at least 1 year (frequency was > 2 times/wk) and abnormal 24-h pH parameters and symptom-association probability (SAP). Exclusion criteria were patients with esophageal or gastric malignancy or histologically proven Barrett's esophagus, gastric or duodenal ulcer, previous esophageal or gastric surgery, extra-esophageal symptoms, patients taking antisecretory or prokinetic drugs at least 30 and 15 d before the procedure, respectively. Forty-six patients (mean age 45.2 ± 13.4 years, range 22-78 years; 20 men) fulfilled the inclusion/exclusion criteria and were evaluated. All these

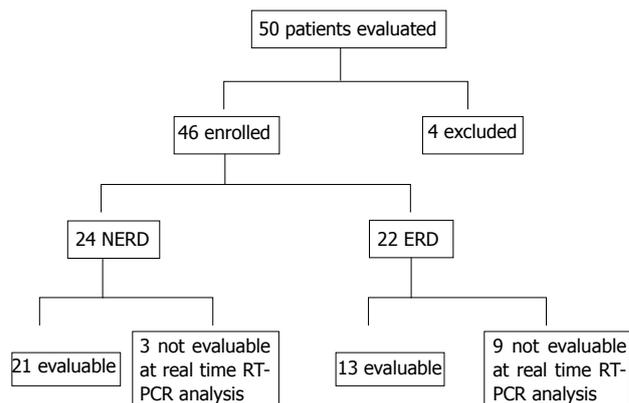


Figure 1 Study profile. RT-PCR: Reverse transcription polymerase chain reaction; NERD: Non-erosive reflux disease; ERD: Erosive reflux disease.

Table 1 Demographic, endoscopic, histological and 24-h esophageal pH monitoring data of the studied population

	NERD	ERD
No. of subjects	21	13
Sex (M/F)	9/12	7/6
Mean age ± SD (yr) (range)	44.2 ± 14.9 (22-73)	54.4 ± 15.7 (29-78)
Endoscopy		
Normal	21	0
A	0	0
B	0	7
C	0	6
D	0	0
Histology		
Normal	21	12
Mild	0	1
Moderate	0	0
Severe	0	0
24-h pH monitoring		
Mean % of acid exposure time ± SD	10.4 ± 1.3	10.7 ± 1.4
Mean number of acid reflux events ± SD	126 ± 20	128 ± 22

M: Male; F: Female; NERD: Non-erosive reflux disease; ERD: Erosive reflux disease.

patients underwent standard endoscopy and biopsy for histological evaluation. Twenty-four had an apparently normal esophageal mucosa at endoscopy (NERD), whereas 22 had ERD. None of the patients had received cyclical therapy with proton pump inhibitors (PPIs) (not more than 8 wk in the past year). This study was single-blinded for the pH, histological, immunostaining and real-time reverse transcription polymerase chain reaction (RT-PCR) evaluations.

The frequency and intensity of symptoms and their impact on quality of life were registered using a structured and validated questionnaire for the diagnosis of GERD^[5], and patients with a score > 3.1 were considered positive. For real-time RT-PCR, only 34 patients (mean age 47.08 ± 16.04 years, range 22-73 years; 16 men) were evaluable (Figure 1). Twenty-one had an apparently normal esophageal mucosa at endoscopy (NERD), whereas 13 had ERD (Table 1).

Patients gave written informed consent to participate in the study, which was approved by the local research ethical committee.

Twenty-four-hour ambulatory pH monitoring

Every patient underwent 24-h esophageal pH monitoring according to standard methodology. To define better the localization of the lower esophageal sphincter (LES) and upper esophageal sphincter (UES), esophageal manometry was performed before pH monitoring, with a water-perfused catheter that incorporated three distal openings, radially oriented for LES pressure recording, and three side-hole recording sites at 5, 10 and 15 cm above the distal openings. Multichannel 24-h pH monitoring was performed using two probes, with one and two antimony sensors, respectively, with a separate skin reference (Zinetics Medical Inc., Salt Lake City, UT, United States). In accordance with manometric findings, the three pH sensors were placed at the gastric level, at 5 cm above the LES and 10 cm below the UES, respectively. Data were stored on a single portable digital recorder (Digitrapper pH 200; Medtronic, Minneapolis, MN, United States). Before each study, the pH probe was calibrated in buffer solutions of pH 7 and pH 1.

During the test day, meal time and composition were standardized. The reflux parameters were assessed according to Johnson and DeMeester^[6]. Of these, only the percentage of time spent at pH < 4.0 over 24 h was evaluated. The pH testing was considered abnormal if pH < 4.0 was present for > 5% of the total 24 h. The SAP was calculated according to Weusten *et al.*^[7] and was considered positive if it exceeded 95%.

Endoscopic evaluation

Patients underwent upper gastrointestinal (GI) endoscopy (videogastroscope Olympus GIF 160) after sedation by intravenous midazolam (2.5 mg), to assess the presence or absence of erosions.

The Los Angeles classification was used to grade esophagitis^[8]. In each subject, eight specimens were taken with standardized biopsy forceps (Olympus FB 24K), from each of the four quadrants, two bites from each quadrant, 5 cm above the squamous-columnar junction (SCJ), from macroscopically intact (non-eroded) esophageal mucosa. The SCJ (or Z-line) was defined as the border between gastric glandular and esophageal squamous epithelium, and it roughly corresponded to the proximal edge of the gastric folds.

Of the eight biopsies taken during endoscopy from each patient, two were used for total RNA extraction, and two for BrdU labeling. Four were oriented to appropriate cellulose acetate supports (Endofilters Bioptica, Milan, Italy), fixed in 4% buffered formalin, and embedded in paraffin, for processing by hematoxylin-eosin for histological and immunohistochemical analysis.

Histological evaluation

Four-micrometer-thick serial sections were cut from each paraffin block and stained with hematoxylin-eosin. For each case, whole longitudinally sectioned samples were examined. Esophagitis was identified and graded according to the classification of Ismail-Beigi *et al.*^[9]: (1) the de-

gree of basal cell hyperplasia, expressed as a percentage of epithelial thickness: none (0%-15%), mild (16%-33%), moderate (34%-67%), severe (> 67%); (2) presence or absence of papillary zone elongation, determined by calculating papillary length as a percentage of epithelial thickness: absent (0%-67%) and present (> 67%); and (3) density of neutrophil and eosinophil infiltration: none (0/high power field), mild (1-2/high power field), moderate 3-10/high power field) and severe (> 10/high power field). The area of one high power field was 0.229 mm.

In vitro BrdU incorporation and immunohistochemical evaluation of S-phase cells

Each biopsy was immersed in 5 mL RPMI 1640 containing non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum (FBS) supplemented with 160 µmol/L BrdU (Sigma-Aldrich, St Louis, MO, United States) and incubated for 4 h at 37 °C in a 5% CO₂/air incubator. Tissues were rapidly rinsed with three washes of cold phosphate buffered saline (PBS), fixed in 10% buffered formalin, and embedded in paraffin. Sections were cut from each paraffin block and picked up on poly-L-lysine-coated slides. Sections were dewaxed, hydrated through decreasing concentrations of ethanol, rinsed in distilled water, and autoclaved in 10 mmol/L sodium citrate buffer (pH 6.0) at 120 °C for 21 min for antigen retrieval. After cooling and washing, the endogenous peroxidase activity was quenched using 3% hydrogen peroxide in absolute methanol for 10 min at room temperature. Sections were incubated with primary mouse anti-BrdU antibody (Bu20a; Abcam, Cambridge, United Kingdom) diluted in 1% bovine serum albumin in PBS overnight at 4 °C, using appropriate dilutions.

Sections were processed according to a non-biotin amplified method (NovoLink™ Polymer Detection System; Novocastra Laboratories, Newcastle Upon Tyne, United Kingdom) and counterstained with hematoxylin.

Quantitative analysis of BrdU immunostaining was performed on contiguous field visualized on the color monitor of a personal computer equipped with a 3 CCD (charge-couple device) color video camera (KY F55B; JVC, Pinebrook, NJ, United States) connected to a light microscope (Leitz DIAPLAN, Wetzlar, Germany). For each case, whole longitudinally sectioned samples were examined. Samples that did not contain at least 1000 cells were excluded. Quantitative evaluation was only carried out on portions of epithelium in between vertically sectioned stromal papillae, and corresponding to 100 µm from the basal layer. BrdU-LI was defined as the ratio of BrdU-positive nuclei to the total number of epithelial cells, and was expressed as a percentage.

Esophageal epithelial cell isolation, RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from esophageal epithelial cells that were isolated as follows. Esophageal biopsy samples were washed in PBS and incubated in 0.5% collagenase type II (C6885; Sigma-Aldrich) in 4-(2-hydroxyethyl)-1-

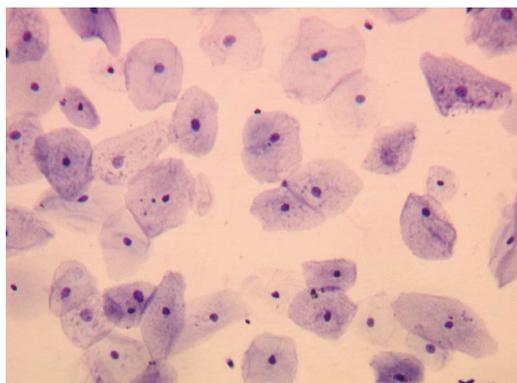


Figure 2 Cytological preparation from esophageal biopsy after epithelial cell isolation. Only epithelial cells were present. Toluidine blue staining (300 ×).

piperazineethanesulfonic acid (HEPES) buffer for 30 min at 37 °C in a shaking bath.

Collagenase activity was blocked by adding the same volume of 20% FBS in HEPES buffer. The digested material was re-suspended and passed through a 40- μ m pore size cell strainer (BD Falcon™, Franklin Lakes, NJ United States) and centrifuged for 5 min at 800 *g*. Cells were washed in PBS and counted in a hemocytometer. From each biopsy sample, an average of 50 000 cells were recovered. Cell morphology was evaluated by seeding cells on poly-L-lysine-coated slides for 2 h at 37 °C. Cells were fixed with 2% paraformaldehyde in PBS for 5 min and stained with 1% toluidine blue in distilled water for 1 min (Figure 2).

Total RNA was extracted from isolated esophageal epithelial cells using TRI Reagent (Ambion, Austin, TX, United States) according to the manufacturer's instructions. Whole cell RNA was quantified spectrophotometrically and 2 μ g RNA for each sample was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States), following the manufacturer's protocol.

The relative expression of cyclin A (CCNA1), cyclin D1 (CCND1) and the housekeeping gene β -glucuronidase were evaluated by real-time polymerase chain reaction (PCR) performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using TaqMan Gene Expression Assay primers and probe kits (Assays catalog number Hs00927505 for CCNA1 and Hs00277039 for CCND1; Applied Biosystems). Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. For each sample, three replicates were analyzed. The relative amounts of the transcripts were calculated with the $2^{-\Delta\Delta CT}$ method against aliquots from a single preparation of calibrator cDNA from the U2OS cell line.

Statistical analysis

Differences between groups were assessed by Student's *t* test. $P < 0.05$ was considered statistically significant. Data were analyzed with SPSS software (SPSS, Chicago, IL, United States).

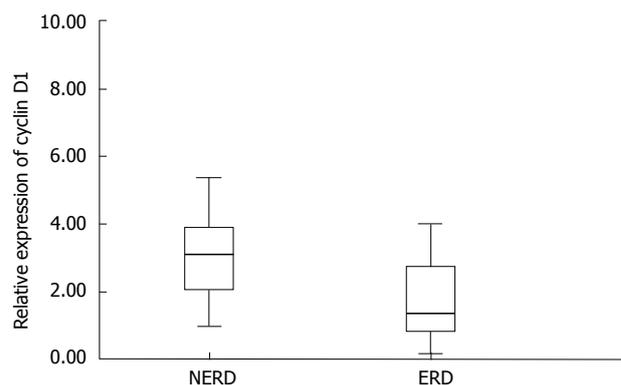


Figure 3 Box plots of relative expression of cyclin D1 mRNA by real-time RT-PCR analysis; median (bold line in box), and interquartile range (upper and lower lines of the box) in human esophageal mucosa of NERD and ERD patients ($P < 0.01$). RT-PCR: Reverse transcription polymerase chain reaction; NERD: Non-erosive reflux disease; ERD: Erosive reflux disease.

RESULTS

At pH monitoring, the percentage time with esophageal pH < 4 in the two groups of patients (NERD and ERD) was $10.4\% \pm 1.3\%$ and $10.7\% \pm 1.4\%$, respectively. No significant differences were found in the mean percentage time between the two groups.

Histological analysis showed that, among 13 patients affected by erosive esophagitis in endoscopic normal mucosa, 12 had a normal pattern, and 1 had mild esophagitis. None of the patients with NERD showed signs of esophagitis (Table 1).

Expression of cyclins A and D was evaluated by real-time RT-PCR from isolated epithelial cells. To check for purity of isolated cells, morphology was assessed after toluidine blue staining (Figure 2). In all samples evaluated, analysis of the isolated cell population revealed the presence of epithelial cells only.

Real-time RT-PCR analysis shows that, in patients with ERD, the relative expression of cyclin D1 mRNA, in esophageal epithelium, was strongly decreased in comparison with that of NERD patients (Figure 3). In particular, the relative expression of cyclin D1 mRNA in NERD epithelium was twofold higher, and showed elevated variability between patients, with respect to ERD epithelium. The relative expression of cyclin D1 mRNA ranged from 0.17 to 8.36 among all patients, with a mean (\pm SD) value of 2.41 ± 1.8 . The mean (\pm SD) cyclin D1 value in 21 NERD patients was 3.44 ± 1.9 , whereas in 13 ERD patients, it was 1.32 ± 0.87 ($P = 0.011$).

Only 25 of the 34 patients enrolled were evaluable for real-time RT-PCR analysis of cyclin A mRNA (18 NERD and 7 ERD). The relative expression of cyclin A mRNA ranged from 0 to 8.13 among all patients with a mean (\pm SD) value of 1.84 ± 2.59 . Real-time RT-PCR analysis showed that, in patients with ERD, the relative expression of cyclin A mRNA in esophageal epithelium was decreased in comparison with that in NERD patients (Figure 4). In particular, the mean (\pm SD) cyclin A value of NERD patients was 2.31 ± 2.87 , whereas in ERD patients, it was 0.66 ± 1.11 . Despite the fact that

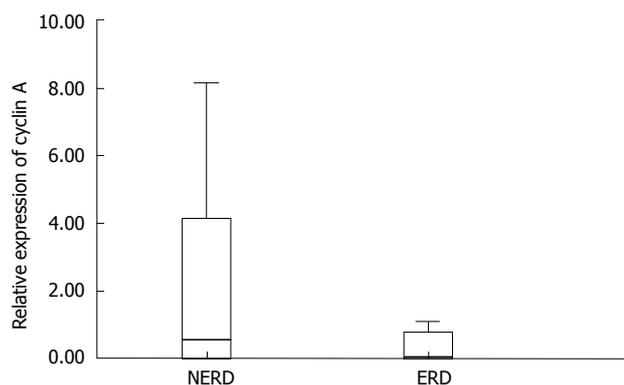


Figure 4 Box plots of relative expression of cyclin A mRNA by real-time RT-PCR analysis values; median (bold line in box), and interquartile range (upper and lower lines of the box) in human esophageal mucosa of NERD and ERD patients. RT-PCR: Reverse transcription polymerase chain reaction; NERD: Non-erosive reflux disease; ERD: Erosive reflux disease.

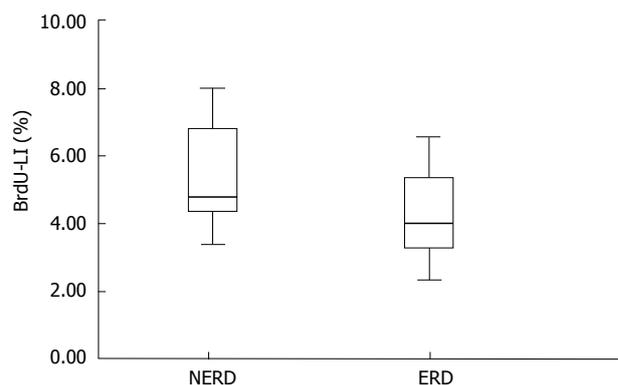


Figure 5 Box plots of BrdU-LI analysis values, median (bold line in the box), and interquartile range (upper and lower lines of the box) in human esophageal mucosa of NERD and ERD patients. NERD: Non-erosive reflux disease; ERD: Erosive reflux disease.

the relative expression of cyclin A mRNA in NERD epithelium was fourfold higher than in ERD epithelium, the difference between the two groups was not statistically significant ($P = 0.158$); both for the low number of cases evaluated, in particular in the ERD group, and for the high variability of the values relative to NERD patients.

Twelve patients were evaluable for BrdU analysis. BrdU-LI ranged from 2.33% to 8%, with a mean (\pm SD) value of $4.95\% \pm 1.67\%$. The mean BrdU-LI of the NERD patients ($n = 7$) was $5.42\% \pm 1.68\%$, whereas in ERD patients ($n = 5$), it was $4.3\% \pm 1.59\%$ (Figure 5). Once again, NERD epithelium showed a greater number of BrdU-positive cells than ERD epithelium did, but the difference between the two groups was not statistically significant ($P = 0.272$).

DISCUSSION

In the present study, we evaluated a series of esophageal biopsies to define the proliferation activity of the epithelium in patients with erosive or non-erosive GERD. In previous investigations, we have demonstrated that, in patients with GERD, cell proliferation evaluated by MIB1 immunostaining was reduced in esophageal mucosa exposed to chronic acid-peptic insult^[2,3]. In particular, patients with NERD and ERD showed a decrease in cell proliferation to 50% and 75%, respectively, compared to normal subjects^[2].

In contrast to our results, Mastracci *et al*^[4] have found that MIB1 immunostaining of GERD patients is significantly greater than in controls. These different data might reflect different sampling conditions that could influence the proliferating activity of the epithelial cells. In particular, Mastracci and co-workers have evaluated specimens that were taken from 2-4 cm to the Z line, and observed a progressive decrease in the Ki-67 LI by increasing the distance from the Z line.

Feagins *et al*^[10] have shown that multiple acid exposures decrease cell proliferation in non-neoplastic,

telomerase-immortalized Barrett's cell lines. This decrease in cell proliferation is the result of a delay in cell cycle progression that is mediated by p53. In agreement with these results, we have recently demonstrated that, in patients with ERD and NERD, long-term PPI therapy increases esophageal cell proliferation^[3]. These data confirm that acid-peptic insults have an antiproliferative effect on esophageal epithelial cells.

In the present study, only patients with at least a 1-year history of GERD were included. Upper endoscopy was performed and biopsies were taken only in apparently normal mucosa at 5 cm above the Z-line. In this way, we studied the behavior of the mucosa exposed to chronic acid insult, but far from erosions, and especially, from reparative changes secondary to the lack of the superficial mucosa, where basal cell hyperplasia has been reported^[11], which can be characterized by increased proliferative activity.

Regardless of these considerations, in the present study, we evaluated proliferative activity of the epithelium in patients with erosive and non-erosive GERD. For this purpose, three proliferation markers were assessed: cyclins A and D relative expression, evaluated by real-time RT-PCR, and *in vitro* BrdU incorporation for immunohistochemical detection of S-phase cells in histological samples.

Cyclins are a family of proteins involved in cell cycle regulation. Cyclin expression rises and falls at various stages of the cell cycle, thus activating specific cyclin dependent kinases (CDKs), which, by phosphorylation of multiple substrates, control a number of critical steps in cell cycle progression^[12]. Cyclin D1 is encoded by the *CCND1* gene located on chromosome 11q13, and in association with CDK4 or CDK6, regulates the transition from G1 to S phase^[13,14]. It is synthesized in response to extracellular mitogenic signals and is maximally expressed in mid-to-late G1-phase^[15]. Cyclin A, in association with CDK1 or CDK2, promotes the transition from G2 to M phase^[16], and is expressed later in the cell cycle, during DNA replication, achieving its maximal levels during late S-phase^[17,18]. Cyclins D1 and A are regarded

as specific markers of the G1 and S phases of the cell cycle, respectively. Therefore, in the present study, we evaluated their mRNA expression to assess the proliferative activity of esophageal epithelial cells. In previous investigations, we have in fact demonstrated that the relative expression of cyclin mRNA is directly related to the cell proliferation rate in breast cancer specimens^[19]. Moreover, to identify S-phase cells specifically, in the present study, DNA-synthesizing cells were detected *in situ* after BrdU incorporation by immunohistochemical analysis with anti-BrdU antibodies.

Our results demonstrated that cyclin D, as a marker of G1 phase, was significantly higher in NERD compared to ERD. Also the S-phase markers evaluated in our study (cyclin A and BrdU) were higher in NERD compared to ERD, although in this case, because of the small number of evaluable samples, the difference was not statistically significant. The reduction in the number of samples analyzed for cyclin A compared to those analyzed for cyclin D was due to the fact that the thickness of the epithelium in ERD was significantly reduced, and therefore, in these samples, it was not always possible to isolate a sufficient number of epithelial cells for molecular analysis.

The limitation of this study was the small number of patients, but this is believed to be the first study to evaluate, at the molecular level, esophageal epithelial cells. This method is clean but it creates considerable tissue loss.

In conclusion, the present study confirmed our previous results regarding the reduction of epithelial proliferative activity in ERD compared with NERD patients. Besides, this study supports the previous data on an antiproliferative effect of acid-peptic injury in esophageal cell epithelium, and reinforces the idea that individuals who develop ERD might be genetically characterized by weaker epithelial cell proliferation. On the other hand, patients with more efficient epithelial proliferation capability could have a lower probability of developing macroscopic mucosal lesions when stressed by acid and pepsin. Further studies are required to understand better the mucosal defense mechanisms, and in particular, those controlling the cellular proliferative activity of esophageal mucosa.

COMMENTS

Background

Cell replication in basal layers has been suggested as one of the causes of mucosal resistance and structural epithelial defense. To elucidate better the different proliferative activity between non-erosive reflux disease (NERD) and erosive reflux disease (ERD), the authors evaluated a series of molecular and immunohistochemical markers of cell proliferation in squamous epithelial cells of patients with gastroesophageal reflux disease (GERD).

Research frontiers

In previous investigations, the authors have demonstrated that, in patients with GERD, cell proliferation evaluated by MIB1 immunostaining is reduced in esophageal mucosa exposed to chronic acid-peptic insult, in contrast with other studies. These different data might reflect different sampling conditions that could influence the proliferating activity of the epithelial cells.

Innovations and breakthroughs

The present study confirmed the previous results with regard to reduction of epithelial cell proliferative activity in ERD compared with NERD patients. It supports previous data on an antiproliferative effect of acid-peptic injury in esophageal cell epithelium, and reinforces the idea that individuals who develop ERD might be genetically characterized by a weaker proliferating epithelial cell capability.

Applications

This paper shows that patients with more efficient epithelial proliferative capability could have a lower probability of developing macroscopic mucosal lesions when stressed by acid and pepsin.

Peer review

The paper by Dr. Calabrese and colleagues discusses activity of cells in esophageal squamous epithelium in patients with NERD and ERD. This is a very well written manuscript that contributes to the knowledge of the molecular mechanisms in patients with GERD.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Zhang DN

Liver hemangioma and vascular liver diseases in patients with systemic lupus erythematosus

Annalisa Berzigotti, Marilena Frigato, Elena Manfredini, Lucia Pierpaoli, Rita Mulè, Carolina Tiani, Paola Zappoli, Donatella Magalotti, Nazzarena Malavolta, Marco Zoli

Annalisa Berzigotti, Lucia Pierpaoli, Carolina Tiani, Paola Zappoli, Donatella Magalotti, Marco Zoli, Department of Internal Medicine, Nephrology and Ageing, University of Bologna, 40138 Bologna, Italy

Marilena Frigato, Elena Manfredini, Rita Mulè, Nazzarena Malavolta, UOS Reumatologia, Policlinico S, Orsola-Malpighi, 40138 Bologna, Italy

Author contributions: Berzigotti A and Frigato M designed the research; Berzigotti A, Manfredini E, Pierpaoli L, Mulè R, and Magalotti D performed the research; Berzigotti A and Tiani C analyzed the data; Berzigotti A and Zappoli P wrote the paper; Malavolta N and Zoli M supervised the entire work.

Supported by Department of Internal Medicine, Nephrology and Ageing of University of Bologna, Italy

Correspondence to: Marco Zoli, MD, Department of Internal Medicine, Nephrology and Ageing, University of Bologna, Policlinico S Orsola-Malpighi, Via Albertoni 15, 40138 Bologna, Italy. marco.zoli@unibo.it

Telephone: +39-051-6362211 Fax: +39-051-6362210

Received: August 9, 2010 Revised: October 28, 2010

Accepted: November 5, 2010

Published online: October 28, 2011

Abstract

AIM: To investigate whether systemic lupus erythematosus (SLE) is associated with benign focal liver lesions and vascular liver diseases, since these have been occasionally reported in SLE patients.

METHODS: Thirty-five consecutive adult patients with SLE and 35 age- and sex-matched healthy controls were evaluated. Hepatic and portal vein patency and presence of focal liver lesions were studied by colour-Doppler ultrasound, computerized tomography and magnetic resonance were used to refine the diagnosis, clinical data of SLE patients were reviewed.

RESULTS: Benign hepatic lesions were common in SLE patients (54% vs 14% controls, $P < 0.0001$), with

hemangioma being the most commonly observed lesion in the two groups. SLE was associated with the presence of single hemangioma [odds ratios (OR) 5.05; 95% confidence interval (CI) 1.91-13.38] and multiple hemangiomas (OR 4.13; 95% CI 1.03-16.55). Multiple hemangiomas were associated with a longer duration of SLE (9.9 ± 6.5 vs 5.5 ± 6.4 years; $P = 0.04$). Imaging prior to SLE onset was available in 9 patients with SLE and hemangioma, showing absence of lesions in 7/9. The clinical data of our patients suggest that SLE possibly plays a role in the development of hemangioma. In addition, a Budd-Chiari syndrome associated with nodular regenerative hyperplasia (NRH), and a NRH associated with hepatic hemangioma were observed, both in patients hospitalized for abdominal symptoms, suggesting that vascular liver diseases should be specifically investigated in this population.

CONCLUSION: SLE is associated with 5-fold increased odds of liver hemangiomas, suggesting that these might be considered among the hepatic manifestations of SLE.

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Key words: Colour-Doppler ultrasound; Portal hypertension; Rheumatic diseases; Portal vein; Hepatic vein thrombosis

Peer reviewer: Dr. Herwig R Cerwenka, Professor, Department of Surgery, Medical University of Graz, Auenbruggerplatz 29, A-8036 Graz, Austria

Berzigotti A, Frigato M, Manfredini E, Pierpaoli L, Mulè R, Tiani C, Zappoli P, Magalotti D, Malavolta N, Zoli M. Liver hemangioma and vascular liver diseases in patients with systemic lupus erythematosus. *World J Gastroenterol* 2011; 17(40): 4503-4508 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4503.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4503>

INTRODUCTION

The spectrum of liver disease in systemic lupus erythematosus (SLE) patients is wide, ranging from benign conditions (such as benign liver lesions and mild chronic hepatitis), to aggressive and potentially lethal disorders (such as vascular liver diseases, including portal and hepatic veins thrombosis, nodular regenerative hyperplasia and idiopathic portal hypertension)^[1,2]. Benign focal liver lesions, such as cavernous hemangioma, have been reported in SLE^[3,4], and vascular liver diseases in SLE have been frequently observed in autopsic series^[5], suggesting an association between these disorders, but data in living SLE patients are scarce and rely on isolated case reports.

Color-Doppler ultrasonography (CDUS) is the initial imaging technique used to screen patients with suspected liver disease, since it allows a non-invasive, real-time evaluation of abdominal organs and vessels, which is repeatable, easy to perform and inexpensive compared with other techniques. Ultrasound allows an accurate identification and characterization of focal hepatic lesions^[6], and of portal vein and hepatic veins patency; in cases of portal or hepatic vein thrombosis, CDUS accuracy is similar to that of computerized tomography (CT)^[7-13].

This case-control study was aimed at investigating, *via* CDUS, whether SLE is associated with focal lesions and vascular liver diseases.

MATERIALS AND METHODS

Patients and controls

We prospectively included in the present study all consecutive patients with an established diagnosis of SLE, namely at least 4 criteria among those published in the guidelines by the American College of Rheumatology^[14]. Exclusion criteria were the presence of previously recognized liver disease from alcohol, hepatitis B virus (HBV) or hepatitis C virus (HCV) virus or autoimmune causes, and personal history of malignancy.

Thirty subjects with SLE, normal aspartate transaminase (AST)/alanine transaminase (ALT) and no recognized liver disease observed at our outpatient Unit, and 5 patients with SLE requiring hospitalization for any cause at our Unit over 24 mo were consecutively enrolled.

Thirty-five age and sex-matched healthy controls without SLE were included. Controls were consecutively recruited among subjects referred for an abdominal ultrasound examination to the Ultrasound Laboratory of our Unit for the following reasons: routine screening ($n = 12$), follow-up of renal or hepatic cyst ($n = 11$), evaluation of gallbladder lithiasis ($n = 6$), and abdominal discomfort ($n = 6$). Healthy state was ensured by specific questions on liver, heart, lung or renal diseases, history of malignancy, and chronic medication intake. Subjects with history of any of these conditions were excluded.

This study was approved by the Senior Staff Committee of University Hospital, a board which regulates non-interventional studies and is comparable to an Institutional Review Board. The nature of the study was

explained to the patients and controls, and a written informed consent was obtained in each case, according to the principles of the Declaration of Helsinki (revision of Edinburgh 2000).

Ultrasound-Doppler examination

After an overnight fast, patients and controls were entered in the ultrasound examination room and invited to remain in the supine position for 10 min. Thereafter, an abdominal color-Doppler ultrasonography (CDUS) examination was performed by an experienced operator by using last-generation duplex equipment (Esaote Ansaldo AU Technos, Genoa, Italy) with a 4.5-7 MHz convex probe provided by a color, power and pulsed Doppler device.

Liver parenchyma and portal and hepatic veins patency were systematically evaluated. Location, number and size of the focal liver lesions were recorded. If present, they were diagnosed as^[6]: (1) typical liver hemangioma: round-shaped, hyperechoic lesion with sharp margins, up to 4 cm in size. No Doppler signal inside the lesion; (2) atypical liver hemangioma: size > 4 cm; hypoechoic lesion or heterogeneous echopattern. No Doppler signal inside the lesion; and (3) focal nodular hyperplasia (FNH): hypo- or slightly hyperechoic lesion < 3 cm with sharp margins and typical color-Doppler findings: central feeding artery and spoke wheel centrifugal vascular pattern.

When ultrasound (US) suggested atypical hemangioma, FNH or lesions of uncertain nature, a definite diagnosis was achieved by magnetic resonance imaging or by multislice CT scan.

Clinical and laboratory data of SLE patients

Routine clinical and laboratory data were collected, and data on the duration of the disease and its treatment were recorded. Systemic lupus erythematosus disease activity index (SLEDAD)^[15] and Systemic lupus international collaborating clinics/American college of rheumatology damage index for systemic lupus erythematosus (SLICC/ACR)^[16] were calculated.

Statistical analysis

Statistical analysis was performed by SPSS 12.0 statistical package (SPSS Inc., Chicago, IL, United States). All results are expressed as mean \pm SD. Comparisons between cases and controls were done by Student's *t* test for unpaired data for continuous normally distributed variables, and by χ^2 test for frequencies; Mann-Whitney test was used for non-normally distributed continuous variables. The strength of the association between SLE and the conditions in study were estimated by odds ratios (OR) and their 95% confidence interval (CI). A *P* value of < 0.05 was considered statistically significant.

RESULTS

Focal hepatic lesions in SLE patients and controls

Table 1 summarizes the main characteristics of the stud-

Table 1 Main clinical, laboratory and ultrasound characteristics of the patients included in the study (*n* = 35)

	Overall (<i>n</i> = 35)	Outpatients (<i>n</i> = 30)	Hospitalized (<i>n</i> = 5)
Age (yr)	50 ± 20	50 ± 17	51 ± 29
Gender (M/F)	2/33	1/29	1/4
SLE duration (yr)	6.9 ± 7.0	6.5 ± 6.6	9.6 ± 9.8
LAC positivity	8/35	7/30	1/5
SLEDAI ¹	8.9 ± 4.2	9.1 ± 4.3	7.6 ± 2.8
ACR/SLICC ¹	2.1 ± 1.6	2.1 ± 1.7	2.2 ± 1.3
Acrocyanosis ¹	24/33	18/28	4/5
Steroid treatment ¹	26/33	22/28	4/5
Duration of steroid therapy (yr) ¹	6.4 ± 6.3	5.9 ± 5.7	9.5 ± 9.4
AST (U/L)	22 ± 9	20 ± 5	34 ± 18
ALT (U/L)	22 ± 9	18 ± 8	24 ± 13
Bilirubin (mg/dL)	0.6 ± 0.4	0.5 ± 0.2	1.1 ± 0.8
GGT (U/L)	32 ± 40	22 ± 20	67 ± 72
ALP (U/L)	190 ± 105	171 ± 78	247 ± 159
Thrombosis of hepatic veins	1	0	1
Normal liver echopattern	9	8	1
NRH	2	0	2
Single hepatic hemangioma	12	9	3
Multiple hepatic hemangioma	7	7	0
Atypical hemangioma	5	2	3
FNH	2	2	0

¹Data available in 33 patients. M: Male; F: Female; SLE: Systemic lupus erythematosus; LAC: Lupus anticoagulant; SLEDAI: Systemic lupus erythematosus disease activity index; ACR: American college of rheumatology damage index for systemic lupus erythematosus; SLICC: Systemic lupus international collaborating clinics; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; NRH: Nodular regenerative hyperplasia; FNH: Focal nodular hyperplasia.

ied patients with SLE.

As shown, 19 patients (54.2%) showed one or more benign focal liver lesion. Hemangioma was the most frequent diagnosis, being observed in 19 cases (54.2%). FNH was observed in 2 cases (5.7%), and in both cases was associated with hemangioma.

Hemangiomas were observed both in outpatients and in hospitalized patients. Among hospitalized patients, a single hemangioma was seen in association with nodular regenerative hyperplasia (NRH); in two additional patients with no abdominal symptoms, admitted for fever in one case and for polyarthralgia in one case, a single hepatic hemangioma of large size in the right lobe (4.5 cm and 6 cm) was diagnosed.

Among outpatients, 16 (53.3%) had focal liver lesions; 9 had a single hepatic hemangioma (in two cases with atypical hypoechoic US aspect requiring CT scan; size 10–22 mm), and 7 (20% of patients of the whole series and 23.3% of outpatients) had multiple typical hepatic hemangiomas (number 2–9; size 10–22 mm) (Figure 1).

Hemangiomas did not demonstrate a preferential location inside the liver, being equally distributed in all segments.

Two patients with multiple hepatic hemangiomas had a FNH associated (confirmed by magnetic resonance imaging). Furthermore, one patient showed previously un-

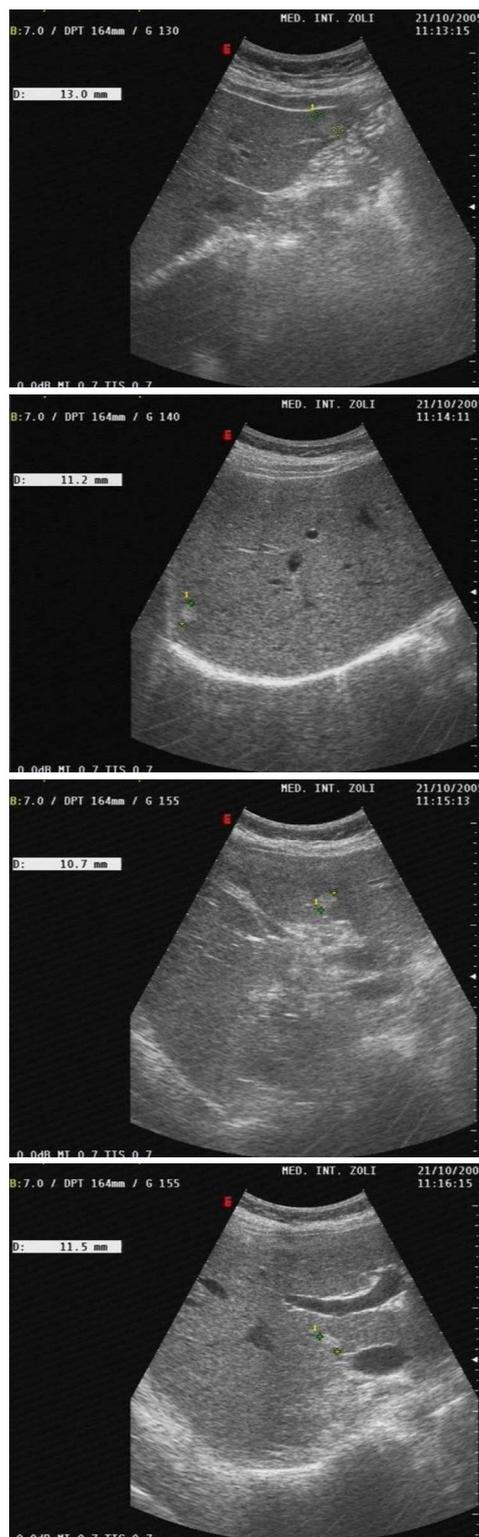


Figure 1 Ultrasonographic appearance of multiple hemangiomas in one of the studied patients. In this 47-year-old woman, 4 hepatic lesions were detected in 4 different segments of the liver.

diagnosed Caroli disease of left liver lobe associated with a single atypical hemangioma of the right lobe. These findings were confirmed by abdominal MR.

Five control subjects (14%) were diagnosed of hemangioma. Two of them had multiple lesions (2 lesions in

Table 2 Clinical and laboratory characteristics of systemic lupus erythematosus patients according to the presence and number of hepatic hemangioma

	Patients with HH (n = 19)	Single HH (n = 12)	Multiple HH (n = 7)	Patients without HH (n = 16)
Age (yr)	51 ± 16	52 ± 17	52 ± 17	49 ± 18
Gender (M/F)	2/17	2/10	0/7	0/16
SLE duration (yr)	7.1 ± 6.4	5.5 ± 6.4	9.9 ± 6.5 ^a	6.8 ± 6.1
Clinical flares (n)	3.5 ± 1.8	2.9 ± 1.4	4.2 ± 2.1	2.7 ± 1.4
SLEDAI ¹	8.3 ± 4.3	8.9 ± 5.1	7.4 ± 2.6	10.0 ± 4.3
ACR/SLICC ¹	2.3 ± 1.9	2.0 ± 1.7	3.0 ± 2.0	1.9 ± 1.4
Acrocyanosis ¹ (%)	94.4	100.0	85.7	43.8 ^b
Steroid treatment (%) ¹	75.0	60.0	100.0	87.5
Yr of steroid Rx (yr) ¹	6.8 ± 6.2	8.2 ± 7.1	5.5 ± 5.6	6.6 ± 6.1
LAC positivity (%)	22.2	18.2	28.6	25.0
AST (U/L)	23 ± 11	25 ± 14	21 ± 4	21 ± 7
ALT (U/L)	18 ± 6	17 ± 6	20 ± 8	19 ± 11
Bilirubin (mg/dL)	0.7 ± 0.5	0.7 ± 0.7	0.7 ± 0.3	0.5 ± 0.2
GGT (U/L)	36 ± 54	42 ± 65	23 ± 9	29 ± 28
ALP (U/L)	213 ± 133	243 ± 134	169 ± 138	168 ± 65

¹Data available in n = 33. ^aP < 0.05 vs patients with single hepatic hemangioma and ^bP = 0.02 vs patients with hepatic hemangioma. M: Male; F: Female; SLE: Systemic lupus erythematosus; SLEDAI: Systemic lupus erythematosus disease activity index; SLICC: Systemic lupus international collaborating clinics; ACR: American college of rheumatology damage index for systemic lupus erythematosus; LAC: Lupus anticoagulant; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; HH: Hepatic hemangioma.

both cases).

SLE was associated with the presence of single hemangioma (OR 5.05; 95% CI 1.91-13.38) and multiple hemangiomas (OR 4.13; 95% CI 1.03-16.55).

Time-course of hemangioma in SLE

In order to ascertain whether hemangiomas formation was associated with SLE onset, we evaluated the clinical files of patients with liver hemangioma to identify imaging prior to SLE development. These data were found in 9/19 cases and consisted of US examination in 7 and CT scan in 2. In 7/9 cases imaging studies documented a normal liver, while in two cases a hemangioma was already present. In the two cases of pre-existent liver hemangioma, the lesion's size at the time of the present study was stable in one, and increased from 15 to 21 mm in one.

Association between clinical variables and liver hemangioma in SLE

We did not find any significant difference between patients with and without hemangiomas for laboratory parameters, age and prevalence of the main clinical manifestation of SLE (glomerulonephritis, neurological symptoms, pulmonary hypertension, and polyserositis) (Table 2).

We observed a significantly higher prevalence of acrocyanosis in patients with hemangioma compared with patients without focal liver lesions: 94% vs 47% (P = 0.02).

We evaluated whether the presence of liver hemangi-

oma was associated with duration of SLE disease, activity of the disease at onset (SLEDAI), damage index at the time of US examination (SLICC) and number of clinical flares of SLE.

Multiple hepatic hemangiomas were associated with a longer duration of SLE disease (9.9 ± 6.5 vs 5.5 ± 6.4, P = 0.049). Patients with hepatic hemangiomas also showed a higher number of clinical flares, but the difference did not reach statistical significance.

As for treatment, the rate and duration of corticosteroid therapy for SLE were similar in patients with and without hemangioma.

Prevalence of vascular liver disease in SLE patients

We observed 2 cases of vascular liver diseases (5.7%). Both were identified in hospitalized patients with abdominal symptoms. The first patient (female, 39 year) was admitted for ascites and showed chronic Budd-Chiari syndrome with clinical and US signs of portal hypertension (namely small esophageal varices at endoscopy and enlargement of portal vein and spleen, and porto-collateral circulation at US), and NRH. A percutaneous biopsy was obtained and confirmed the radiological diagnosis. The second patient (female, 47 year) was admitted for a biliary colic, and CDUS identified a diffuse and severe alteration of liver echopattern associated with a 4 cm hyperechoic nodule. On CT scan she was diagnosed of probable NRH associated with a 4 cm hemangioma; portal and hepatic veins were patent. The patient refused liver biopsy.

DISCUSSION

The main result of the present study is the demonstration of an association between SLE and liver hemangioma. The prevalence of liver hemangioma was 54.2% in our SLE patients; this figure is more than twice the maximum expected in the general adult population (0.4%-20%)^[17], and was significantly higher than the 14% observed in healthy control subjects. Accordingly, in this study SLE was associated with increased odds (5 fold increase) of hemangioma occurrence.

This data suggests that SLE may be directly implicated in the formation of benign vascular neoplasia. Some additional findings from our study support this hypothesis: hemangioma seemed to appear after SLE onset in 7 patients; it increased in size in one; hemangiomas were multiple in 40% of patients, and patients with multiple hemangiomas had a long-lasting disease.

Hepatic hemangioma is a benign vascular neoplasia of endothelial origin. Even if the pathogenetic mechanisms leading to its formation have not been fully elucidated yet, it has been proposed that hemangiomas are caused by unregulated angiogenesis due to an imbalance between angiogenic and angiostatic factors^[18]. This hypothesis is supported by the observation that hemangiomas may increase in dimension over time during pregnancy and estrogen therapy^[19], as a consequence of estrogens-enhanced neo-

angiogenesis^[20].

It has been shown that SLE patients have increased circulating levels of estrogens^[21] and other angiogenic factors such as vascular endothelial growth factor (VEGF) and IL-18, which are associated with activity of disease^[22,23]. It could be speculated that an activation of angiogenesis might lead to liver hemangiomas formation in the course of the disease. Regrettably we lack evidence to support this hypothesis, since VEGF and other pro-angiogenic cytokines were not dosed in our patients.

In two patients of our series, hemangiomas were associated with FNH. FNH is a benign hepatocellular lesion which has been reported in 0.6%-3.0% of the general population^[17]. It has been showed that FNH represents an abnormal adaptive responsive of liver parenchyma to local hemodynamic disturbances^[24]. The association of hemangioma and FNH is frequent^[25,26], and several authors have speculated that both lesions may have causative factors in common, including focal disturbance of the hepatic blood supply that somehow facilitates the hyperplastic development of these benign lesions^[27].

In the present series of SLE patients, we found 2 cases of vascular diseases of the liver, which were diagnosed in patients hospitalized for abdominal symptoms (ascites and increase of hepatic enzymes, and suspect of biliary colic). Vascular diseases of the liver, such as hepatic and portal vein thrombosis, are life-threatening events occurring more often in patients with congenital or acquired prothrombotic condition^[28]. SLE is a well recognized prothrombotic condition and vascular liver diseases have been reported in this setting^[1,2]; our experience is in line with these previous reports, and suggests that US examination should be performed in cases of abdominal symptoms in SLE patients to specifically rule out vascular liver diseases.

The present study suffers from some limitations. We lack histological confirmation for the imaging findings since most lesions were found in asymptomatic patients, in whom biopsy was not performed. Still, in patients without chronic hepatic diseases, imaging techniques are considered sufficient to diagnose benign liver lesions^[29].

The clinical observations from our series suggest an association between SLE and liver hemangioma formation. Future studies are needed to assess the mechanisms leading to this association.

In conclusion, this study shows that the liver is a frequent site of abnormal findings in SLE patients. SLE is associated with an increased likelihood of liver hemangiomas and multiple hepatic hemangiomas, which can be associated with FNH and vascular liver diseases. Vascular disorders can be found in patients with SLE, and should be actively looked for in SLE patients with abdominal symptoms.

COMMENTS

Background

Benign focal liver lesions, such as cavernous hemangioma, and vascular liver diseases have been reported in systemic lupus erythematosus (SLE), suggest-

ing an association between these disorders.

Research frontiers

Only a few case reports and autoptic series have been published hitherto regarding the prevalence of benign liver lesions and vascular liver diseases in patients with SLE.

Innovations and breakthroughs

In this study, the authors found that the prevalence of hepatic hemangioma is very high in patients with SLE. Moreover, liver hemangiomas in this population were often multiple, and associated in some instance with focal nodular hyperplasia and vascular liver diseases.

Applications

The clinical observations from this study suggest an association between SLE and liver hemangioma formation. Vascular hepatic disorders can be found in patients with SLE, and should be actively looked for in SLE patients with abdominal symptoms.

Peer review

Although this study on liver findings in SLE patients does not lead to direct therapeutic consequences, it evaluates an interesting aspect of this disease. Histological proof would of course be desirable, but in general, biopsy cannot be justified in this context.

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S- Editor Tian L L- Editor Rutherford A E- Editor Xiong L

***Helicobacter pylori* infection in bleeding peptic ulcer patients after non-steroidal antiinflammatory drug consumption**

Francesco Manguso, Elisabetta Riccio, Germana de Nucci, Maria Luisa Aiezza, Gerardino Amato, Linda Degl'Innocenti, Maria Maddalena Piccirillo, Gianfranco De Dominicis, Tara Santoro, Elena Trimarco, Antonio Balzano

Francesco Manguso, Elisabetta Riccio, Germana de Nucci, Tara Santoro, Antonio Balzano, Complex Operating Unit of Gastroenterology, AORN A Cardarelli, 80131 Napoli, Italy
Maria Luisa Aiezza, Elena Trimarco, Complex Operating Unit of Pharmacy, AORN A Cardarelli, 80131 Napoli, Italy
Gerardino Amato, Linda Degl'Innocenti, Maria Maddalena Piccirillo, Complex Operating Unit of Microbiology, AORN A Cardarelli, 80131 Napoli, Italy

Gianfranco De Dominicis, Complex Operating Unit of Pathology, AORN A Cardarelli, 80131 Napoli, Italy

Author contributions: Manguso F, Riccio E and Balzano A conceived the study and wrote the article; de Nucci G and Santoro T collected data and reviewed the final version; Aiezza ML and Trimarco E determined the pharmacological history, collected data, and reviewed the final version; Amato G, Degl'Innocenti L, and Piccirillo MM performed the serological and microbiological tests, and reviewed the final version; De Dominicis G performed the histological evaluation and reviewed the final version.

Correspondence to: Dr. Francesco Manguso, Complex Operating Unit of Gastroenterology, AORN A Cardarelli, Via A Cardarelli 9, 80131 Napoli, Italy. manguso@alice.it

Telephone: +39-81-7474034 Fax: +39-81-7473018

Received: January 27, 2011 Revised: June 9, 2011

Accepted: June 16, 2011

Published online: October 28, 2011

Abstract

AIM: To establish the prevalence of *Helicobacter pylori* (*H. pylori*) infection in patients with a bleeding peptic ulcer after consumption of non-steroidal antiinflammatory drugs (NSAIDs).

METHODS: A very early upper endoscopy was performed to find the source of upper gastrointestinal bleeding and to take biopsy specimens for analysis of

H. pylori infection by the rapid urease (CLO) test, histological examination, and bacterial culture. IgG anti-CagA were also sought. The gold standard for identifying *H. pylori* infection was positive culture of biopsy specimens or contemporary positivity of the CLO test and the presence of *H. pylori* on tissue sections.

RESULTS: Eighty patients, 61 males (76.3%), mean age 61.2 ± 15.9 years, were consecutively enrolled. Forty-seven (58.8%) patients occasionally consumed NSAIDs, while 33 (41.3%) were on chronic treatment with low-dose aspirin (LD ASA). Forty-four (55.0%) patients were considered infected by *H. pylori*. The infection rate was not different between patients who occasionally or chronically consumed NSAIDs. The culture of biopsy specimens had a sensitivity of 86.4% and a specificity of 100%; corresponding figures for histological analysis were 65.9% and 77.8%, for the CLO test were 68.2% and 75%, for the combined use of histology and the CLO test were 56.8% and 100%, and for IgG anti-CagA were 90% and 98%. The highest accuracy (92.5%) was obtained with the culture of biopsy specimens.

CONCLUSION: Patients with a bleeding peptic ulcer after NSAID/LD ASA consumption frequently have *H. pylori* infection. Biopsy specimen culture after an early upper gastrointestinal tract endoscopy seems the most efficient test to detect this infection.

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Key words: *Helicobacter pylori*; *Helicobacter pylori* infection; Low-dose aspirin; Non-steroidal antiinflammatory drugs; Peptic ulcer hemorrhage; Endoscopy

Peer reviewer: Hanna Gregorek, Assistant Professor, PhD,

Department of Microbiology and Clinical Immunology, The Children's Memorial Health Institute, Al Dzieci Polskich 20, Warsaw 04-730, Poland

Manguso F, Riccio E, de Nucci G, Aiezza ML, Amato G, Degl'Innocenti L, Piccirillo MM, De Dominicis G, Santoro T, Trimarco E, Balzano A. *Helicobacter pylori* infection in bleeding peptic ulcer patients after NSAID consumption. *World J Gastroenterol* 2011; 17(40): 4509-4516 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4509.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4509>

INTRODUCTION

Acute upper gastrointestinal (GI) bleeding is a life-threatening emergency frequently observed in patients admitted to tertiary care hospitals, with peptic ulcer bleeding accounting for approximately 50% of cases^[1]. A high number of these emergency admissions for upper GI bleeding are attributable to non-steroidal anti-inflammatory drug (NSAID) use, especially in older patients^[2,3].

The fact that peptic ulcers can be effectively treated by acid suppression strongly suggests that it is largely a disease of acid hypersecretion^[3-5], although there is much evidence for a role of *Helicobacter pylori* (*H. pylori*) infection^[6], and NSAID-induced injury^[3]. Published data about the combined role of *H. pylori* infection and NSAID use in patients with peptic ulcer bleeding are conflicting. *H. pylori* infection has been demonstrated in a variety of studies, with a considerable degree of consistency, to increase the risk of NSAID-related GI injury^[7]. Moreover, a recent meta-analysis indicated that prophylactic *H. pylori* eradication may help to reduce the risk of both gastric and duodenal ulcers and their complications, including bleeding, in chronic users of non-steroidal antiinflammatory drugs (NSAIDs)^[8]. Other studies on the interaction between *H. pylori* infection and low-dose aspirin (LD ASA) use in patients with a history of upper GI bleeding demonstrated the protective role of *H. pylori* eradication on rebleeding. In these patients, *H. pylori* eradication may allow the use of LD ASA instead of other antithrombotic drugs^[9]. On the other hand, some studies evidenced a lower rate of *H. pylori* infection in patients with bleeding peptic ulcers than in patients with uncomplicated peptic disease. In particular, a negative interaction between *H. pylori* infection and NSAID use was postulated, indicating a lower risk of bleeding in ulcer patients taking NSAIDs^[10].

H. pylori infection can be diagnosed by invasive techniques requiring endoscopy and biopsy (histological examination, culture, and rapid urease test) and by non-invasive tests (serology, urea breath test, detection of *H. pylori* antigen in stool specimen). With the exception of the culture test, a single test has not reached acceptable accuracy for the diagnosis of *H. pylori* infection^[11]. The rapid urease test and histological examination may indicate the presence of any urease-producing or helix-shaped bac-

teria, respectively. Moreover, serological tests are markers of exposure to *H. pylori* but do not indicate whether active infection is ongoing^[11,12]. The results of the urea breath test are influenced by factors related to the patient, the bacteria, and the test itself^[13]. Rapid gastric emptying, contamination with oral commensals, achlorhydria, and gastric atrophy may cause false positive results, while false negative results can occur through suppression of urease activity if the breath test is performed too soon after antibiotic or acid suppression therapy. The detection of *H. pylori* antigen in stools has some limitations related to bowel movements: a short transit time could favor elimination of unaltered antigens, while constipation could lead to degradation of the antigens^[13]. Finally, in patients with recent upper GI bleeding the diagnosis of *H. pylori* infection can be challenging^[14,15]. In fact, the hemorrhage itself (given the pH buffering effect of blood in the GI tract), the use of proton pump inhibitors and antibiotics may influence the results of invasive and non-invasive tests for *H. pylori*^[15-17].

Our study was planned to give further information about the prevalence of *H. pylori* infection in occasional and chronic NSAID/LD ASA users admitted for peptic ulcer bleeding and submitted to very early upper endoscopy with a concomitant search for *H. pylori*.

MATERIALS AND METHODS

Ethics

The study was approved by the Institutional Review Board of the A. Cardarelli Hospital of Naples, Italy, and was conducted in compliance with the Declaration of Helsinki (1964 and following amendments), current Good Clinical Practices and the applicable European and local regulatory requirements.

Study design

This was a single-center, observational, prospective, registered study (EMOFANS Study: ACTRN12607000521426) carried out in the A. Cardarelli Hospital, a high volume hospital dedicated to emergencies. This study was planned to obtain information about the prevalence of *H. pylori* infection in patients regularly or occasionally consuming NSAIDs/LD ASA who were admitted for peptic ulcer disease complicated by hemorrhage. Taking into account our previous data, we calculated that it would be possible to recruit a total of 80 consecutive patients with the characteristics required by the protocol within a period of 12 mo. The primary objective of the study was to establish the prevalence of *H. pylori* infection in patients consecutively admitted to the emergency unit with upper GI bleeding from complicated peptic ulcer, who had been on chronic treatment or occasionally consumed NSAIDs/LD ASA. A secondary objective was to compare the efficiency of invasive (culture of biopsy specimens, *H. pylori* on tissue sections, rapid urease test) and non-invasive (IgG anti-CagA) techniques for the detection of *H. pylori* infection.

Table 1 Forrest classification

1 Actively bleeding ulcer
1a: Spurting
1b: Oozing
2 Non-actively bleeding ulcer
2a: Non-bleeding visible vessel
2b: Ulcer with surface clot
2c: Ulcer with red or dark blue spots
3 Ulcer with clean base

Inclusion and exclusion criteria

We recruited patients who fulfilled the following criteria: (1) male or female patients, of any ethnic origin, 18 years or more of age, who provided written informed consent prior to any study-related procedures and who were, in the opinion of the investigator, able to understand and to follow the protocol and likely to comply with all the requirements of the study; (2) patients with peptic ulcer disease complicated by hemorrhage (hematemesis, melena, hematochezia, or with other clinical signs of blood loss, i.e., hemodynamic instability with hypotension and tachycardia) in the 72 h before admission; (3) patients on chronic treatment with NSAIDs/LD ASA or who had received occasional treatment with NSAIDs in the 30 d before admission. Treatment with LD ASA was defined as the continuous use of up to 300 mg of aspirin per day for prophylaxis against vascular occlusive diseases^[18]; and (4) patients with an ulcer, defined as a lesion with loss of mucosal integrity and continuity of ≥ 5 mm with an apparent depth of ≥ 1 mm, as measured using gastric biopsy forceps as standard. The exclusion criteria were: (1) patients who had received treatment with antibiotics or proton pump inhibitors within the 4 wk prior to potential enrolment in order to avoid false negative *H. pylori* results; (2) a history of previous major upper GI surgery; (3) patients with upper GI neoplastic ulcer; and (4) patients already hospitalized for other reasons.

Data collection

The patients' details were collected in a database and included: (1) demographic data; (2) comorbidities according to the Charlson Comorbidity Index^[19]; (3) clinical and biochemical parameters; (4) occasional or regular use of NSAIDs/LD ASA; (5) other treatments taken; (6) endoscopy findings; (7) histology findings; (8) serological findings; and (9) microbiology results. The American Society of Anesthesiology classification of physical status^[20] was calculated for each patient at admission prior to the endoscopy. Lesions were localized and classified according to the Forrest classification (Table 1)^[21], and the Rockall risk score was also calculated after endoscopy^[22].

Helicobacter pylori detection

The study plan included very early upper endoscopy, defined as within 6 h of arrival at the hospital^[23], or as soon as possible after hemodynamic stabilization, to evaluate the source of upper GI bleeding and to take biopsies in

eligible subjects. Gastric biopsies were obtained during endoscopy after successful hemostasis if needed. Biopsy specimens were taken from the antrum and gastric body to search for *H. pylori*, according to guidelines^[24], and from all suspicious lesions. In particular, two specimens from the antrum as well as two specimens from the gastric body were used to perform the rapid urea (CLO) test (GASTREX, Warsaw, Poland) with separate kits. Moreover, two biopsy specimens from the antrum as well as two specimens each from the anterior and posterior parts of the gastric body were cultured for *H. pylori*. Finally, two biopsy specimens from the antrum and two specimens from the body were taken to detect *H. pylori* infection on tissue sections after modified Giemsa staining^[25]. At the same time a blood sample was collected and IgG antibodies against CagA protein (DIA.PRO, Diagnostic Bioprobes Srl, Milan, Italy) were analyzed by enzyme immunoassays. CagA antibody titers (> 5 U/mL) were classified as positive according to the manufacturer's instructions.

The CLO test was carried out at room temperature, with the sample examined at 24 h, and considered positive when the appropriate color change (yellow to red) occurred. With regards to culture of biopsy specimens, primary isolation was performed on commercial selective Pylori agar (BioMérieux, 43263, Marcy-L'Étoile, France). Following primary selective isolation, *H. pylori* strains were identified by usual phenotypic tests (Gram stain and by oxidase, catalase and urease tests). To overcome the instability of *H. pylori* in biopsy material during its transport from the collection site to the laboratory, which is a limiting factor for culture and susceptibility testing, the biopsy specimens for the bacterial culture were immediately placed in an appropriate transport medium (Portagerm-Pylori, BioMérieux, 42041, Marcy-L'Étoile, France). The biopsy histology was interpreted by a GI pathologist blind to the patient's information and the results of the other *H. pylori* tests.

The gold standard for identifying *H. pylori* infection was a positive culture of biopsy specimens or contemporary positivity for the CLO test and the presence of *H. pylori* on tissue sections, in accordance with current guidelines^[26].

Statistical analysis

Continuous data are expressed as means and standard deviations and compared by an independent samples *t* test. Categorical variables were analyzed by Pearson's chi-square test or Fisher's exact test. All tests of significance were two-sided. A *P*-value less than 0.05 was considered statistically significant. The PASW (Predictive Analytics Software) Statistics for Windows (Release 18.0.0-Jul 30, 2009; SPSS Inc., Chicago, Ill., United States) was used for the statistical analyses. Sensitivity, specificity, positive and negative predictive values, and false positive and false negative rates were determined using StatsDirect statistical software (release 2.7.8, March 15, 2010). The diagnostic accuracy was calculated as follow: Overall Ac-

Table 2 Demographic, clinical and endoscopic characteristics of all patients with bleeding from peptic ulcers after consumption of nonsteroidal anti-inflammatory drugs, grouped according to whether they did or did not have *Helicobacter pylori* infection *n* (%)

	Patients (<i>n</i> = 80)	<i>Helicobacter pylori</i> negative (<i>n</i> = 36)	<i>Helicobacter pylori</i> positive (<i>n</i> = 44)	<i>P</i> -value
Males	61 (76.3)	28 (77.8)	33 (75.0)	0.771
Age (yr) (mean ± SD)	61.2 ± 15.9	60.6 ± 18.3	61.7 ± 13.9	0.767
Smoker				0.703
Non-smoker	43 (53.8)	21 (58.3)	22 (50.0)	
Current	26 (32.5)	10 (27.8)	16 (36.4)	
Ex-smoker	11 (13.8)	5 (13.9)	6 (13.6)	
Symptoms on presentation				0.721
Hematemesis	11 (13.8)	4 (11.1)	7 (15.9)	
Melena	58 (72.5)	26 (72.2)	32 (72.7)	
Hematemesis and melena	11 (13.8)	6 (16.7)	5 (11.4)	
Initial mean hemoglobin (g/dL)	9.2 ± 2.3	8.7 ± 2.2	9.6 ± 2.4	0.076
American Society of Anesthesiology class				0.600
1-2	62 (77.5)	26 (72.2)	36 (81.8)	
3	15 (18.8)	8 (22.2)	7 (15.9)	
4	3 (3.8)	2 (5.6)	1 (2.3)	
Complete Rockall score				0.183
0-2	39 (48.8)	19 (52.8)	20 (45.5)	
3-5	34 (42.5)	12 (33.3)	22 (50)	
6-8	7 (8.8)	5 (13.9)	2 (4.5)	
¹ Comorbidity	27 (33.8)	12 (33.3)	15 (34.1)	0.943
Occasional consumption of NSAIDs	47 (58.8)	19 (52.8)	28 (63.6)	0.326
Patients on chronic LD ASA	33 (41.3)	17 (47.2)	16 (36.4)	0.326
Other antiplatelet drugs	5 (6.3)	2 (5.6)	3 (6.8)	0.999
Anticoagulants	2 (2.5)	2 (5.6)	0 (0)	0.199
Other drugs	39 (48.8)	15 (41.7)	24 (54.5)	0.252
Locations of ulcers				0.515
Duodenum alone	41 (51.3)	21 (58.3)	20 (45.5)	
Stomach alone	29 (36.3)	11 (30.6)	18 (40.9)	
Stomach and duodenum	10 (12.5)	4 (11.1)	6 (13.6)	
Forrest				0.684
1a	2 (2.5)	1 (2.8)	1 (2.3)	
1b	12 (15.0)	6 (16.7)	6 (13.6)	
2a	4 (5.0)	1 (2.8)	3 (6.8)	
2b	3 (3.8)	1 (2.8)	2 (4.6)	
2c	16 (20.0)	10 (27.8)	6 (13.6)	
3	43 (53.8)	17 (47.2)	26 (59.1)	

NSAID: Nonsteroidal anti-inflammatory drug; LD ASA; Low dose aspirin. ¹According to the Charlson Comorbidity Index. Because of rounding, not all percentages total 100.

curacy = (True Positive + True Negative)/(True Positive + False Positive + False Negative + True Negative).

RESULTS

Study population

Eighty consecutive patients (61 male, 19 female; mean age 61.2 ± 15.9 years (range, 21-85)) with upper GI bleeding from complicated peptic ulcer disease and on treatment with NSAIDs/LD ASA before admission, were enrolled. All patients were admitted to the emergency unit of the A. Cardarelli Hospital of Naples between January and December 2008. The characteristics of the study population are summarized in Table 2. No patients had a history of *H. pylori* eradication. In 67 (83.8%) patients endoscopic examinations took place within 6 h of arrival at the hospital, while in the remaining subjects endoscopies were delayed because of the patients' condition and were performed within 24 h. The site of the ulcers was duodenal in 41 patients (51.3%),

gastric in 29 (36.3%), and in both segments in the remaining 10 patients (12.5%). In 14 cases (17.5%) the ulcers were classified as F1, in 23 (28.8%) as F2, and in 43 (53.8%) as F3 (Table 2). Six (7.5%) patients suffered rebleeding. None required surgery for bleeding or died during hospitalization. All patients were given proton-pump inhibitors intravenously in the emergency area and orally thereafter.

Consumption of non-steroidal antiinflammatory drugs

Most of the patients had occasionally consumed NSAIDs, particularly for a fever or moderate pain. In detail, 36 patients (45.0%) had occasionally consumed only one NSAID before their bleeding event, with one of these on concomitant chronic treatment with an antiaggregant (ticlopidine). Nine (11.3%) and two (2.5%) patients had consumed two and three NSAIDs in sequence, respectively. Thirty three patients (41.3%) were on chronic treatment with LD ASA for primary or secondary prevention of cardiovascular diseases., 18

Table 3 Patients' distribution on the basis of occasional or chronic consumption of nonsteroidal anti-inflammatory drugs

Occasional NSAID use	
One NSAID	35 (43.8)
Two NSAIDs (in sequence)	9 (11.3)
Three NSAIDs (in sequence)	2 (2.5)
NSAID + ticlopidine	1 (1.3)
Chronic NSAID use	
LD ASA alone	18 (22.5)
LD ASA + another NSAID ¹	8 (10.0)
LD ASA + two other NSAIDs (in sequence) ¹	1 (1.3)
LD ASA + ticlopidine	3 (3.8)
LD ASA + clopidogrel + NSAID ¹	1 (1.3)
LD ASA + LMWH	1 (1.3)
LD ASA + LMWH + 2 NSAIDs (in sequence) ¹	1 (1.3)

Data are expressed as number (percentage). NSAID: Non-steroidal anti-inflammatory drug; LD ASA: Low dose aspirin; LMWH: Low-molecular-weight heparin. ¹NSAIDs consumed occasionally. Because of rounding, not all percentages total 100.

Table 4 Frequency of positive diagnostic tests for *Helicobacter pylori* in culture-positive or culture-negative patients

	Culture-positive (n = 38)	Culture-negative (n = 42)
CLO test + tissue section-positive	19 (23.8)	6 (7.5)
CLO test positive	5 (6.3)	9 (11.3)
Tissue section-positive	4 (5.0)	8 (10.0)

Data are expressed as number (percentage).

(22.5%) on treatment with LD ASA alone, eight (10.0%) had occasionally consumed another NSAID, and one (1.3%) had taken two other NSAIDs in sequence. Four (5.0%) patients were on treatment with ticlopidine or clopidogrel (one of whom had occasionally consumed a NSAID), and two (2.5%) were receiving low-molecular-weight heparin (one of whom had consumed two NSAIDs in sequence) (Table 3). Thirty-nine (48.8%) patients were on treatment with other drugs considered not harmful to the intestinal mucosa. Twenty-eight out of 47 (59.6%) patients who occasionally consumed NSAIDs and 16/33 (48.5%) on chronic treatment with LD ASA were considered infected by *H. pylori*, with no statistically significant difference between the two groups ($P = 0.326$).

Diagnostic tests for *Helicobacter pylori*

Among the 80 bleeding patients, 38 (47.5%) had positive cultures of biopsy specimens, 37 (46.3%) had positive histopathological findings, 39 (48.8%) had a positive CLO test, and 36 (45.0%) were positive for IgG anti-CagA. The frequency of positive diagnostic tests for *H. pylori* in culture-positive or culture-negative patients is summarized in Table 4. Contemporaneous positivity of the CLO test and the presence of *H. pylori* on tissue sections were found in 25 (31.3%) patients. In particular, among 42 patients who had a negative culture of biopsy specimens, 6 (14.3%) had contemporary positivity of the CLO test and the presence of *H. pylori* on tissue sections. On the other hand, among 55 patients who did

not have contemporary positivity of the CLO test and the presence of *H. pylori* on tissue sections, 19 (34.5%) had a positive culture of biopsy specimens. In accordance with the pre-established gold standard, 44 (55.0%) patients were considered infected by *H. pylori*. Among 44 infected, 25 (56.8%) had contemporary positivity of the CLO test and the presence of *H. pylori* on tissue sections, and 38 (86.4%) had a positive culture of biopsy specimens. With regards to IgG anti-CagA, among the infected and non-infected patients 26 (59.1%) and 10 (27.8%), respectively, had antibody titers > 5 U/mL.

Sensitivity, specificity, positive and negative predictive values, false positive rate, false negative rate and accuracy for all techniques are shown in Table 5. Culture of biopsy specimens had a sensitivity of 86.4% and a specificity of 100%. In this analysis, the sensitivities and specificities of the remaining tests were 65.9% and 77.8%, respectively, for histological analysis; 68.2% and 75.0%, respectively, for the CLO test; 56.8% and 100%, respectively, for the combined use of histology and the CLO test; and 90.0% and 98.0%, respectively, for the anti-CagA test. The highest accuracy (92.5%) was obtained with the culture of biopsy specimens.

The 80 patients with bleeding ulcers were divided into two groups for further analysis on the basis of presence or absence of *H. pylori* infection. These two groups were identical with regards demographic, clinical and endoscopic parameters (Table 2).

DISCUSSION

The reported prevalence of *H. pylori* infection in healthy persons (without GI illness) among many studies ranges from a minimum of 11% to a maximum of 69%, with some of the variability depending on the socioeconomic status of the country of the patients investigated^[27,28]. The prevalence of *H. pylori* infection in patients with peptic ulcer disease is not well established as yet, especially because the prevalence of non-NSAID non-*H. pylori* ulcer is rising in the West, with current good evidence that 20%-40% of peptic ulcers are not associated with *H. pylori* infection or the use of NSAIDs^[29]. In one study it was found that, after excluding NSAID users, only 61% of patients with peptic ulcers had *H. pylori* infection^[30]. Data about *H. pylori* infection in patients with peptic ulcer disease complicated by hemorrhage, chronically or occasionally treated with NSAIDs/LD ASA are scarce. Such data might be difficult to collect, especially because of the choice of appropriate tests to detect *H. pylori* infection, and the timing of their performance in patients who bleed.

In our study *H. pylori* infection was found in 55% of patients with peptic ulcer disease complicated by hemorrhage after consumption of NSAIDs/LD ASA, who were not on treatment with antibiotics or proton-pump inhibitors. The Italian National Project for Gastrointestinal Bleeding (PNED) study reported a prevalence of *H. pylori* infection of 44.3% among patients who had bleeding from a non-variceal upper GI source, when the

Table 5 Performance of tests for *Helicobacter pylori* infection

	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	Accuracy (%)	False positive (%)	False negative (%)
Culture of biopsy specimens	38/44 86.4 (72.7-94.8)	36/36 100 (90.3-100)	38/38 100 (90.8-100)	36/42 85.7 (71.5-94.6)	74/80 -92.5	0/36 0	6/44 -13.6
<i>Helicobacter pylori</i> on tissue sections	29/44 65.9 (50.1-79.5)	28/36 77.8 (60.9-89.9)	29/37 78.4 (61.8-90.2)	28/43 65.1 (49.1-79.0)	57/80 -71.3	8/36 -22.2	15/44 -34.1
Rapid urease test	30/44 68.2 (52.4-81.4)	27/36 75.0 (57.8-87.9)	30/39 76.9 (60.7-88.9)	27/41 65.9 (49.4-79.9)	57/80 -71.3	9/36 -25	14/44 -31.8
<i>Helicobacter pylori</i> on tissue sections and rapid urease test	25/44 56.8 (41.0-71.7)	36/36 100 (90.3-100)	25/25 100 (86.3-100)	36/55 65.5 (51.4-77.8)	61/80 -76.3	0/36 0	19/44 -43.2
Anti-CagA	26/44 59.1 (43.3-73.7)	26/36 72.2 (54.8-85.8)	26/36 72.2 (54.8-85.8)	26/44 59.1 (43.3-73.7)	52/80 -65	10/26 -38.5	18/44 -40.9

CI: Confidence interval; NPV: Negative predictive value; PPV: Positive predictive value.

presence of the infection was determined by histological evaluation performed during the patients' stay in hospital; 36% of the patients were on treatment with NSAIDs^[31]. In our study, 46.3% of patients had positive histopathological findings, confirming the results of the PNED study^[31]. Another study performed in patients who bled from peptic ulcers (57.4% users of NSAID and/or antiplatelet drugs) found, by histological examination and the CLO test, an overall prevalence of *H. pylori* infection of 53.7%. In detail, the prevalences according to the histological examination and the CLO test were 42.3% and 44.8%, respectively^[32]. In a study by Schilling *et al* the CLO test was positive in 50% of patients, while *H. pylori* infection was detected by the ¹³C-urea breath test and histological examination (gold standard) in 62% of the cases^[33]. Our study showed positive CLO test results in 48.8% of cases.

Data about the use of culture of biopsy specimens for the detection of *H. pylori* in bleeding patients are scarce. Three studies performed between 1998 and 2000 involving a total of 314 patients showed percentages of *H. pylori*-positive patients from 24.7% to 69.1%^[34-36]. In another study involving children with upper GI bleeding, *H. pylori* infection was considered to be present when histology and/or culture were positive; unfortunately, data about the culture test alone were not presented, but *H. pylori* infection was detected in 48.8% of patients, with 29.8% of the children on treatment with NSAIDs^[37]. In our study 47.5% of patients had a positive culture of biopsy specimens.

We used a restrictive gold standard to consider a patient infected by *H. pylori*. In view of its absolute specificity, if culture alone was positive the patient was considered *H. pylori*-positive^[26]. Moreover, because a patient with at least two positive tests should be considered as *H. pylori*-positive^[26], we used as adjunctive gold standard, the contemporary positivity of the CLO test and the presence of *H. pylori* on tissue sections. In fact, the concomitant positivity of the rapid urease test and of the histological examination indicates, with the highest probability, the presence of helical urease-producing bacteria. Six patients whose cultures were negative showed contemporary positivity for the CLO test and the presence

of *H. pylori* on tissue sections. This finding demonstrates that culture tests are responsible for a number, albeit low, of false negative results. On the other hand, by using the second gold standard, only 25 (31.3%) patients would have been considered infected by *H. pylori*, even though 37 (46.3%) had positive histopathological findings and 39 (48.8%) had a positive CLO test. Moreover, among 55 patients who did not have contemporary positivity of the CLO test and the presence of *H. pylori* on tissue sections, 19 had a positive culture of biopsy specimens. These results indicate that by using the criterion of at least two positive tests to consider patients as infected by *H. pylori*, many false negative results can be expected. In our study the combined use of three invasive tests performed during a very early upper endoscopy was adequate for the diagnosis of *H. pylori* infection. With regards to the non-invasive test, 36 (45%) patients were positive for IgG anti-CagA. This test showed a low sensitivity and specificity confirming that it is a rather inaccurate diagnostic method which cannot be recommended as the first diagnostic test for *H. pylori* infection.

Patients with complicated peptic ulcer disease are candidates for testing for *H. pylori* infection. Indeed, accurate and early diagnosis of *H. pylori* infection is a critical clinical problem in these patients. The discovery of the link between the *H. pylori* bacterium and peptic ulcer is one of the greatest breakthroughs in medical history, but it is surprising that so far a large bulk of data has led to discordant results in patients with hemorrhagic complicated disease, treated or not with NSAIDs. It has been suggested that the rate of *H. pylori* infection is lower in patients with bleeding peptic ulcer than in patients with uncomplicated peptic disease, and that there is a negative interaction between *H. pylori* infection and NSAID use^[38,10]. Our study demonstrated that many patients with peptic ulcer disease complicated by hemorrhage and consuming NSAIDs/LD ASA are actually infected (55%), and that the infection may be detected with appropriate tests performed during a very early endoscopy. Culture of biopsy specimens appears to be more efficient than other techniques at detecting *H. pylori* infection (accuracy 92.5%). We believe that the discordant data in the literature are due to the different cohorts of

patients studied, the kinds of invasive/non-invasive test used, the timing at which the tests were performed, the contemporary use of proton-pump inhibitors or antibiotics, and resources available in the context in which the patient is admitted. We had two ideal conditions for eliminating some of these sources of variability: (1) the presence of a rota of gastroenterologists skilled in diagnostic and therapeutic measures available 24 h a day, 7 d a week (not as a 24-h “on call” service), and able to enroll all consecutive patients; and (2) close local collaboration between specialist gastroenterologists and microbiologists, with the possibility of methodologically sound performance of the culture tests, which is a tedious, time-consuming procedure that can be influenced by the transport conditions from the endoscopy room to the laboratory and the speed of processing, because the viability of the organism is reduced by exposure to atmospheric oxygen. Finally, we did not find a statistically significant difference in the percentage of *H. pylori* infections between patients who occasionally consumed NSAIDs and those on chronic treatment with LD ASA, indicating that chronic consumption does not modify the infection rate.

In conclusion, faced with a person with a bleeding peptic ulcer we suggest that invasive methods should be used to identify *H. pylori* infection. The accuracy of results of biopsy specimen culture in patients with peptic ulcer bleeding remains very high, and the sensitivity and specificity of this method do not seem to be affected by blood in the stomach or by the use of NSAIDs or LD ASA, when performed after a very early upper endoscopy.

ACKNOWLEDGMENTS

We acknowledge the great deal of work performed by the medical, biology, pharmacology, and nursing staff of the A. Cardarelli hospital.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) has been considered as a major cause of the development of peptic ulcer disease. Several studies have reported that the prevalence of *H. pylori* infection may be underestimated in patients with bleeding peptic ulcers. Moreover, knowledge regarding the detection of *H. pylori* infection in patients with peptic ulcer disease complicated by hemorrhage, chronically or occasionally treated with non-steroidal anti-inflammatory drugs (NSAIDs) is limited. Numerous invasive and non-invasive diagnostic methods are available for the detection of *H. pylori*. Effectiveness values of these tests may vary depending on the brand of test used, age of the population tested, treatment used and, probably, the bleeding situation.

Research frontiers

In this article, the authors assess the prevalence of *H. pylori* infection in patients with peptic ulcer disease complicated by hemorrhage after consumption of NSAIDs.

Innovations and breakthroughs

More than 50% of patients with peptic ulcer disease complicated by hemorrhage after consumption of NSAIDs are infected by *H. pylori*.

Applications

In these patients the authors recommend searching for *H. pylori* infection by using the culture of biopsy specimens after an early upper gastrointestinal tract

endoscopy.

Peer review

This is an interesting and well presented study, in which the authors using an approach that overcomes the previous conditions negatively influencing results presented in the literature, clearly showed that invasive methods should be used to identify *H. pylori* infection in a patient with a bleeding peptic ulcer.

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S- Editor Sun H L- Editor Cant MR E- Editor Zhang DN

Impact of liver steatosis on response to pegylated interferon therapy in patients with chronic hepatitis B

Fehmi Ateş, Mehmet Yalnız, Saadet Alan

Fehmi Ateş, School of Medicine, Department of Gastroenterology, Mersin University, 33070 Mersin, Turkey

Mehmet Yalnız, School of Medicine, Department of Gastroenterology, Firat University, 23119 Elazığ, Turkey

Saadet Alan, Department of Pathology, Malatya Government Hospital, 44300 Malatya, Turkey

Author contributions: Ateş F and Yalnız M designed this research; Alan S performed pathologic investigation; Ateş F, Yalnız M and Alan S wrote this article.

Correspondence to: Dr. Fehmi Ateş, School of Medicine, Department of Gastroenterology, Mersin University, 33070 Mersin, Turkey. drfehmiates@hotmail.com

Telephone: +90-533-5296453 Fax: +90-324-3374305

Received: January 15, 2011 Revised: February 21, 2011

Accepted: February 28, 2011

Published online: October 28, 2011

Abstract

AIM: To evaluate the impact of liver steatosis upon response to given therapy in chronic hepatitis B (CHB) patients.

METHODS: 84 consecutive CHB patients treated with 48-wk PEGylated interferon (PEG-IFN) therapy were enrolled. Baseline characteristics and sustained viral response (SVR) to PEG-IFN therapy were evaluated.

RESULTS: Mean body mass index (BMI) was 27.36 ± 4.4 kg/m². Six (7.1%) had hypertension and three (3.5%) had diabetes mellitus. Steatosis was present in 22.6% (19/84) of liver biopsy samples. Age, BMI, and triglyceride levels of the patients with hepatic steatosis were significantly higher than those without hepatic steatosis ($P < 0.05$). SVR to PEG-IFN therapy was 21.4% (18/84). Sixteen of these 18 CHB patients with SVR (88.9%) did not have any histopathologically determined steatosis. On the other hand, only two of the 19 CHB patients with hepatic steatosis had SVR (10.5%). Although the SVR rate observed in patients without steatosis (16/65, 24.6%) was higher compared

to those with steatosis (2/19, 10.5%), the difference was not statistically significant ($P > 0.05$).

CONCLUSION: Occurrence of hepatic steatosis is significantly high in CHB patients and this association leads to a trend of decreased, but statistically insignificant, SVR rates to PEG-IFN treatment.

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Key words: Chronic hepatitis B; Hepatic steatosis; Pegylated interferon therapy

Peer reviewer: Mireia Miquel, MD, PhD, Liver Unit, Gastroenterology Service, Parc Taulí s/n, Sabadell 08201, Spain

Ateş F, Yalnız M, Alan S. Impact of liver steatosis on response to pegylated interferon therapy in patients with chronic hepatitis B. *World J Gastroenterol* 2011; 17(40): 4517-4522 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4517.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4517>

INTRODUCTION

Fatty livers, defined by the accumulation of lipid droplets, mainly triglycerides, in hepatocytes, are vulnerable to factors associated with further hepatic injury by their increased sensitivity to oxidative stress and to cytokine-mediated hepatic damage. This alone may not only lead to chronic liver disease, but can also influence the progression of chronic liver diseases with different etiologies and the response to given therapy. Steatosis, together with obesity and type 2 diabetes mellitus (DM), is also a proposed risk factor for the development of hepatocellular carcinoma^[1].

Steatosis has been observed in the majority of chronic alcoholics and is also a common histopathological feature of chronic hepatitis C (CHC) infection. In patients with CHC, steatosis of the liver, accompanied by metabolic

and viral factors, increases the severity of fibrosis and unfavorably influences the response to given therapy^[2-4]. Steatosis, however, may co-exist with other chronic liver diseases, in addition to alcoholic liver diseases and hepatitis C, because of the increasing prevalence of obesity and metabolic syndrome.

The number of studies reporting co-existence of steatosis and chronic hepatitis B (CHB), a major cause of chronic liver disease worldwide, is increasing. The impact of superimposed non-alcoholic fatty liver disease in patients with CHB, however, is less clear. There are only a few studies on this topic^[5,6]. The components of metabolic syndrome [obesity, hypertension (HTA), and dyslipidemia] are associated with the presence of nonalcoholic steatohepatitis in patients with CHB, and the presence of hepatic fibrosis seems to be associated with known host and viral factors, as well as the presence of abdominal obesity^[7].

We aimed to determine the frequency and risk factors of liver steatosis in patients with CHB, and to investigate its correlation with the response to given PEGylated interferon (PEG-IFN) therapy.

MATERIALS AND METHODS

Patients

Twenty-one hepatitis B e antigen (HBeAg) (+) and 63 HBeAg (-) ($n = 84$) consecutive patients with CHB, who were diagnosed by liver biopsy, and received 48-wk PEG-IFN therapy were enrolled in the study between December 2006 and July 2009. Patients were given either PEG-IFN α -2a or 2b. Forty patients received PEG-IFN α -2a 180 μ g sc and 44 patients received Peg-IFN α -2b 1.5 μ g/kg sc once a week. Sixty of the patients were male (71.4%) and 24 were (28.6%) female, their mean age was 38.6 ± 10.9 years and their age range was 18-61 years.

Before inclusion, the patients were informed and their written consents were obtained. The study protocol was approved by the local Ethics Committee, and the study was performed in accordance with the ethical standards laid down in an appropriate version of the 1975 Declaration of Helsinki.

Inclusion criteria

In the serum samples of the patients enrolled to the study, the hepatitis B surface antigen (HBsAg) had to have been present for more than 6 mo and, within the last 6 mo, at least two different measurements must have shown an elevation of alanine aminotransferase (ALT) $> \text{ULN} \times 2$. Using polymerase chain reaction (PCR), it was found that HBV DNA levels were > 10000 copies/mL in cases with HBeAg (-) and > 100000 copies/mL in cases with HBeAg (+). The liver biopsies of all patients were consistent with the diagnosis of CHB.

Exclusion criteria

Patients who met at least one of the following were excluded from the study: patients co-infected with other viruses, such as hepatitis A, C, D, E, Cytomegalovirus, Epstein-Barr virus and HIV; patients with toxic hepatitis;

patients with another liver disease; alcohol consumers (more than 20 g per day); patients who were taking antiviral drugs or interferon before the biopsy.

Body mass index (BMI) was calculated by dividing the body weight (kg) by the square of height (m). Based on the BMI values, $< 25 \text{ kg/m}^2$ was considered as normal, $25\text{-}30 \text{ kg/m}^2$ as overweighted, and $> 30 \text{ kg/m}^2$ as obese.

Serum analyses

Fasting blood samples were obtained 1 d before the liver biopsy, and ALT, aspartate aminotransferase, γ glutamyltransferase, glucose (GLU), cholesterol, and triglyceride levels were measured.

Virological analyses

For the analyses, HbsAg, HBeAg, Anti-HBe, Anti-HBc ARCHITECT chemiluminescent microparticle immunoassay kits (Abbott Park, Wiesbaden-Delkenheim, Germany) and ARCHITECT i2000 system were used. HBV DNA levels were studied quantitatively using an HBV RG PCR Kit (sensitivity: 100 copies/mL) and Rotor-Gene 3000 (Corbett Research) device. Sustained viral response (SVR) was defined as the fall in HBV DNA to undetectable levels (< 100 copies/mL) 6 mo after (week 72) the end of 1-year PEG-IFN α therapy and disappearance of HBeAg in cases with HBeAg (+).

Histological evaluation

All percutaneous liver biopsies were performed by two experienced gastroenterologists using a 16-gauge needle. All histological analyses were performed by one experienced pathologist who was blinded to the study. Necroinflammation was determined by scoring according to Knodell's histological activity index (HAI): portal inflammation (0-4), lobular degeneration and focal necrosis (0-4), periportal \pm bridging necrosis (0-10)^[8]. The stage of fibrosis was classified from "no fibrosis" (Stage 0) to cirrhosis (Stage 4). Grading of hepatosteatosis was semi-quantitatively performed according to hepatocyte involvement: None:0, Mild: 0%-10%, Moderate:10%-30%, Marked: 30%-60%, and Severe: $> 60\%$ ^[9].

Statistical analysis

While the numerical data of the patients were presented as mean \pm SD, categorical variables were presented together with frequency and percentages. The intergroup differences of numerical variables were investigated using Student's t test, while the differences of categorical values were investigated using the χ^2 test. Variables that were found to be significant in univariate analysis ($P < 0.05$) were subjected to a multivariate logistic regression model to be investigated. All analyses were performed using a statistical software program (SPSS version 15.0).

RESULTS

Patient characteristics

Among 84 patients enrolled to the study, six (7.1%) had HTA and three (3.5%) had DM. The mean BMI value was

Table 1 Demographic and clinical characteristics of the patients (mean \pm SD)

Parameter	n (%)
Male	60 (71.4)
Female	24 (28.6)
Age (yr)	38.6 \pm 10.9
HBeAg (+)	21 (25.0)
HBeAg (-)	63 (75.0)
Hypertension	6 (7.1)
Diabetes mellitus	3 (3.5)
BMI (kg/m ²)	27.4 \pm 4.40
< 25	28 (33.3)
25-30	34 (40.5)
> 30	22 (26.2)
Glucose (mg/dL)	101.4 \pm 26.0
Cholesterol (mg/dL)	182.7 \pm 29.3
Triglyceride (mg/dL)	131.0 \pm 55.4
AST (IU/L)	103.3 \pm 39.5
ALT (IU/L)	136.8 \pm 47.2
GGT (IU/L)	49.9 \pm 41.1
ALP (IU/L)	91.9 \pm 26.5
HBV-DNA (copies/mL \times 10 ⁴)	5502.9 \pm 11889.7

HBeAg: Hepatitis B e antigen; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ glutamyl transferase; ALP: Alkaline phosphatase; HBV: Hepatitis B virus.

Table 2 Histological characteristics of the patients

Parameter	n (%)
HAI score	
0-3	5 (5.9)
4-8	56 (66.7)
9-12	22 (26.2)
13-18	1 (1.2)
Stage of fibrosis	
0	0 (0.0)
1	7 (8.3)
2	42 (50.0)
3	35 (41.7)
4	0 (0.0)
Steatosis	
None (0)	65 (77.4)
Mild (< 10%)	7 (8.3)
Moderate (10%-30%)	7 (8.3)
Marked (30%-60%)	4 (4.8)
Severe (> 60%)	1 (1.2)

HAI: Histological activity index.

27.36 \pm 4.4 kg/m². When the patients were evaluated according to their BMI, 28 (33.3%) were grouped as normal (< 25 kg/m²), 34 (40.5%) as overweighted (25-30 kg/m²), and 22 (26.2%) as obese (> 30 kg/m²). The demographic, clinical, and laboratory data of the patients are presented in Tables 1 and 2.

Incidence of liver steatosis and related factors

Hepatosteatois was histologically present in 19 of 84 patients with CHB (22.6%). For patients with hepatosteatois, 36.8% (7/19) showed mild, 36.8% (7/19) moderate, 21.1% (4/19) marked, and 5.3% (1/19) severe hepatosteatois. The factors that were statistically cor-

Table 3 Comparison between patients with and without hepatosteatois (mean \pm SD)

Parameter	Steatosis (+) (n = 19)	Steatosis (-) (n = 65)	P value
Male	14 (73.07)	46 (70.8)	NS
Female	5 (26.3)	19 (29.2)	NS
Age (yr)	50.5 \pm 8.7	35.2 \pm 8.9	< 0.01
BMI (kg/m ²)	32.9 \pm 3.1	25.7 \pm 3.3	< 0.01
\geq 25	18 (94.7)	38 (58.5)	< 0.01
Glucose (mg/dL)	102.7 \pm 27.7	96.7 \pm 19.0	NS
Cholesterol (mg/dL)	192.2 \pm 28.0	178.0 \pm 27.4	NS
Triglyceride (mg/dL)	188.3 \pm 52.0	114.2 \pm 44.2	< 0.01
AST (IU/L)	90.7 \pm 34.8	107.0 \pm 40.3	NS
ALT (IU/L)	128.3 \pm 18.9	139.2 \pm 52.5	< 0.01
GGT (IU/L)	49.8 \pm 29.8	50.0 \pm 44.0	NS
ALP (IU/L)	91.8 \pm 26.3	91.9 \pm 26.7	NS
HBV-DNA (copies/mL \times 10 ⁴)	5261.6 \pm 2394.9	5684.2 \pm 13245.8	NS
HBeAg (+)	4 (21.1)	17 (26.2)	NS
HBeAg (-)	15 (78.9)	48 (73.8)	NS
Advanced fibrosis (score \geq 3)	8 (42.1)	27 (41.5)	NS
Advanced HAI (score \geq 9)	6 (31.5)	17 (26.2)	NS
SVR	2 (10.5)	16 (24.6)	NS

BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ glutamyl transferase; ALP: Alkaline phosphatase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HAI: Histological activity index; SVR: Sustained viral response. NS: Non significant.

related with hepatosteatois were patient's age, BMI, and triglyceride (TG) levels. In the hepatosteatois group, the age (50.5 \pm 8.7 years *vs* 35.2 \pm 8.9 years, $P < 0.01$), BMI (32.9 \pm 3.1 kg/m² *vs* 25.7 \pm 3.3 kg/m², $P < 0.01$), and TG levels (188.3 \pm 52.0 mg/dL *vs* 114.2 \pm 44.2 mg/dL, $P < 0.01$) were higher compared to the group without hepatosteatois. No significant correlation was found between hepatosteatois and other parameters, such as HBeAg status, stage of fibrosis, HAI score, or HBV DNA level ($P > 0.05$). In multivariate analysis, it was found that advanced age, increased BMI, and elevated TG are independent predictors of the presence of hepatosteatois (Table 3).

Factors associated with SVR

Among 84 patients with CHB who received PEG-IFN therapy, 21.4% (18/84) showed SVR. The rate of SVR was 23.8% (5/21) in cases with HBeAg (+) and 20.6% (13/63) in cases with HBeAg (-). 88.8% (16/18) of CHB patients with SVR did not have any histopathologically determined steatosis. On the other hand, only two of the 19 CHB patients with liver steatosis - one with mild steatosis and the other one with moderate steatosis - had an SVR (10.5%). Although the SVR rate observed in patients without hepatosteatois (16/65, 24.6%) was higher compared to those with hepatosteatois (2/19, 10.5%), the difference was not statistically significant ($P > 0.05$). Using multivariate analysis, it was found that only ALT elevation was an independent predictor of SVR. No significant difference was found between SVR (+) and SVR (-) groups in terms of other parameters (Table 4). There

Table 4 Comparison between patients with and without sustained viral response (mean ± SD)

Parameter	SVR (+) (n = 18)	SVR (-) (n = 66)	P value
Male	13 (72.2)	47 (71.2)	NS
Female	5 (27.8)	19 (28.8)	NS
Age (yr)	35.3 ± 8.0	39.7 ± 10.8	NS
BMI (kg/m ²)	26.5 ± 3.1	27.9 ± 4.5	NS
≥ 25	11 (61.1)	45 (68.2)	NS
Glucose (mg/dL)	97.4 ± 28.0	102.4 ± 25.5	NS
Cholesterol (mg/dL)	162.1 ± 23.7	175.6 ± 30.2	NS
Triglyceride (mg/dL)	109.7 ± 50.8	136.8 ± 28.5	NS
AST (IU/L)	117.2 ± 55.4	97.8 ± 28.5	NS
ALT (IU/L)	199.8 ± 82.0	122.3 ± 49.8	< 0.01
GGT (IU/L)	62.9 ± 69.9	46.4 ± 28.6	NS
ALP (IU/L)	93.4 ± 38.8	91.5 ± 22.4	NS
HBV-DNA (copies/mL × 10 ⁴)	4961.6 ± 2245.1	5779.5 ± 13129.5	NS
HBeAg (+)	5 (27.8)	16 (24.2)	NS
HBeAg (-)	13 (72.2)	50 (75.8)	NS
Advanced fibrosis (score ≥ 3)	7 (38.9)	28 (42.4)	NS
Advanced HAI (score ≥ 9)	5 (27.8)	18 (27.8)	NS
Hepatosteatois	2 (11.1)	17 (25.8)	NS

BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ glutamyl transferase; ALP: Alkaline phosphatase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HAI: Histological activity index; SVR: Sustained viral response. NS: Non significant.

Table 5 Comparison between hepatitis B e antigen (+) patients with and without sustained viral response n (%)

	SVR (+) (n = 5)	SVR (-) (n = 16)	P value
Hepatosteatois (+)	1 (20)	3 (23)	NS
Hepatosteatois (-)	4 (80)	13 (77)	NS

SVR: Sustained viral response. NS: Non significant.

Table 6 Comparison between hepatitis B e antigen (-) patients with and without sustained viral response

	SVR (+) (n = 13)	SVR (-) (n = 50)	P value
Hepatosteatois (+)	1 (7.7)	14 (7.0)	NS
Hepatosteatois (-)	12 (92.3)	36 (93.0)	NS

SVR: Sustained viral response. NS: Non significant.

was no difference in patients with or without steatosis regarding treatment response between the types of PEG-interferon (data not shown).

Subgroup analysis according to the HBeAg status

In HBeAg (-) patients, SVR was seen 7.7% (1/13) patients with hepatosteatois, while it was 92.3% (12/13) without hepatosteatois. Although the SVR rate was higher in the patients without hepatosteatois, the difference was not statistically significant (Table 5).

In HBeAg (+) patients, SVR was seen in 20% (1/5) patients with hepatosteatois while it was 80% (4/5) patients without hepatosteatois. This difference was also not statistically significant (Table 6).

HBeAg seroconversion was seen in only four (19%) patients; all four patients had undetectable HBV DNA levels. None of our patients had HBsAg seroconversion.

DISCUSSION

This study investigated the incidence and clinical importance of liver steatosis in patients with CHB. The factors associated with liver steatosis were determined. In patients who were given 48 wk PEG-IFN α therapy, the effects of liver steatosis and other factors upon persistent viral response were also investigated. In previous studies, the incidence of hepatosteatois in patients with CHB was reported to range between 4.5% and 76%^[9-14]. Especially in the studies where the alcohol consumers are not excluded, patients with CHB showed higher rates of hepatosteatois^[15,16]. In the present study, hepatosteatois was histopathologically determined in 22.6% of CHB patients, and this prevalence is similar to that of the general population. Cases with other accompanying liver diseases, such as hepatitis C or alcohol consumers, were excluded and, thereby, misleading results were avoided.

In the development of non-alcoholic hepatosteatois, insulin resistance constitutes the main mechanism^[17]. Insulin resistance leads to hyperinsulinemia and an increase in free fatty acid concentrations, resulting with TG accumulation in hepatocytes^[18]. Insulin might also play an important role in the development of fibrosis accompanied by hepatosteatois by activating the profibrogenic pathways^[19]. The cause and clinical importance of hepatosteatois accompanying CHB are not well defined. In previous studies, non-alcoholic hepatosteatois seen in patients with CHB was related to advanced age, large waist circumference, high fasting GLU and C-peptide levels, HTA, or dyslipidemia^[10,20]. In the present study, we found that advanced age, higher BMI, and elevated TG levels were independent risk factors of hepatosteatois in patients with CHB. Hepatosteatois and fibrosis scores, however, were not correlated.

The virus by itself might be the cause of the hepatosteatois as seen in some CHC patients with hepatic steatosis^[21]. However, we did not find any correlation between hepatosteatois and viral factors, such as HBeAg status, HBV DNA level, and HAI. Taken together, the presence of steatosis correlates with some host factors (advanced age, high BMI, and TG levels), but not with viral genotype or viral load. Accordingly, the results of the present study support the finding that metabolic factors, rather than viral factors, are more determinant for hepatosteatois encountered in cases with CHB^[22] and that, whereas the association between steatosis and HCV is specific, this not the case in HBV-infected patients.

Hepatosteatois is related to metabolic factors, and hepatitis C virus infection *per se* leads to hepatosteatois

directly in different genotypes (in genotype 2 and 3)^[21]. The presence of liver steatosis in chronic viral hepatitis B might vary according to different genotypes, as reported in CHC^[3,4,20]. In the present study, HBV genotyping could not be performed due to lack of laboratory resources. Nevertheless, hepatitis B infection in Turkey is accepted to be virtually all genotype D (almost 100%); hence, a genotype effect is not expected, and analyzing the genotype is not recommended as cost-effective in such studies.

Treatment of CHB is a big challenge. The response rates are still low despite novel therapy strategies. Besides the viral factors, other accompanying conditions might hamper the success of a therapy. Hepatosteatois encountered in other chronic liver diseases not only has the potential to influence the progression of diseases, but is also suggested to diminish the response to the given therapy^[23]. In the literature, there is only one study that retrospectively investigated the effect of co-existent steatosis upon the response to treatment in CHB patients^[24]. That study reported that the presence of steatosis does not have any effect on the outcome of the treatment. In the present study, persistent viral response to 48 wk of PEG-IFN was 21.4%, which was consistent with previous studies^[25,26]. As a support for the study of Moucari *et al.*^[25], only ALT elevation was an independent predictor of SVR. Strikingly, 88.9% (16/18) of CHB patients with SVR did not have any histopathologically determined steatosis. On the other hand, only two of the 19 (10.5%) CHB patients with liver steatosis had SVR. The high SVR rates obtained in patients without hepatosteatois compared to those with hepatosteatois, however, were not significant statistically. The fact that hepatosteatois has no statistically significant effect on SVR may be due to our small number of patients.

It would be better if the homeostasis model assessment (HOMA) could also be determined. However, HOMA was designed to determine the relationship between chronic viral hepatitis and the presence of steatosis with respect to the effect upon treatment of viral hepatitis. The role of risk factors of steatosis, including the GLU HOMA index upon the course of chronic viral hepatitis B patients with steatosis, was beyond the scope of this study. This will be the subject of future studies.

In conclusion, hepatosteatois is encountered frequently in patients with CHB. This association leads to a trend of decreased, but statistically insignificant, SVR rate to PEG-IFN treatment, both in HBeAg (+) and HBeAg (-) patients. Hepatic steatosis, a risk-free, benign condition in healthy subjects, might become a dangerous co-factor of disease progression when it is present in patients affected by another liver disease. It might affect the response to antiviral treatment and the significant negative effect of hepatosteatois on response to therapy in CHB patients should be demonstrated using larger prospective studies. Advanced age, BMI, and high levels of TG are independent risk factors of hepatic steatosis development. Treatment strategies against obesity and TG elevations would have positive effects on CHB progression and the response to the given therapy. Hence,

combating steatosis and its associated factors might aid in increasing the response to therapy in CHB patients.

COMMENTS

Background

Fatty livers encountered frequently in clinical practice may co-exist with other chronic liver diseases, and can influence the progression of the chronic liver diseases with different etiologies. The number of studies reporting co-existence of steatosis and chronic hepatitis B (CHB) is increasing. The impact of superimposed non-alcoholic fatty liver disease in patients with CHB, however, is less clear.

Research frontiers

Fatty livers are more vulnerable to factors associated with further hepatic injury because of their increased sensitivity to oxidative stress and cytokine-mediated hepatic damage, which may lead to chronic liver disease. The presence of a fatty liver can influence the progression of the chronic liver diseases with different etiologies and the response to given therapy. Steatosis of the liver in patients with chronic hepatitis C increases the severity of fibrosis and unfavorably influences the response given to therapy. Nevertheless, the association of liver steatosis and CHB, a major cause of chronic liver disease worldwide, is less clear.

Innovations and breakthroughs

Hepatosteatois is not infrequent in patients with CHB. Advanced age, body mass index (BMI), and high levels of triglyceride (TG) are independent risk factors for the development of hepatic steatosis in patients with chronic viral hepatitis B. This coexistence leads to a trend of decreased, but statistically insignificant, sustained viral response rate to PEGylated interferon treatment both in hepatitis B e antigen (HBeAg) (+) and HBeAg (-) patients.

Applications

Hepatic steatosis, a risk-free, benign condition in healthy subjects, might become a dangerous co-factor of disease progression when it is present in patients affected by another liver disease. It might affect the response to antiviral treatment and the significant negative effect of hepatosteatois upon response to therapy in CHB patients should be demonstrated using larger prospective studies. Given the importance of advanced age, BMI, and high levels of TG as being independent risk factors for development of hepatic steatosis, treatment strategies against obesity and TG elevations would have positive effects on CHB progression and the response to given the therapy. Hence, combating steatosis and its associated factors might aid in increasing the response to therapy in CHB patients.

Peer review

This study, even is observational, is fairly interesting.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Zheng XM

YFa and analogs: Investigation of opioid receptors in smooth muscle contraction

Krishan Kumar, Ritika Goyal, Annu Mudgal, Anita Mohan, Santosh Pasha

Krishan Kumar, Ritika Goyal, Annu Mudgal, Santosh Pasha, Peptide Synthesis Laboratory, Institute of Genomics and Integrative Biology, Delhi 110007, India

Krishan Kumar, Anita Mohan, University School of Basic and Applied Sciences, GGSIP University, Sector-16 C, Dwarka, Delhi 110075, India

Krishan Kumar, Department of Chemistry, Motilal Nehru College, University of Delhi, Delhi 110021, India

Author contributions: Kumar K, Mohan A and Pasha S designed the study; Kumar K performed the majority of experiments; Goyal R, Kumar K and Pasha S analyzed the data; Kumar K wrote the first draft of manuscript; Goyal R and Mudgal A contributed to the final version of the manuscript.

Supported by Council of Scientific and Industrial Research, Delhi

Correspondence to: Dr. Santosh Pasha, Scientist "G", Peptide Synthesis Laboratory, Institute of Genomics and Integrative Biology, Mall Road, Delhi 110007, India. spasha@igib.res.in

Telephone: +91-11-27667439 Fax: +91-11-27667471

Received: April 19, 2011 Revised: June 16, 2011

Accepted: June 23, 2011

Published online: October 28, 2011

Abstract

AIM: To study the pharmacological profile and inhibition of smooth muscle contraction by YFa and its analogs in conjunction with their receptor selectivity.

METHODS: The effects of YFa and its analogs (D-Ala₂) YFa, Y (D-Ala₂) GFMKKKFMRF amide and Des-Phe-YGGFMKKKFMRF amide in guinea pig ileum (GPI) and mouse vas deferens (MVD) motility were studied using an isolated tissue organ bath system, and morphine and DynA (1-13) served as controls. Acetylcholine was used for muscle stimulation. The observations were validated by specific antagonist pretreatment experiments using naloxonazine, naltrindole and norbinalorphimine norBNI.

RESULTS: YFa did not demonstrate significant inhibi-

tion of GPI muscle contraction as compared with morphine (15% vs 62%, $P = 0.0002$), but moderate inhibition of MVD muscle contraction, indicating the role of κ opioid receptors in the contraction. A moderate inhibition of GPI muscles by (Des-Phe) YFa revealed the role of anti-opiate receptors in the smooth muscle contraction. (D-Ala-2) YFa showed significant inhibition of smooth muscle contraction, indicating the involvement of mainly δ receptors in MVD contraction. These results were supported by specific antagonist pretreatment assays.

CONCLUSION: YFa revealed its side-effect-free analgesic properties with regard to arrest of gastrointestinal transit. The study provides evidences for the involvement of κ and anti-opioid receptors in smooth muscle contraction.

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Key words: Opioid receptor; Guinea pig ileum; Mouse vas deferens; Smooth muscle contraction; Gastrointestinal motility

Peer reviewer: Edward J Ciaccio, PhD, Research Scientist, Department of Medicine, HP 804, Columbia University, 180 Fort Washington Avenue, New York, NY 10032, United States

Kumar K, Goyal R, Mudgal A, Mohan A, Pasha S. YFa and analogs: Investigation of opioid receptors in smooth muscle contraction. *World J Gastroenterol* 2011; 17(40): 4523-4531 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4523.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4523>

INTRODUCTION

Centrally acting μ -opioid agonists are still the most widely used analgesics for the relief of severe pain, but their

utility is limited by a number of well-known side effects, including tolerance, physical dependence, respiratory depression, and adverse gastrointestinal effects. To rectify these complications, the effects of opioid drugs on gastrointestinal transit have been extensively studied using rat models. Transit arrest is a common effect of opioids in mammals but the underlying secretomotor changes appear to vary between species^[1]. Studies on gut muscle contractility have demonstrated that the circular muscle contractility plays a dominant role in segmentation and peristaltic propulsion of the gut^[2]. Also, the drug sensitivity of circular muscle contraction differs from that of longitudinal muscle contraction^[3,4].

The efforts to study opioids and opioid-receptor pharmacology have largely relied upon the availability of highly subtype-selective agonists and antagonists. Although immunohistochemical studies have revealed that the opioid receptor subtypes, μ , δ and κ , are present in the neural tissue of the rat enteric nervous system (ENS), but not in smooth muscle cells^[5,6], various other studies have indicated their involvement in intestinal smooth muscle movement. *In vivo* studies using the charcoal meal method have indicated that μ and δ receptor activation causes slow transit in rats, but κ receptor activation has negligible effect^[7-9]. On the contrary, Mitolo-Chieppa *et al.*^[10] have reported the involvement of κ -opioid receptors in inhibiting gut motility. An *in vitro* study has indicated that activation of both μ and δ receptors has an inhibitory influence on the peristaltic reflex of the rat ileum^[11]. Similarly, *in vitro* studies using electrical stimulation have indicated an inhibitory influence of δ receptors (but not of μ receptors) on longitudinal muscle contractions in the rat jejunum^[12,13]. Thus, the ambiguity regarding the role of κ -opioid receptors in gastrointestinal and vas deferens motility still persists. Keeping in mind these findings and current efforts to develop peripherally acting opioid analgesics directed towards different opioid receptor profiles (e.g., δ agonists or mixed μ agonist/ δ antagonists)^[14,15], we designed the methionine-enkephalin-Arg6-Phe7 (MERF)-based chimeric opioid peptide analogs, which have an affinity for multiple opioid receptors, to study tolerance behavior and other side effects of opioids.

MERF peptide has overlapping sequences of Met-enkephalin and FMRF amide, belongs to the opioid family^[16], and is comprehensively distributed in the central nervous system of different mammals^[17]. Conversely, peptides of the NPPF [Neuropeptide FF (FLFQQR-Fa)/FMRFa family antagonize morphine-induced supraspinal analgesia^[18] and may function as endogenous anti-opioid agents^[19]. NPPF has also been perceived to exhibit opioid effects along with a role in tolerance. The intriguing relationship between opioid and anti-opioid activity of the peptide can be attributed to the FMRF amino acid sequence at the C terminus of MERF. Along these lines, a chimeric peptide YFa (YGGFMKKKFMRF

amide) of met-enkephalin and FMRFa was designed to determine the role of endogenous amphipathic sequences like MERF in analgesia, and its modulation^[20]. YFa administered intraperitoneally induces naloxone-reversible antinociception, suggesting the involvement of opioid receptors in mediation of its antinociceptive effects. Moreover, YFa-potentiated morphine induced antinociception and attenuated the development of tolerance to morphine analgesia, suggesting its possible role in pain modulation^[21]. mRNA expression studies have revealed that YFa produces κ receptor specific antinociception without any tolerance^[22], and it further induced cross tolerance to 20 mg/kg morphine analgesia after 4 d pretreatment with 80 mg/kg YFa^[23]. The results of these studies have been substantiated by forskolin-stimulated cAMP inhibition and Eu-GTP- γ S binding studies^[24].

In addition to YFa, its analogs (D-Ala2) YFa, Y (D-Ala2) GFMKKKFMRF amide, and Des-Phe (YGGFMKKKFMRF amide) have also been studied. (D-Ala2) YFa (1 mg/mouse) administered intracerebroventricularly (icv) with 5.86 nmol/L morphine (2 mg/mouse, icv) produced an additive antinociceptive effect, suggesting its modulatory role in opioid (morphine) analgesia^[21]. Furthermore, mRNA studies have indicated that (D-Ala2) YFa acts mainly through δ receptors and partially through κ and μ opioid receptors^[25], suggesting that D-Ala2 substitution in YFa leads to changes in its receptor selectivity from κ to δ subtype. Des-Phe (YGGFMKKKFMRF amide) demonstrates the loss of mRNA expression of μ opioid receptor and shows κ opioid receptor agonist activity at a higher concentration (unpublished observations). Thus, the observed tolerance-free antinociception of YFa and its analogs prompted us to examine their other pharmacological properties so as to understand the role of opioid receptors in inhibition of gut motility and vas deferens contraction.

In our previous study, we observed early onset of antinociceptive effect (5 min) by chimeric peptide, YFa, which could be a result of direct opioid receptor stimulation and/or due to release of endogenous opioid peptides. In the present study, *in vitro* guinea pig ileum (GPI) and mouse vas deferens (MVD) assays were performed. These assays provided a more physiologically favorable environment for the ligand-receptor interaction to understand the peripheral action of the peptides, because these peripheral opioid responses are important for some of their therapeutic properties such as analgesia and side effects like constipation. The effect of opioid receptor activation in these isolated organ preparations is to reduce smooth muscle contraction *via* inhibition of excitatory neurotransmitter release, which is revealed by measuring the inhibitory action on electrically stimulated contraction of the ileal and vas deferens muscles.

MATERIALS AND METHODS

Peptide synthesis

Peptides YFa, (D-Ala2) YFa, (Des-Phe) YFa and Dynor-

phin A (Tyr1-Gly2-Gly3-Phe4-Leu5-Arg6-Arg7-Ile8-Arg9-Pro10-Lys11-Leu12-Lys13) [DynA(1–13)], were synthesized by the solid-phase method on an ACT-90 peptide synthesizer (Advanced ChemTech, Louisville, KY, United States) using the standard chemistry of 9-flourenylmethoxycarbonyl amino acids (Novabiochem, Laufelfigen, Switzerland) and 1-hydroxybenzotriazole/diisopropylcarbodiimide activation method on Rink amide-MBHA and Wang resin. The peptides were purified by RP-C18 column (mBondapak 10 mm, 7.8 mm × 300 mm; Waters, Milford, MA, United States) on semi-preparative reverse-phase HPLC (Waters 600) with a 40-min linear gradient from 10% to 90% acetonitrile (containing 0.05% trifluoroacetic acid) in water. The mass analysis of the peptides was done in linear positive ion mode by MALDI-TOF/TOF (Bruker Daltonics Flex Analysis, Germany) with 2, 5-dihydroxybenzoic acid as the matrix. The peptide sequence was confirmed by automated peptide sequencing (Procise 491; Applied Biosystems, Carlsbad, CA, United States).

Chemicals

All the chemicals including naloxonazine, naltrindole, norBNI and acetylcholine were purchased from Sigma (St. Louis, MO, United States). Morphine was obtained from AIIMS (New Delhi, India). All the peptides were dissolved in Mili-Q water.

Animals

Male guinea pigs, 300-400 g (AIIMS), were housed, two per cage, kept on a 12-h light/dark cycle, and fed standard rat chow and water *ad libitum*. Male albino mice were obtained from Maulana Azad Medical College (Delhi, India). Animals were housed in temperature-controlled room (25 °C ± 1 °C) and exposed to a 12-h light/dark cycle. The animals were handled according to the guidelines of The Committee for the Purpose of Control and Supervision of Experiments on Animals, India, and the Animal Ethical Committee of the Institute of Genomics and Integrative Biology (Delhi, India).

Bioassay

The experimental procedures were essentially those used previously^[26-28]. For GPI and MVD bioassay, tissue strips were obtained from adult male guinea pigs weighing 300-400 g and male Swiss albino mice weighing 25-30 g. All the animals were sacrificed by intraperitoneal administration of overdose thiopentone (200 mg/kg). Tissues were suspended under 1 g tension in a 10-mL organ bath chamber containing Tyrode solution at 37 °C and bubbled with 95% O₂ and 5% CO₂. The tissues were connected to an isotonic force transducer connected to eight channel organ baths (AD Instruments, Sydney, NSW, Australia) and allowed to equilibrate for 30-45 min. All the tissues were stimulated by chemical method using acetylcholine^[29,30]. Only the tissue preparations that responded to 2 × 10⁻⁴ mol/L acetylcholine by producing contractions of more than 1.5 g tension, were used. Preparations were

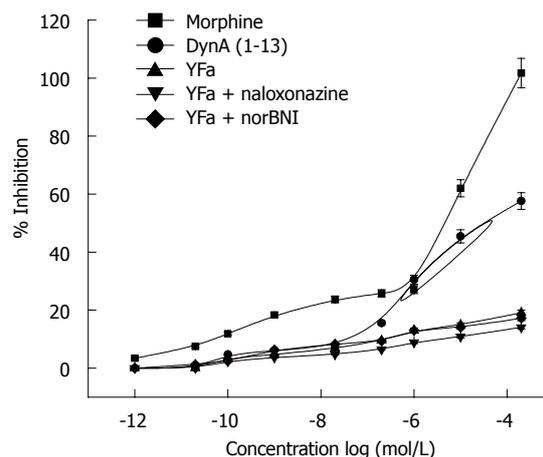


Figure 1 Guinea pig ileum assay of YFa. Morphine and DynA (1-13) were used as controls. Values represent mean ± SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and $P = 0.0002$.

equilibrated for at least 1 h with washes every 10 min before exposure to drugs. At the start of each experiment, a maximum response to acetylcholine (10⁻⁶ mol/L) was obtained in each tissue to check its suitability and the responses to opioid antagonists were expressed as percentages of the maximum acetylcholine. Each experiment was repeated with at least four separate tissue preparations obtained from different animals. Naloxonazine and naltrindole, specific antagonists of μ and δ opioid receptors, were used as negative controls.

Data analysis

GPI and MVD muscle contraction was measured as tension in grams. The inhibition percentage was calculated by taking acetylcholine contraction as 100% in all the tissues. All the assays were performed in triplicate and data were analyzed by Student's *t* test and one-way ANOVA in ORIGIN version 7.1. The data of each ligand were compared with morphine and DynA(1-13) separately, and $P < 0.05$ was considered statistically significant.

RESULTS

Effect of YFa on guinea pig ileum muscle contraction

In THE GPI assay (Figure 1), YFa demonstrated negligible inhibition of ileal muscle contraction, even at the highest concentration. Morphine, which interacts through μ opioid receptors, exhibited a highly significant inhibition rate of 62% ($P = 0.001$) at 10⁻⁵ mol/L and 101% at 2 × 10⁻⁴ mol/L. However, DynA (1-13), a known κ receptor agonist, showed a moderate inhibition of 57% ($P = 0.004$) at the highest dose of 2 × 10⁻⁴ mol/L.

Effect of YFa on mouse vas deferens muscle contraction

In MVD preparations (Figure 2), YFa exhibited a moderate inhibition of 24% ($P = 0.001$) at 2 × 10⁻⁷ mol/L and 45% at 10⁻⁵ mol/L. The maximum inhibitory response rate was 68% ($P = 0.001$), which was significantly lower

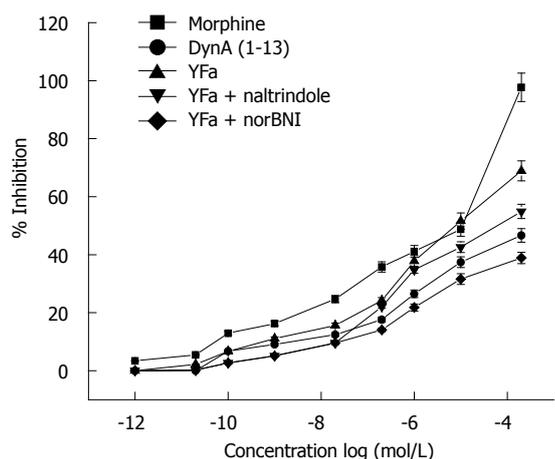


Figure 2 Mouse vas deferens assay of YFa. Morphine and DynA (1-13) were used as controls. Values represent mean \pm SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and $P = 0.001$.

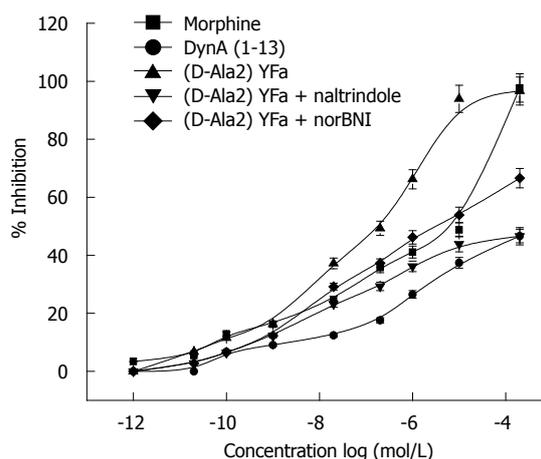


Figure 4 Mouse vas deferens assay of (D-Ala2) YFa. Morphine and DynA (1-13) were used as controls. Values represent mean \pm SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and $P = 0.0006$.

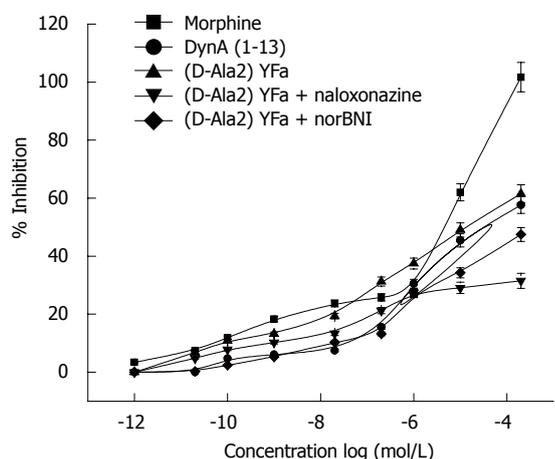


Figure 3 Guinea pig ileum assay of (D-Ala2) YFa. Morphine and DynA (1-13) were used as controls. Values represent mean \pm SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and $P = 0.0002$.

than that of morphine (101%) but higher than DynA (1-13) (47%, $P = 0.004$). IC_{50} of YFa (7.10 $\mu\text{mol/L}$, $P = 0.001$) was nearly half that of morphine (13.41 $\mu\text{mol/L}$, $P = 0.001$), (Table 1). Vas deferens preparations pretreated with κ receptor specific antagonist norBNI showed a 44% reversibility of inhibitory activity, whereas, with naltrindole, the δ receptor specific antagonist, the activity was declined by only 20%.

Effect of (D-Ala2) YFa on guinea pig ileum muscle contraction

In contrast to YFa, (D-Ala2) YFa treatment resulted in moderate inhibition of GPI muscle contraction (Figure 3). It showed escalating behavior in inhibition from a value of 31.27% ($P = 0.0006$) at 2×10^{-7} mol/L to 61.51% at 2×10^{-4} mol/L. The IC_{50} of (D-Ala2) YFa was 12 $\mu\text{mol/L}$ ($P = 0.001$) (Table 1) compared with that of morphine (4.44 $\mu\text{mol/L}$, $P = 0.001$) (Table 1), which again indicat-

Table 1 IC_{50} values for inhibition of smooth muscle contraction induced by YFa and its analogs in guinea pig ileum and mouse vas deferens assays

Guinea pig ileum ($n = 9$) ^a			Mouse vas deferens ($n = 9$) ^a		
Agonist	IC_{50} ($\mu\text{mol/L}$)	P value	Agonist	IC_{50} ($\mu\text{mol/L}$)	P value
Morphine	4.40	0.001	Morphine	13.41	0.001
Dyn A (1-13)	33.8	0.004	Dyn A (1-13)	ns	-
YFa	ns	-	YFa	7.10	0.001
D (Ala-2) YFa	12.0	0.001	D (Ala-2) YFa	0.20	0.0006
(Des-Phe) YFa	14.9	0.0002	(Des-Phe) YFa	ns	-
MERF-COOH	3.71	0.005	MERF-COOH	5.51	0.0005
MERF-NH2	ns	-	MERF-NH2	ns	-

^a $P < 0.05$ for guinea pig ileum and mouse vas deferens assays was considered statistically significant. The values represent mean \pm SE for three experiments performed in triplicate. IC_{50} values were calculated by one-way ANOVA and data were statistically significant. MERF: Methionine-enkephalin-Arg6-Phe7. ns: Non-significant.

ed a moderate interaction with GPI muscle. Antagonist pretreatment of ileal tissue with naloxonazine exhibited a 50% decline in inhibition of muscle contraction, whereas only a 20% reversibility was observed with pretreatment with norBNI.

Effect of (D-Ala2) YFa on mouse vas deferens muscle contraction

(D-Ala2) YFa demonstrated a considerable inhibition of MVD muscle contraction (Figure 4), which increased progressively from 11.49% ($P = 0.0006$) at 10^{-10} mol/L to 37.22% at 2×10^{-8} mol/L ($P = 0.0006$). The maximum inhibitory response of 96% ($P = 0.0006$) at 2×10^{-4} mol/L was comparable to that of morphine (97%, $P = 0.0006$), but significantly higher than that of DynA (1-13) (46%). D-(Ala2) YFa showed an IC_{50} of 0.20 $\mu\text{mol/L}$ ($P = 0.0006$) (Table 1), demonstrating the selective interaction of the peptide with δ opioid receptors, which are substantially present in MVD muscles.

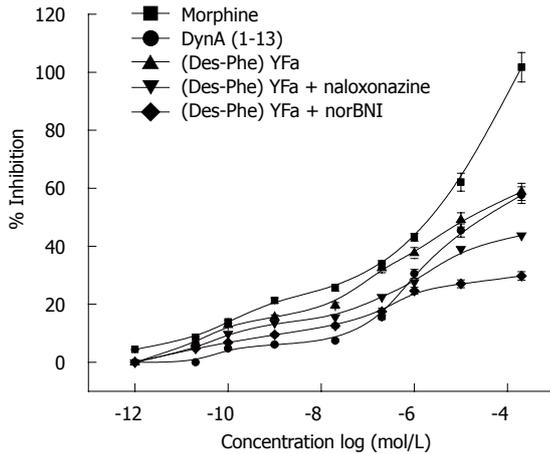


Figure 5 Guinea pig ileum assay of (Des-Phe) YFa. Morphine and DynA (1-13) were used as controls. Values represent mean \pm SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and $P = 0.0002$.

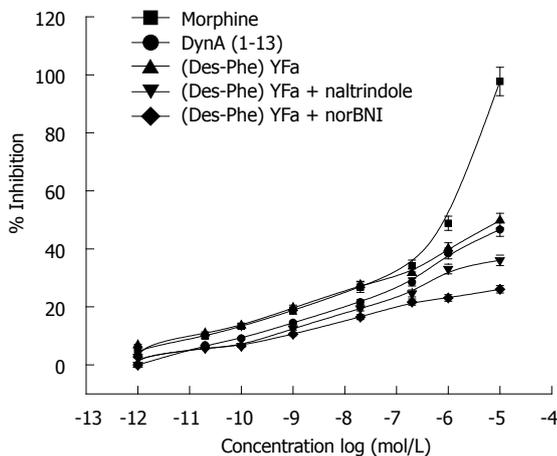


Figure 6 Mouse vas deferens assay of (Des-Phe) YFa. Morphine and DynA(1-13) were used as controls. Values represent mean \pm SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and $P = 0.001$.

Pretreatment with naltrindole or norBNI resulted in a 52% ($P = 0.0006$) and 30% ($P = 0.0006$) reversibility of inhibition of MVD muscle contraction. DynA (1-13) also showed a weak inhibition of MVD contraction with a non-significant IC_{50} value.

Effect of (Des-Phe) YFa on guinea pig ileum muscle contraction

Moderately significant inhibition was observed with (Des-Phe) YFa treatment comparable to that of DynA(1-13) (Figure 5). The inhibitory response was stronger than that of YFa but weaker than that of morphine. A two-fold increase in inhibition from 15.54% ($P = 0.0002$) to 32.49% ($P = 0.0002$) was noted as concentration increased from 10^{-9} mol/L to 2×10^{-7} mol/L with IC_{50} at 14.9 μ mol/L ($P = 0.0002$) (Table 1). The specific-antagonist-pretreated GPI preparations demonstrated that the reversibility in inhibitory activity of (Des-Phe) YFa was higher with nor-

BNI (50%, $P = 0.0002$) than with naloxonazine (26%, $P = 0.0002$).

Effect of (Des-Phe) YFa on mouse vas deferens muscle contraction

(Des-Phe) YFa treatment resulted in a weak inhibition of MVD muscle contraction (Figure 6). The inhibitory response was comparable to that of dynorphin with a maximum response of 49.78% ($P = 0.001$) at 2×10^{-4} mol/L. Pretreatment with norBNI resulted in a 48% ($P = 0.001$) reversibility of inhibitory activity, while naltrindole pretreatment led to a 28% reversibility ($P = 0.001$).

Effect of MERF-COOH on guinea pig ileum muscle contraction

MERF-COOH, an endogenous opioid receptor agonist, has been reported to bind to all three subtypes of opioid receptors. GPI assay (Figure 7) showed a dose-dependent response that was comparable to that of morphine at all concentrations. Analogous to morphine and MERF-COOH, exhibited a steady rise in inhibition at a dose of 10^{-5} mol/L (58.57%, $P = 0.005$ and 62.10%, $P = 0.001$, respectively), and further demonstrated a sudden (almost twofold) elevation in inhibition profile by 95.27% ($P = 0.005$) at the highest dose. The IC_{50} value (3.71 μ mol/L, $P = 0.005$) (Table 1) was comparable to that of morphine (4.40 μ mol/L, $P = 0.001$). Moreover, a similar reversibility (50%) in inhibition profile was noted in the GPI preparations pretreated with norBNI and naloxonazine.

Effect of MERF-COOH on mouse vas deferens muscle contraction

In the MVD assay (Figure 8), MERF-COOH demonstrated a significant inhibition of MVD muscle contraction. At 10^{-6} mol/L concentration, it exhibited a comparable inhibition profile to morphine, whereas at higher concentrations, the trend varied. MERF-COOH exhibited an IC_{50} value of 5.51 μ mol/L ($P = 0.001$), which was less than half that of morphine (13.41 μ mol/L, $P = 0.001$) (Table 1). Moreover, the peptide showed a 72.57% ($P = 0.001$) inhibition at the highest dose. The δ - and κ -specific antagonist pretreatment of MVD preparations exhibited a similar degree of reversibility (35%) of inhibitory activity with naltrindole and norBNI, respectively.

Effect of MERF-NH₂ on guinea pig ileum and mouse vas deferens smooth muscle contraction

Contrary to MERF-COOH, MERF-NH₂ treatment resulted in a weak inhibition of GPI (41%, $P = 0.001$) and MVD (31%, $P = 0.0005$) muscle contraction at the highest concentration of 10^{-4} mol/L (Figures 9 and 10). The specific-antagonist-pretreated preparations of GPI (naloxonazine and norBNI) and MVD (naltrindole and norBNI) did not show any significant reversibility in inhibition profile.

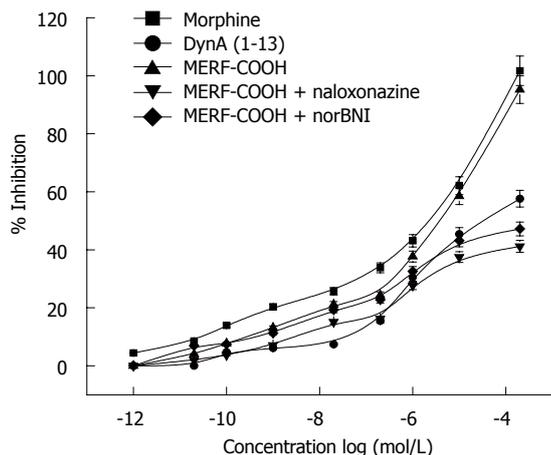


Figure 7 Guinea pig ileum assay of methionine-enkephalin-Arg6-Phe7-COOH. Morphine and DynA (1-13) were used as controls. Values represent mean \pm SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and $P = 0.005$. MERF: Methionine-enkephalin-Arg6-Phe7.

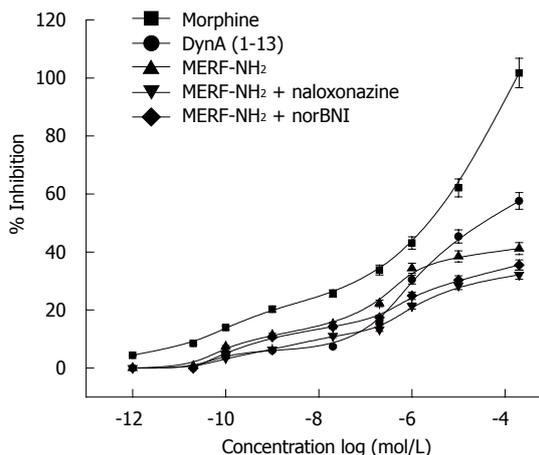


Figure 9 Guinea pig ileum assay of methionine-enkephalin-Arg6-Phe7-NH₂. Morphine and DynA (1-13) were used as controls. Values represent mean \pm SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and $P = 0.001$. MERF: Methionine-enkephalin-Arg6-Phe7.

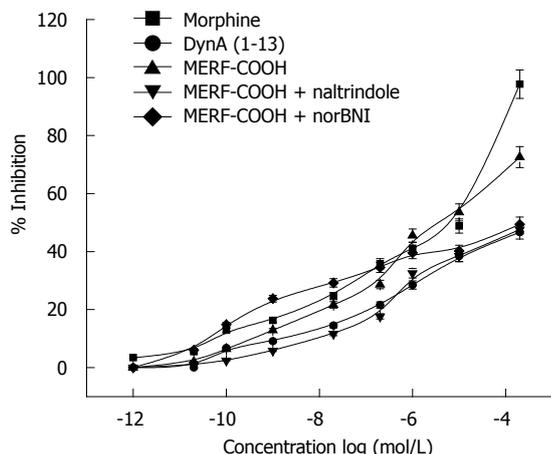


Figure 8 Mouse vas deferens assay of methionine-enkephalin-Arg6-Phe7-COOH. Morphine and DynA (1-13) were used as controls. Values represent mean \pm SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and $P = 0.001$. MERF: Methionine-enkephalin-Arg6-Phe7.

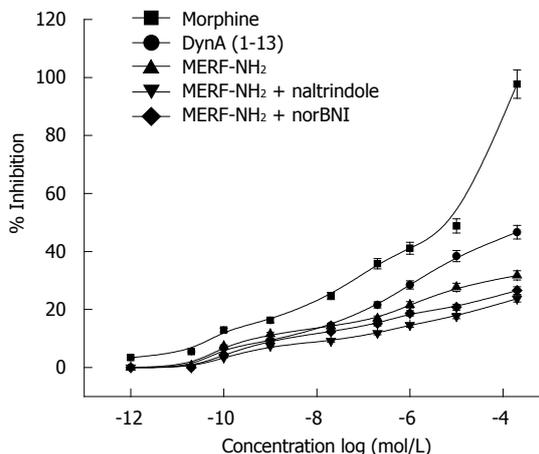


Figure 10 Mouse vas deferens assay of methionine-enkephalin-Arg6-Phe7-NH₂. Morphine and DynA (1-13) were used as controls. Values represent mean \pm SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and $P = 0.0005$. MERF: Methionine-enkephalin-Arg6-Phe7.

DISCUSSION

This study examines the effects of YFa and its analogs on GPI and MVD motility, in conjunction with their receptor selectivity. It is well documented that μ opioid receptors are primarily responsible for constipation, along with inhibition of nitric oxide generation^[31]. In the gastrointestinal tract, activation of μ opioid receptors results in the inhibition of gut motility that leads to constipation, whereas similar receptors in the central nervous system mediate the analgesic actions of opioids^[32]. The μ -receptor-selective drug, morphine, significantly restricted the smooth muscle contractions in lower intestine, indicating the presence of μ opioid receptors in the ileal muscle. Therefore, by inhibiting gastric flow and reducing propulsive peristalsis of the intestine, morphine decreases the rate of intestinal transit. Reduction in gut

secretion and increase in intestinal fluid absorption further contribute to the constipating effect^[33].

In 1993, Smith and Leslie^[34] reported the δ subtype of opioid receptors as the major form in MVD, with a smaller number of μ receptors. Alternatively, in 1999, Pound^[35] reported that morphine induced significant inhibition of MVD muscle contraction, which indicated the presence of separate μ opioid receptors. Furthermore, functional interactions between μ and δ opioid receptors, for several biochemical and pharmacological responses have also been reported by various groups^[36-39]. These functional interactions of μ receptors could be rationalized on the basis of their indirect activation by δ receptors^[40]. Collectively, these findings reveal that δ opioid receptors are prominent in MVD and there exists some cooperation between μ and δ opioid receptors that supports the hypothesis of synergistic interactions

between these two receptors.

Although the presence of μ receptors in the gut and MVD is well supported in the literature, the role of κ receptors is still ambiguous. Here, we used YFa and its analogs as probes to unravel these hidden aspects. Our previous studies on YFa have revealed its κ -receptor-selective nature. However, at higher concentrations, it also interacts with μ receptors^[22-24]. In the present study, YFa showed a negligible inhibition of GPI contraction. This could be due to non-availability of κ opioid receptors or to the counteracting effect of the anti-opioid side (FMRF-amide) of the peptide, through its interaction with the anti-opiate receptors, by increasing sensitivity to cholinergic stimulation upon acetylcholine release^[41-43].

To investigate further the reason behind these observations, an analog of YFa, (Des-Phe) YFa, was designed and studied. Upon modification, (Des-Phe) YFa retained its κ -receptor-selective antinociceptive nature but removal of Phe from the C terminus resulted in loss of RF-amide interaction with anti-opiate receptor, hence nullifying the counteractive effect of anti-opiate moiety (RFa) in YFa. (Des-Phe) YFa exhibited a significant inhibition of GPI muscle contractions, comparable to those of dynorphin. In comparison with YFa, a threefold increase in inhibition was observed as a result of the modification. Therefore, the counteractive effect of the anti-opioid side of YFa could be the reason for the non-significant inhibitory effect of YFa. This observation emphasizes the existence of κ -receptor-mediated contractions, in addition to the known μ receptor involvement in GPI muscle contractions^[10,44-47]. Reversibility of contraction by pretreatment with κ -receptor-specific antagonist nor-BNI confirmed the κ -receptor-mediated interaction in GPI.

To substantiate the role of the anti-opiate moiety in the effect of YFa, analogs of MERF were studied. As mentioned earlier, MERF is a well-documented peptide belonging to the opioid family. Here, we studied the inhibitory profiles of two slight modifications of this peptide, MERF-COOH and MERF-NH₂, the latter of which has a C-terminal RFamide residue that interacts with the anti-opiate receptors. As expected, in the GPI assay, MERF-COOH led to a 100% inhibition and MERF-NH₂ treatment resulted in a negligible response. This complete reversal in properties confirms the role of counteractive effects of anti-opioid moieties in antinociception.

In the MVD assay, YFa demonstrated moderately significant inhibition of vas deferens contractions, in a dose-dependent fashion. This observation firmly suggests the involvement of the κ receptors in the observed effect, which corroborates the earlier reports suggesting the involvement of κ receptors in MVD muscle contraction^[10,44,48]. Moreover, the maximum inhibitory response was found to be stronger than that of dynorphin (κ -receptor-specific agonist), suggesting the involvement of other receptors also, which may be due to the saturation of κ opioid receptors. The role of κ receptors was substantiated by antagonist pretreatment studies

that showed a higher reversibility of contraction by κ -receptor than δ -receptor antagonist. The role of anti-opiate receptor is still not evident in MVD, therefore, that possibility was not considered in this case.

Recently, we have reported that (D-Ala2) YFa mediates its action primarily through δ opioid receptors and partially through μ and κ opioid receptors^[25]. In the present study, (D-Ala2) YFa demonstrated a moderate inhibition of GPI muscle contraction, comparable to that of dynorphin, suggesting the involvement of μ and κ receptors in the observed effect. Specific antagonist pretreatment studies have emphasized the role of μ receptors and naloxonazine (μ -receptor antagonist) pretreatment resulted in a 50% reversibility in inhibition. The IC₅₀ value of (D-Ala2) YFa of 12 μ m, which was much lower than that of DynA (1-13) (33.8 μ m), substantiated the role of μ receptors in (D-Ala2) YFa-mediated GPI contraction.

Furthermore, in the MVD assay, (D-Ala2) YFa demonstrated a significantly greater inhibition than that of morphine at all concentrations up to 10⁻⁵ mol/L. As expected, this suggests the involvement of δ and μ receptors in MVD muscle contraction. Pretreatment with naltrindole resulted in an almost 50% reduction in inhibition that suggested the involvement of δ receptors, which was further demonstrated by the IC₅₀ value of (D-Ala2) YFa of 0.2 μ mol/L ($P = 0.01$). However, significant inhibition of MVD muscle contraction by morphine (μ -receptor specific) and (D-Ala2) YFa (δ -receptor specific) further signifies that some cooperation may exist between μ and δ receptors in vas deferens preparations^[40], or the δ receptors may regulate μ -receptor function *via* heterodimerization^[49]. Further studies on heterodimerization of opioid receptors (μ , δ and κ) are required to elucidate their synergistic behavior and are currently in progress in our laboratory.

In conclusion, YFa and its analogs can be viewed as promising candidates to understand the role of opioid receptors in gastrointestinal transit and MVD motility. Although the precise mechanism by which anti-opiate receptors normalize the effects mediated by opioid receptors in GPI and MVD contraction is currently not clear, we provide convincing evidences that anti-opioid receptors are involved in the phenomenon. We also confirmed the presence of κ receptors in GPI and MVD muscles. Furthermore, the present findings provide a systematic approach to advance the researches on opioids due to the similar nature of opioid receptors in GPI and human intestines.

COMMENTS

Background

To date, centrally acting μ -receptor-specific agonists are the most widely used analgesics but their relieving effect is accompanied by a number of side effects including tolerance and adverse gastrointestinal effects.

Research frontiers

Opioids mediate their effects through various receptors (μ , κ and δ) present in the central nervous system, but the presence of similar receptors in the en-

teric nervous system leads to disturbances in gastrointestinal transit. Previous studies have reported the presence of μ and δ receptors in the gut and vas deferens, whereas the role of κ receptors is still ambiguous. In this study, the authors demonstrated the role of κ receptors and anti-opioid receptors using methionine-enkephalin-Arg6-Phe7 (MERF) peptide analogs.

Innovations and breakthroughs

The study reported YFa, an analgesic peptide molecule, free of gastrointestinal inhibition effect.

Applications

By understanding the roles of various opioid receptors in gastrointestinal transit, this study will provide a systematic approach to advance the researches on opioids.

Terminology

Guinea pig ileum (GPI) and mouse vas deferens (MVD) assays are the well reported methods for screening the drugs/molecules for smooth muscle contractions.

Peer review

Overall, the present work is a useful study which is potentially helpful for establishing the connection between opioid agents and smooth muscle contraction in humans. One goal is to develop pharmacological means to counteract undesirable effects of chronic administration of opioids in patients.

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S- Editor Zhang SJ L- Editor Kerr C E- Editor Zhang DN

Difference between CKD-EPI and MDRD equations in calculating glomerular filtration rate in patients with cirrhosis

Yu-Wei Chen, Han-Hsiang Chen, Tsang-En Wang, Ching-Wei Chang, Chen-Wang Chang, Chih-Jen Wu

Yu-Wei Chen, Han-Hsiang Chen, Chih-Jen Wu, Division of Nephrology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei 10449, Taiwan

Yu-Wei Chen, Han-Hsiang Chen, Tsang-En Wang, Ching-Wei Chang, Chen-Wang Chang, Chih-Jen Wu, Mackay Medicine, Nursing and Management College, Taipei 10449, Taiwan

Han-Hsiang Chen, National Taipei College of Nursing, Taipei 10449, Taiwan

Tsang-En Wang, Ching-Wei Chang, Chen-Wang Chang, Division of Gastroenterology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei 10449, Taiwan

Chih-Jen Wu, Graduate Institute of Medical Science, Taipei Medical University, Taipei 11031, Taiwan

Author contributions: Chen YW, Chen HH, Chang CW and Wu CJ designed the research; Chen YW, Chang CW and Chang CW performed the research; Chen YW and Chen HH contributed new reagents/analytic tools; Chen YW, Wang TE and Chang CW analyzed the data; and Chen YW wrote the paper.

Correspondence to: Dr. Chih-Jen Wu, Division of Nephrology, Department of Internal Medicine, Mackay Memorial Hospital, No. 92, sec 2, Zhongshan N Rd, Zhongshan Dist, Taipei 10449, Taiwan. yw.chen.mmh@gmail.com

Telephone: +886-2-25433535 Fax: +886-2-25433642

Received: January 6, 2011 Revised: June 15, 2011

Accepted: June 22, 2011

Published online: October 28, 2011

Abstract

AIM: To evaluate the difference between the performance of the (CKD-EPI) and Modification of Diet in Renal Disease (MDRD) equations in cirrhotic patients.

METHODS: From Jan 2004 to Oct 2008, 4127 cirrhotic patients were reviewed. Patients with incomplete data with respect to renal function were excluded; thus, a total of 3791 patients were included in the study. The glomerular filtration rate (GFR) was estimated by the 4-variable MDRD (MDRD-4), 6-variable MDRD (MDRD-6), and CKD-EPI equations.

RESULTS: When serum creatinine was 0.7-6.8 mg/dL and 0.6-5.3 mg/dL in men and women, respectively, a significantly lower GFR was estimated by the MDRD-6 than by the CKD-EPI. Similar GFRs were calculated by both equations when creatinine was > 6.9 mg/dL and > 5.4 mg/dL in men and women, respectively. In predicting in-hospital mortality, estimated GFR obtained by the MDRD-6 showed better accuracy [81.72%; 95% confidence interval (CI), 0.94-0.95] than that obtained by the MDRD-4 (80.22%; 95%CI, 0.96-0.97), CKD-EPI (79.93%; 95%CI, 0.96-0.96), and creatinine (77.50%; 95%CI, 2.27-2.63).

CONCLUSION: GFR calculated by the 6-variable MDRD equation may be closer to the true GFR than that calculated by the CKD-EPI equation.

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Key words: Chronic Kidney Disease Epidemiology Collaboration; Estimated glomerular filtration rate; Liver cirrhosis; Modification of Diet in Renal Disease; Renal function

Peer reviewer: Yogesh K Chawla, Dr., Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Chen YW, Chen HH, Wang TE, Chang CW, Chang CW, Wu CJ. Difference between CKD-EPI and MDRD equations in calculating glomerular filtration rate in patients with cirrhosis. *World J Gastroenterol* 2011; 17(40): 4532-4538 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4532.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4532>

INTRODUCTION

Routine tests for serum creatinine (Scr) have been found to significantly improve the prognostic accuracy of

Child-Pugh score and to be an independent predictor of survival in patients with end-stage liver disease^[1]. In the early 2000s, the Model for End-stage Liver Disease (MELD) score emerged as a simple and more objective score than Child-Pugh score, with Scr as one of the 3 variables included [the other 2 being international normalized ratio (INR) and serum bilirubin]^[2-4]. Unlike those of the Child-Pugh score, the 3 variables of the MELD score are selected on the basis of statistical analysis and not empirical analysis. Different from INR and serum bilirubin, which are the basic markers of liver function, Scr is essentially a marker of renal function; and highlights the prognostic significance of the interactions between liver and renal functions in patients with cirrhosis^[5].

Kidney injury is an ominous and common event in cirrhotic patients^[6]. Although Scr shows a strong prognostic value in patients with cirrhosis, it is considered an insensitive predictor in such patients because of reduced muscle mass, protein-deficient diet, severe hyperbilirubinemia, and diminished hepatic biosynthesis of serum creatinine, all of which lead to an overestimation of creatinine clearance as compared with inulin clearance^[7]. Therefore, Scr level and creatinine-based equations also tend to overestimate glomerular filtration rate (GFR) in patients with cirrhosis.

Recently, a new creatinine-based equation known as the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation has been proposed as a more accurate formula than the Cockcroft and Modification of Diet in Renal Disease (MDRD) equations^[8]. However, the CKD-EPI equation has not been tested in patients with cirrhosis. The aim of the present study was to evaluate the difference between the performance of the MDRD and CKD-EPI equations when evaluating renal function in a broader population of patients with cirrhosis than liver transplant registries.

MATERIALS AND METHODS

Patient information and data collection

A retrospective, cross-sectional, single-center study design was used, and the study protocol was approved by the local ethics committee. Patients diagnosed with cirrhosis were selected from those admitted to Mackay Memorial Hospital between January 2004 and October 2008.

Of a total of 228 345 admitted patients, the records of 4127 patients with cirrhosis were reviewed. Patients who survived and were followed up in the outpatient department were defined as survivors, and the most recent laboratory data available for them were collected. Patients whose records indicated death any time during the hospital stay were defined as non-survivors (cases of in-hospital mortality), and laboratory data for these patients were those collected during the admission in which death occurred. In the case of patients with multiple admissions, the records before those of the last admission were excluded. Demographic data, Child-Pugh

scores, and information regarding underlying comorbidities were obtained from the most recent laboratory examinations. Patients with incomplete data with respect to Child-Pugh score and renal function or with cirrhosis due to congenital abnormality were excluded; thus, a total of 3791 patients were included in the study. None of the included patients had received liver transplants. The data on renal function in the common populace were based on the results of health examinations conducted among the residents of Taipei city, Taiwan, which were recently published as part of an epidemiologic study conducted at our institution^[9].

Laboratory methods

We calibrated serum creatinine values using the modified Jaffe method (Beckman Coulter, Inc. UniCel[®] Dx_C 800 Synchron[®] Clinical System) which were further standardized using the isotope dilution mass spectrometry (IDMS) reference method at Mackay Memorial Hospital Laboratory

Equations

The GFR was calculated according to the listed formulae: MDRD-4 = $175 \times (\text{Scr})^{-1.154} \times (\text{Age})^{-0.203} \times (0.742 \text{ if female}) \times (1.178 \text{ if black})$ ^[10], MDRD-6 = $170 \times (\text{Scr})^{-0.999} \times (\text{Age})^{-0.176} \times (0.762 \text{ if patient is female}) \times (1.180 \text{ if black}) \times (\text{SUN})^{-0.170} \times (\text{Albumin})^{0.318}$ ^[10], CKD-EPI = $141 \times \min(\text{Scr}/\kappa, 1)^\alpha \times \max(\text{Scr}/\kappa, 1)^{-1.209} \times 0.993^{\text{Age}} \times 1.018$ (if female) $\times 1.159$ (if black)^[8], where MDRD-4 is the 4-variable MDRD, MDRD-6 is the 6-variable MDRD, age is given in years, albumin in g/dL, Scr is serum creatinine (mg/dL), SUN is serum urea nitrogen concentration (mg/dL), κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr/ κ or 1, and max indicates the maximum of serum creatinine/ κ or 1.

Statistical analysis

Continuous variables are summarized as mean \pm standard deviation unless otherwise stated. We initially compared the demographic data and laboratory variables of survivors, non-survivors, and the common populace using the analysis of variance (ANOVA) test and chi-square test. Student's *t* test was used to assess differences in estimated GFR (eGFR) by CKD-EPI between cirrhotic patients and the common populace, and the difference in eGFR in cirrhotic patients calculated by MDRD6 or CKD-EPI, respectively. Logistic regression analyses were conducted to investigate the accuracy of predicting in-hospital mortality by the different creatinine-based equations. The results of these analyses were used to construct a receiver-operating characteristic (ROC) curve from which we sought the optimum cut-off point for predicting successful sites. The optimum cutoff point was defined as the point on the ROC curve closest to the point (0.1), where the false-positive rate was zero and the sensitivity was 100%. The area under the curve (AUC) and 95% confidence interval (CI) were

Table 1 Demographic and laboratory data of 3791 cirrhotic patients and 4292 common populace *n* (%)

Variables (<i>n</i> , %)	Survived cirrhotic patients (<i>n</i> = 2337)	Expired cirrhotic patients (<i>n</i> = 1454)	Common populace (<i>n</i> = 4292)	<i>P</i> value
Age (yr)	59.03 ± 14.03	63.61 ± 13.62	52.11 ± 12.13	< 0.001
Gender (male/female)	1620/717	990/464	2270/2022	< 0.001
Albumin (3.5-5 g/dL)	3.24 ± 0.68	2.48 ± 0.55	4.50 ± 0.29	< 0.001
BUN (8-12 mg/dL)	17.31 ± 14.77	60.81 ± 40.77	13.39 ± 3.78	< 0.001
Creatinine (0.4-1.2 mg/dL)	1.20 ± 1.06	2.93 ± 1.99	0.89 ± 0.20	< 0.001
eGFR (MDRD4)	79.27 ± 35.43	36.75 ± 33.55	81.72 ± 16.38	< 0.001
eGFR (MDRD6)	65.70 ± 30.28	26.76 ± 24.63	69.65 ± 13.16	< 0.001
eGFR (CKD-EPI)	78.50 ± 29.82	36.03 ± 30.23	88.31 ± 15.78	< 0.001
Total bilirubin (0.3-1.2 mg/dL)	2.24 ± 3.58	9.76 ± 10.68		< 0.001
INR	1.36 ± 0.43	2.70 ± 2.53		< 0.001
Hepatoma	647 (27.69)	717 (49.31)		< 0.001
Ascites	817 (34.96)	1018 (70.01)		< 0.001
Hepatic encephalopathy	431 (18.44)	649 (44.64)		< 0.001
Child-Pugh points	7.12 ± 1.97	10.37 ± 2.09		< 0.001

BUN: Blood urea nitrogen; eGFR: Estimated glomerular filtration rate; MDRD: Modification of Diet in Renal Disease; MDRD4: 4-variable MDRD; MDRD6: 6-variable MDRD; CKD-EPI: The Chronic Kidney Disease Epidemiology Collaboration; INR: International normalized ratio.

calculated. A *P* value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software (version 17.0, SPSS Inc., Chicago, IL, United States).

RESULTS

Patient characteristics

Table 1 shows the demographic data, clinical characteristics, and laboratory data of patients with cirrhosis and the common populace. Older age, poorer renal function, and worse nutritional status were noted in the patients with cirrhosis than in the common populace. Thus, the average eGFR in patients with cirrhosis was significantly lower than that in the common populace, irrespective of the equation used for calculation (MDRD-4, MDRD-6, or CKD-EPI equation).

Difference between the performance of the MDRD-4, MDRD-6, and CKD-EPI equations in patients with cirrhosis and the common populace

Figure 1 shows the application of the 3 creatinine-based equations for calculating GFR in the common populace. The slope of the CKD-EPI equation was similar to that of the MDRD-4 equation when the Scr level was > 0.8 mg/dL and > 0.6 mg/dL in men and women, respectively, but less steep below the knots, which leads to less overestimation of GFR by the CKD-EPI equation at a lower Scr level^[8]. Figure 2 shows the application of the CKD-EPI equation in calculating GFR in both the patients with cirrhosis and the common populace. At the same Scr level, the CKD-EPI equation tended to estimate a significantly lower value of GFR in patients with cirrhosis when the Scr level was 0.8-1.2 mg/dL and 0.5-1.1 mg/dL in men and women, respectively. Figure 3 shows the eGFR obtained by the 3 creatinine-based equations in patients with cirrhosis. The eGFRs obtained by the 3 equations were similar when the Scr level was > 6.9 mg/dL and > 5.4 mg/dL in men and women, respective-

ly. Interestingly, significantly lower eGFR was obtained by the MDRD-6 equation than by the CKD-EPI equation when the Scr level was 0.7-6.8 mg/dL and 0.6-5.3 mg/dL in men and women, respectively. When the Scr level was < 0.5 mg/dL in men (1.8% of men with cirrhosis) and < 0.4 mg/dL in women (1.4% of women with cirrhosis), a lower eGFR was obtained by the CKD-EPI equation than by the MDRD-6 equation.

Prediction of in-hospital mortality by the different methods of renal function evaluation

The eGFR obtained by the MDRD-6 equation showed better accuracy (81.72%; 95% CI, 0.94-0.95) in predicting in-hospital mortality than that obtained by the MDRD-4 equation (80.22%; 95% CI, 0.96-0.97) and CKD-EPI equation (79.93%; 95% CI, 0.96-0.96). In general, eGFR showed a better prognostic value as a surrogate of renal function than Scr level (accuracy, 77.50%; 95% CI, 2.27-2.63). In the ROC curve (Figure 4), the cutoff point for eGFR obtained by the MDRD-6 equation was 41 (AUC, 0.85; 95% CI, 0.84-0.87). Interestingly, the cutoff point for Scr level was 1.3 mg/dL (AUC, 0.83; 95% CI, 0.81-0.84), which was lower than 1.5 mg/dL, a value suggested to indicate renal failure in patients with cirrhosis and the threshold value for the diagnosis of hepatorenal syndrome.

DISCUSSION

This retrospective, cross-sectional, single-center study involved a broader population of patients with cirrhosis than liver transplant registries to obtain eGFR using different creatinine-based equations. A significantly lower eGFR was obtained by the MDRD-6 equation than by the CKD-EPI equation when the Scr level was 0.7-6.8 mg/dL and 0.6-5.3 mg/dL in men and women, respectively. In view of the overall overestimation of GFR by the creatinine-based equations in patients with cirrhosis, eGFR obtained by the MDRD-6 equation may be closer to the

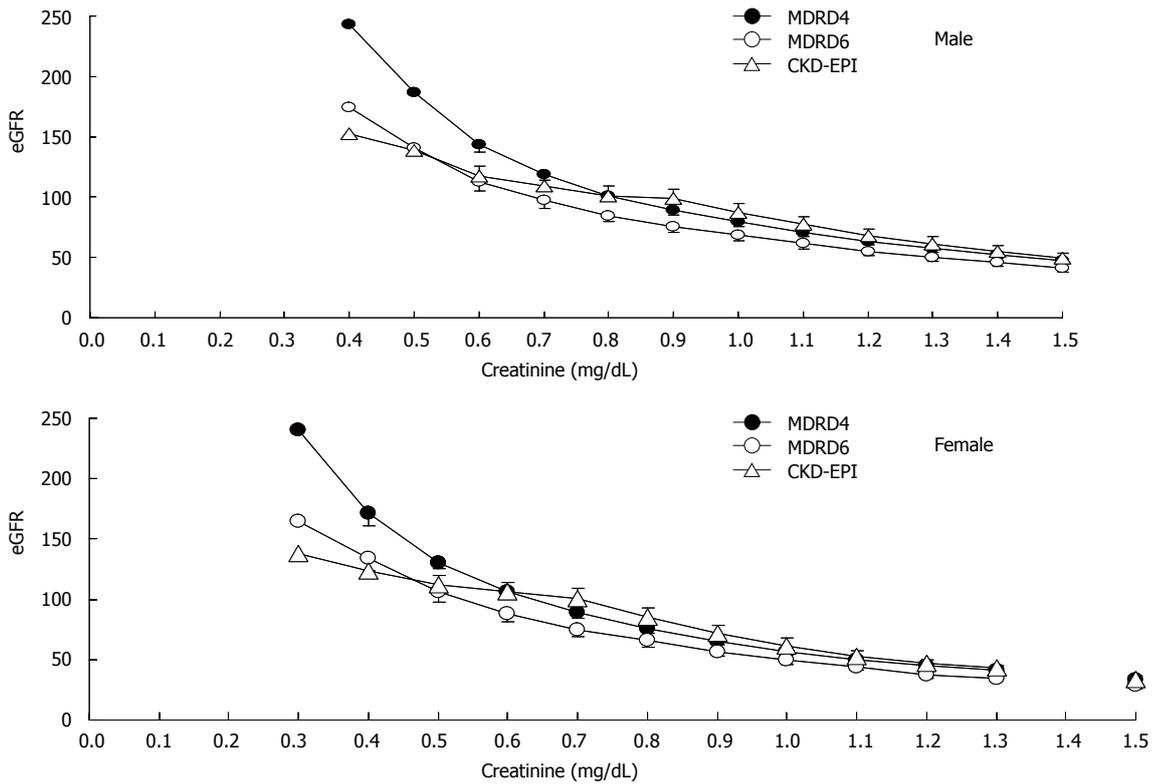


Figure 1 Estimated glomerular filtration rate obtained by the 4-variable Modification of Diet in Renal Disease, 6-variable Modification of Diet in Renal Disease, and the Chronic Kidney Disease Epidemiology Collaboration equations in the common populace. eGFR: Estimated glomerular filtration rate; MDRD: Modification of Diet in Renal Disease; MDRD4, 4-variable MDRD; MDRD6: 6-variable MDRD; CKD-EPI: The Chronic Kidney Disease Epidemiology Collaboration.

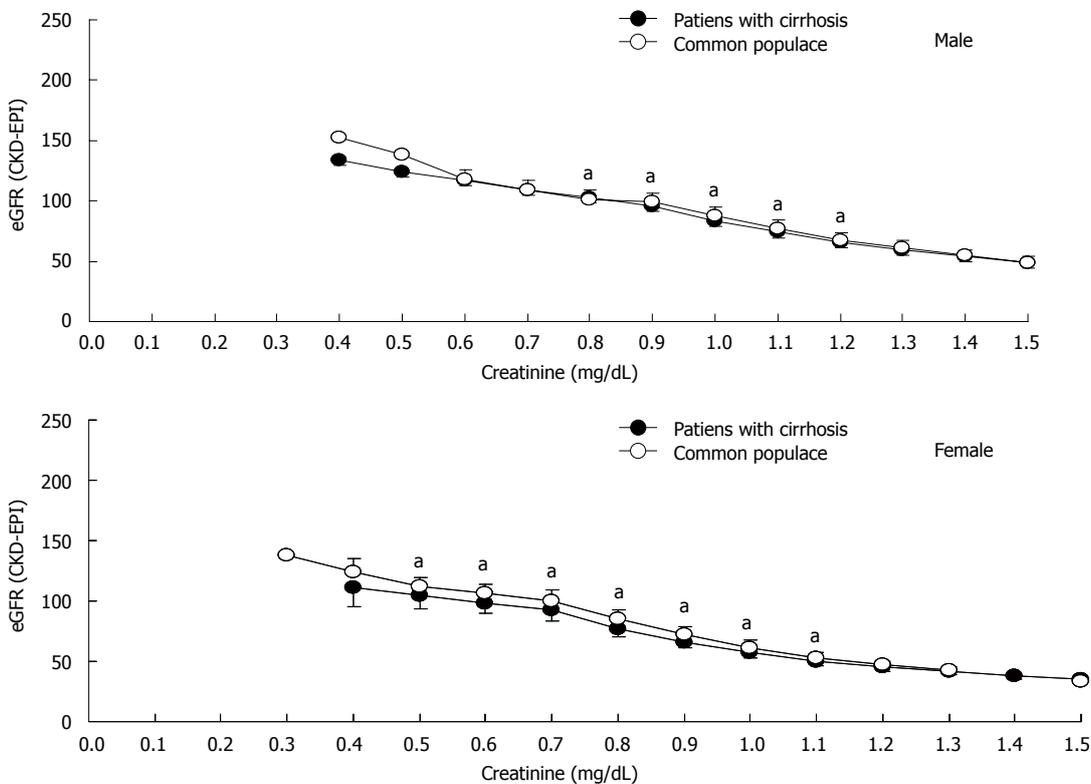


Figure 2 Estimated glomerular filtration rate obtained by the Chronic Kidney Disease Epidemiology Collaboration equation in patients with cirrhosis and the common populace. eGFR: Estimated glomerular filtration rate; CKD-EPI: The Chronic Kidney Disease Epidemiology Collaboration. ^a*P* < 0.05, eGFR between the CKD-EPI vs 6-variable MDRD equations.

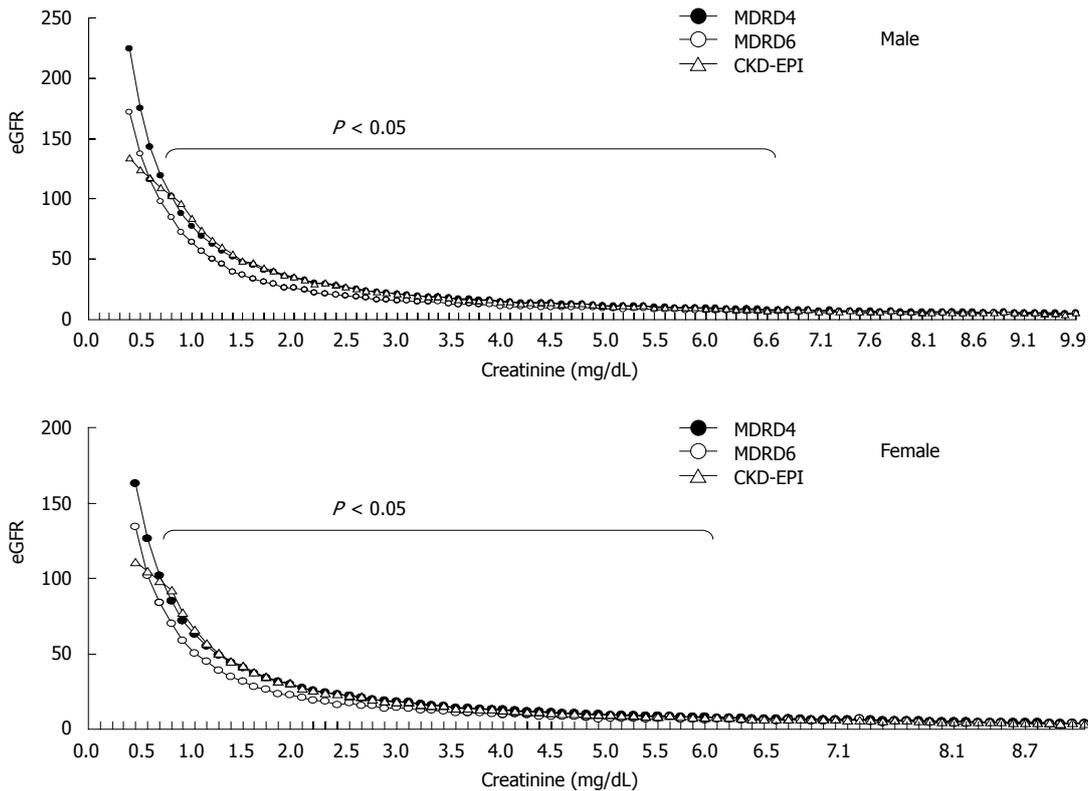


Figure 3 Estimated glomerular filtration rate obtained by the 4-variable Modification of Diet in Renal Disease, 6-variable Modification of Diet in Renal Disease, and the Chronic Kidney Disease Epidemiology Collaboration equations in patients with cirrhosis. eGFR: Estimated glomerular filtration rate; MDRD: Modification of Diet in Renal Disease; MDRD4: 4-variable MDRD; MDRD6: 6-variable MDRD; CKD-EPI: The Chronic Kidney Disease Epidemiology Collaboration.

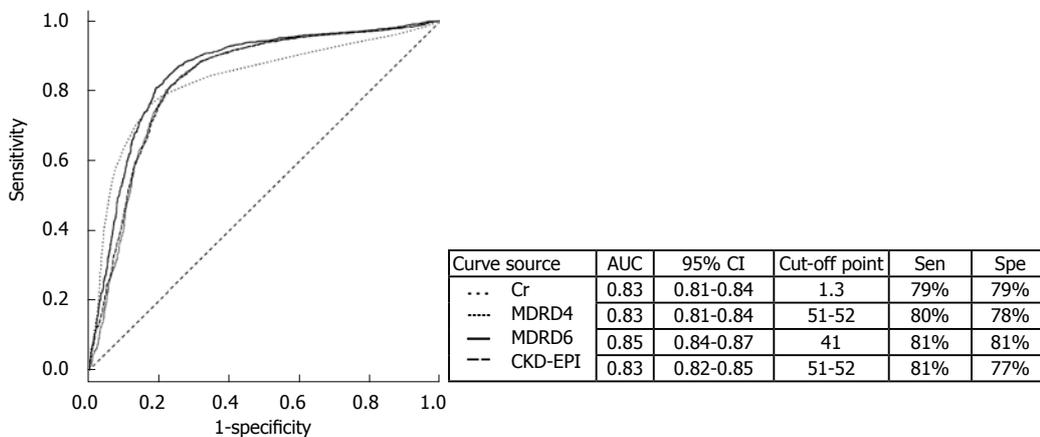


Figure 4 Receiver-operating characteristic curve of serum creatinine, 4-variable Modification of Diet in Renal Disease equation, 6-variable Modification of Diet in Renal Disease equation, and the Chronic Kidney Disease Epidemiology Collaboration equation for predicting in-hospital mortality. Cr: Creatinine; MDRD: Modification of Diet in Renal Disease; CKD-EPI: The Chronic Kidney Disease Epidemiology Collaboration; AUC: Area under curve; CI: Confidence interval; Sen: Sensitivity; Spe: Specificity.

true GFR than that obtained by the CKD-EPI equation. The use of eGFR obtained by the MDRD-6 equation as a surrogate of renal function offered better accuracy in predicting in-hospital mortality than that of eGFR obtained by the MDRD-4 equation, CKD-EPI equation, or Scr level.

The prognostic significance of renal function in patients with cirrhosis is reflected by the inclusion of Scr in the MELD score, which predicts short-term mortality

(3 mo) and is used for the prioritization of transplant recipients in the United States^[2,5,11]. However, it has recently been suggested that Scr weighs too heavily on the MELD score^[12]: the assumption that mortality is constant at the Scr level of < 1 mg/dL is likely to be false. On the other hand, Scr level and creatinine-based equations tend to overestimate GFR, and creatinine clearance from the time of urine collection also leads to overestimation of GFR. As a result, a modified MELD score

with a lower weighting for Scr than that in the current MELD score has been proposed and has been shown to be slightly superior^[12]. However, even after these adjustments, Scr is still a determinant of prognosis.

The creatinine-based Cockcroft and MDRD equations are widely used to estimate GFR in the general population, and MDRD is considered the gold standard in nephrology^[5,13]. However, both the Cockcroft and MDRD equations tend to overestimate GFR: a series has shown that only 66% of estimates were within 30% of the measured GFR^[14,15]. Unfortunately, most of the cited studies evaluated GFR in patients in liver transplant registries, who tend to have more advanced cirrhosis and decreased GFR, in part, due to the liver disease and malnourishment. The present study included a broader population that may have been better nourished or not as ill as that in previous studies.

The CKD-EPI equation, a newly developed equation for estimating GFR, has been proposed to be more accurate than the MDRD equation, especially when GFR is high. Moreover, it shows less bias, improved precision, and greater accuracy^[8]. Our study results agreed with this fact since the slope of the CKD-EPI equation was less steep when the Scr level was < 0.8 mg/dL and < 0.6 mg/dL in men and women, respectively. When the CKD-EPI equation was applied at the same Scr level in patients with cirrhosis and the common populace, a lower GFR was calculated in the former than in the latter. This result was probably related to the older age of the patients with cirrhosis, with the same Scr level. When the CKD-EPI, MDRD-4, and MDRD-6 equations were applied in the case of patients with cirrhosis, the performance of the CKD-EPI and MDRD-4 equations was similar to that in the common populace. However, a significantly lower GFR was estimated by the MDRD-6 equation than by the CKD-EPI equation when the Scr level was 0.7-6.8 mg/dL and 0.6-5.3 mg/dL in men and women, respectively. This result was probably related to the higher blood urea nitrogen (BUN) and lower albumin level-the additional 2 variables used in the MDRD-6 equation-in patients with cirrhosis. Although the CKD-EPI equation also yielded a lower eGFR than the MDRD-6 equation when the Scr level was < 0.5 mg/dL and < 0.4 mg/dL in men and women, respectively, the value was only found in 1.8% men and 1.4% women in all the study subjects. In view of the overall overestimation of GFR by the creatinine-based equations in patients with cirrhosis, eGFR obtained by the MDRD-6 equation seemed to be closer to the true GFR than that obtained by the CKD-EPI equation.

Creatinine shows a significant prognostic value in patients with cirrhosis^[2,5,11]. Theoretically, the creatinine-based equations show a similar prognostic value. However, the Cockcroft equation is less accurate than the MDRD equation since it incorporates body weight, which is markedly biased in patients with edema and/or ascites^[16]. The MDRD-4 (simplified MDRD) equation is usually and most often used to calculate GFR, since it is considered as accurate as the original MDRD-6 equation^[17]. However, its usefulness has not been proved in

healthy individuals, and its accuracy may be low in specific clinical settings^[15,18]. Therefore, the MDRD-6 equation is considered the best, possibly because it incorporates BUN and albumin level, the 2 variables which are abnormal in patients with cirrhosis^[18]. Our data also showed that eGFR obtained by the MDRD-6 equation was more accurate than that obtained by the MDRD-4 equation, CKD-EPI equation, or even Scr level in predicting in-hospital mortality. It is most likely that the improved predication due to BUN and albumin, in particular serum albumin is an excellent predictor of mortality. Thus, the use of eGFR obtained by the MDRD-6 equation as a surrogate of renal function offers a better prognostic value than that of eGFR obtained by the other equations. However, the accuracy of the MDRD equation has only been estimated on a large scale, in patients with chronic kidney disease. This suggests that a specific formula should be derived for patients with cirrhosis.

The present study has several limitations. First, there was no comparison between the CKD-EPI equation and the gold standard for GFR estimation such as that using ¹²⁵I-iothalamate or inulin. Thus, the true performance of the CKD-EPI equation in patients with cirrhosis could not be evaluated. Second, due to the variation in assay of BUN and albumin across labs which is not standardized, these results may not be useful in other populations. Third, the study was retrospective and cross-sectional in nature, and therefore, a prospective, cohort study is needed to test and verify our conclusions.

In conclusion, in view of the overall overestimation of GFR in patients with cirrhosis by creatinine-based equations, GFR calculated by the MDRD-6 equation may be closer to the true GFR than that calculated by the CKD-EPI equation and, hence, more suitable as a surrogate of renal function. However, a formula specifically derived for calculating GFR in patients with cirrhosis is warranted.

COMMENTS

Background

The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation has been proposed to be more accurate than the Cockcroft and Modification of Diet in Renal Disease (MDRD) equations. However, the CKD-EPI equation has not been tested in patients with cirrhosis.

Research frontiers

This is a retrospective study. Glomerular filtration rate (GFR) calculated by the 6-variable MDRD equation is closer to the true GFR than that calculated by the CKD-EPI equation. The 6-variable MDRD equation is a better way of calculating GFR in cirrhotic patients.

Innovations and breakthroughs

To our knowledge, this is the first study to evaluate the difference between the performance of the CKD-EPI and MDRD equations in cirrhotic patients. Although the CKD-EPI equation been proposed to be more accurate than the MDRD equation in the general population, the 6-variable MDRD equation remains the best way to calculate GFR in cirrhotic patients.

Applications

GFR should be calculated by the 6-variable MDRD equation in cirrhotic patients.

Peer review

It is a good study. However the authors have adequately mentioned the limitation.

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S- Editor Tian L L- Editor Webster JR E- Editor Zhang DN

Closure of a persistent sphincterotomy-related duodenal perforation by placement of a covered self-expandable metallic biliary stent

Antonios Vezakis, Georgios Fragulidis, Constantinos Nastos, Anneza Yiallourou, Andreas Polydorou, Dionisios Voros

Antonios Vezakis, Georgios Fragulidis, Constantinos Nastos, Anneza Yiallourou, Andreas Polydorou, Dionisios Voros, 2nd Department of Surgery, Endoscopy Unit, Aretaieion Hospital, University of Athens, Athens 11528, Greece

Author contributions: Vezakis A and Polydorou A performed the endoscopies and contributed to study conception and design; Vezakis A, Fragulidis G, Nastos C and Yiallourou A contributed to research, analysis and interpretation of data; Vezakis A, Fragulidis G and Polydorou A wrote the paper; all authors revised the paper critically for important intellectual content; Voros D gave the final approval of the version to be published.

Correspondence to: Antonios Vezakis, MD, 2nd Department of Surgery, Endoscopy Unit, Aretaieion Hospital, University of Athens, 76 Vas Sofias Ave, Athens 11528, Greece. avezakis@hotmail.com

Telephone: +30-210-7286157 Fax: +30-210-9605145

Received: January 7, 2011 Revised: March 23, 2011

Accepted: March 30, 2011

Published online: October 28, 2011

Abstract

Retroperitoneal duodenal perforation as a result of endoscopic biliary sphincterotomy is a rare complication, but it is associated with a relatively high mortality risk, if left untreated. Recently, several endoscopic techniques have been described to close a variety of perforations. In this case report, we describe the closure of a persistent sphincterotomy-related duodenal perforation by using a covered self-expandable metallic biliary (CEMB) stent. A 61-year-old Greek woman underwent an endoscopic retrograde cholangiopancreatography (ERCP) and sphincterotomy for suspected choledocholithiasis, and a retroperitoneal duodenal perforation (sphincterotomy-related) occurred. Despite initial conservative management, the patient underwent a laparotomy and drainage of the retroperitoneal space. After that, a high volume duodenal fistula developed. Six weeks after the initial ERCP, the patient underwent a repeat endoscopy and placement of a CEMB stent with an indwelling nasobiliary drain. The fistula healed

completely and the stent was removed two weeks later. We suggest the transient use of CEMB stents for the closure of sphincterotomy-related duodenal perforations. They can be placed either during the initial ERCP or even later if there is radiographic or clinical evidence that the leakage persists.

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Key words: Endoscopic sphincterotomy; Complications; Retroperitoneal perforation; Duodenal perforation; Metallic stent

Peer reviewer: Ji Kon Ryu, Professor, Department of Internal Medicine, Seoul National University College of Medicine, 28 Yeongeong-dong, Jongno-gu, Seoul 110-744, South Korea

Vezakis A, Fragulidis G, Nastos C, Yiallourou A, Polydorou A, Voros D. Closure of a persistent sphincterotomy-related duodenal perforation by placement of a covered self-expandable metallic biliary stent. *World J Gastroenterol* 2011; 17(40): 4539-4541 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4539.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4539>

INTRODUCTION

Retroperitoneal duodenal perforation as a result of endoscopic biliary sphincterotomy (ES) is a well-recognized complication. Although it is reported to have an incidence of 0.3% to 1.3%, it is associated with a relatively high mortality rate of 7% to 14%^[1-4]. The overriding question is whether or not immediate surgical exploration is required or if a trial of non-operative management is safe. Recently, several endoscopic techniques have been described to close a variety of perforations^[5,6]. This is, to the best of our knowledge, the first report of a delayed closure of a sphincterotomy-related duodenal perforation by placement of a covered self-expandable metallic bili-

ary (CEMB) stent.

CASE REPORT

A 61-year-old lady with a history of previous laparoscopic cholecystectomy underwent endoscopic retrograde cholangiopancreatography (ERCP) for biliary colic, abnormal liver function tests and a grossly dilated common bile duct (20 mm). The suspected diagnosis was either choledocholithiasis or sphincter of Oddi dysfunction type I. ES was performed over a guidewire using an ERBE VIO 200 S diathermy (ERBE Elektromedizin, Germany) at Endocut mode. Due to an unusual direction of the papilla, the ES was carried out laterally towards the 9 o'clock position. After insertion and removal of the balloon catheter, a visible laceration was recognized just posteriorly to the ES. The presence of free air in the retroperitoneum was immediately apparent (Figure 1). The patient was treated initially with broad spectrum antibiotics and nasogastric drainage. The following day an abdominal computed tomography (CT) scan was performed (Figure 2). A large amount of retroperitoneal air was identified, but no leak of contrast was found. Over the following days the patient deteriorated, became pyrexial and unstable, and a laparotomy was carried out on the 15th post-ERCP day. The retroperitoneal space was explored with debridement of necrotic tissue and placement of drains.

The patient's condition was improved postoperatively, but a high volume duodenal fistula (500-1500 mL/24 h) developed, which was refractory to conservative treatment.

Four weeks later an endoscopy was performed. The procedure was technically difficult because of an edematous duodenum, but the laceration was found at the sphincterotomy site (Figures 3 and 4). A CEMB stent (Wallstent, Boston Scientific) and subsequently a nasobiliary drainage catheter were placed (Figures 5 and 6). The proximal 5 mm uncovered portion of the Wallstent had been cut prior to insertion. The fistula had healed completely a week later and the stent was removed at 2 wk. The nasobiliary drain was left in place for five days, although the output was minimal. A cholangiography and contrast study before removal of the nasobiliary drain showed no existence of leak.

The patient was discharged home 2 mo post-ERCP and remains well 8 mo later.

DISCUSSION

Perforation during ES is usually retroperitoneal in location. It results from an extension of the incision beyond the intramural portion of the bile duct. It is widely believed that perforation is more likely to occur if the incision strays beyond the usual recommended sector (11 to 1 o'clock)^[7]. Due to the low incidence of perforation, the risk factors are not well defined. The risk appears to be increased in patients with Billroth II anastomosis and when needle-knife sphincterotomy is performed^[3]. The incision in these situations is not well controlled and therefore the chances of perforation are higher.

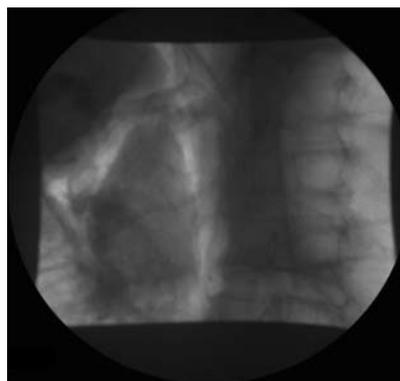


Figure 1 Free gas in the retroperitoneal space.



Figure 2 Computed tomography scan showing the presence of air in the retroperitoneal space and subcutaneous emphysema.

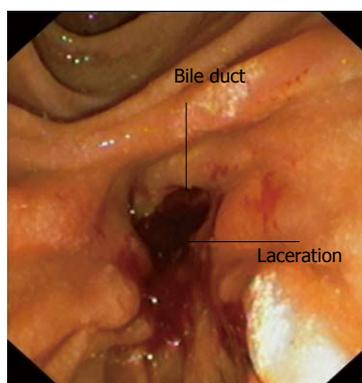


Figure 3 The laceration is evident just below the lower end of the bile duct.

No consensus exists on management guidelines, because ES-related retroperitoneal perforations are rare and the clinical consequences vary enormously. In general, non-operative management of these perforations is possible despite the presence of extensive retroperitoneal air, provided the patient remains well clinically. If the patient develops abdominal pain, fever and appears toxic clinically, surgical exploration should be considered^[8]. Delayed diagnosis can lead to severe morbidity^[9]. Patients with biliary stenting appear to have a lower rate of operative intervention. This is likely related to the higher rate of guidewire-induced injuries in these patients, who are much less likely to require operative intervention^[8].

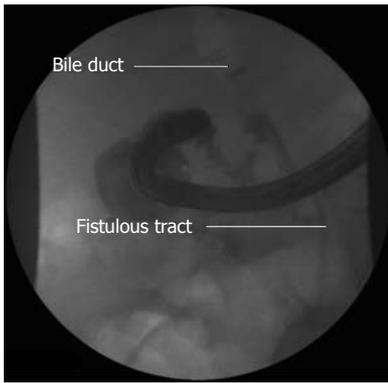


Figure 4 Injection of contrast from the endoscope enables visualization of the fistulous tract. The bile duct is also delineated with the presence of gas.



Figure 5 The covered self-expandable metallic biliary stent covers the laceration.



Figure 6 Covered self-expandable metallic biliary stent and nasobiliary catheter in place.

It is possible that biliary stenting has a protective effect by diverting bile into the duodenum instead of into the retroperitoneum. Endoscopic closure of ERCP-related duodenal perforations by using endoclippping devices^[10], approximation sutures^[5] or duodenal stents^[6] have also been described.

The above described endoscopic techniques are applied after the recognition of the perforation during the initial ERCP. In our case, a repeat ERCP was performed 6 wk

after the initial procedure, in order to repair the perforation and drain the bile duct. A repeat endoscopy after a duodenal perforation is technically demanding, carries the risk of extending the laceration, and excessive skill is required.

CEMB stents have the advantage of covering the laceration and permit free flow of bile into the duodenum instead of into the retroperitoneal space. Additionally, they protect against the leakage of pancreatic and gastric fluid. The additional use of a nasobiliary drainage catheter may reduce bile flow to the duodenum and allows checking of the healing process by performing contrast studies. Fully covered metallic self-expandable biliary stents should be preferred because they can easily be removed after a short period. In our case, a fully covered stent was not available and we modified a partially covered as mentioned previously.

In conclusion, we suggest the transient use of CEMB stents for the closure of sphincterotomy-related duodenal perforations. They can be placed either during the initial ERCP or even later, if there is radiographic or clinical evidence that the leakage persists.

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S- Editor Tian L L- Editor Logan S E- Editor Xiong L

Neoadjuvant plus adjuvant chemotherapy benefits overall survival of locally advanced gastric cancer

Xin-Zu Chen, Kun Yang, Jie Liu, Xiao-Long Chen, Jian-Kun Hu

Xin-Zu Chen, Kun Yang, Jie Liu, Jian-Kun Hu, Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Xiao-Long Chen, Faculty of Medicine, West China Medical School, Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: Chen XZ designed this research and wrote the paper; Yang K, Liu J and Chen XL performed research; Hu JK is responsible for the academic inspection.

Correspondence to: Jian-Kun Hu, MD, PhD, Professor, Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, No. 37 Guoxue Xiang Street, Chengdu 610041, Sichuan Province, China. hujkwch@126.com

Telephone: +86-28-85422878 Fax: +86-28-85164035

Received: February 24, 2011 Revised: April 3, 2011

Accepted: April 10, 2011

Published online: October 28, 2011

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Key words: Gastric cancer; Adjuvant chemotherapy; Neoadjuvant chemotherapy; Surgery; Survival

Peer reviewer: Robert C Moesinger, MD/FACS, Northern Utah Surgeons, Adjunct Assistant Professor, Department of Surgery, University of Utah, 4403 Harrison Blvd No. 1635, Ogden, UT 84403, United States

Chen XZ, Yang K, Liu J, Chen XL, Hu JK. Neoadjuvant plus adjuvant chemotherapy benefits overall survival of locally advanced gastric cancer. *World J Gastroenterol* 2011; 17(40): 4542-4544 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i40/4542.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i40.4542>

Abstract

Neoadjuvant chemotherapy (NAC) has drawn more attention to the treatment of locally advanced gastric cancer (AGC) in the current multidisciplinary treatment model. EORTC trial 40954 has recently reported that NAC plus surgery without postoperative adjuvant chemotherapy could not benefit the locally AGC patients in their overall survival. We performed a meta-analysis of 10 studies including 1518 gastric cancer patients. Stratified subgroups were NAC plus surgery and NAC plus both surgery and adjuvant chemotherapy (AC), while control was surgery alone. The results showed that NAC plus surgery did not benefit the patients with locally AGC in their overall survival [odds ratio (OR) = 1.20, 95% CI 0.80-1.80, $P = 0.37$] and the number needed to treat (NNT) was 74. However, the NAC plus both surgery and AC had a slight overall survival benefit (OR = 1.33, 95% CI 1.03-1.71, $P = 0.03$) and NNT was 14, which is superior to the NAC plus surgery. Therefore, we recommend that combined NAC and AC should be used to improve the overall survival of the locally AGC patients.

TO THE EDITOR

We have read with great interest the excellent article by Li *et al*^[1]. Gastric cancer is still one of the most common malignancies worldwide and about 80% patients with gastric cancer have advanced diseases^[2]. Surgery is known as the only potentially curative treatment for this disease at resectable stages, while chemotherapy could play an important role in improving the prognosis of the patients^[2,3]. Neoadjuvant chemotherapy (NAC) has drawn more attention to the treatment of locally advanced gastric cancer (AGC) in the current multidisciplinary treatment model. The EORTC trial 40954 observed the effect of the NAC without adjuvant chemotherapy (AC) following surgery for locally AGC (UICC stage III and IV-cM0). The protocol did not benefit the survival of the patients, but the R0 resection rate was significantly increased^[4]. However, the fluorouracil-containing AC for AGC has shown significant survival benefit compared with surgery alone^[5-7]. Therefore, we consider that combined NAC and AC may benefit locally AGC patients in overall survival.

The meta-analysis performed by Li *et al*^[1], including 14

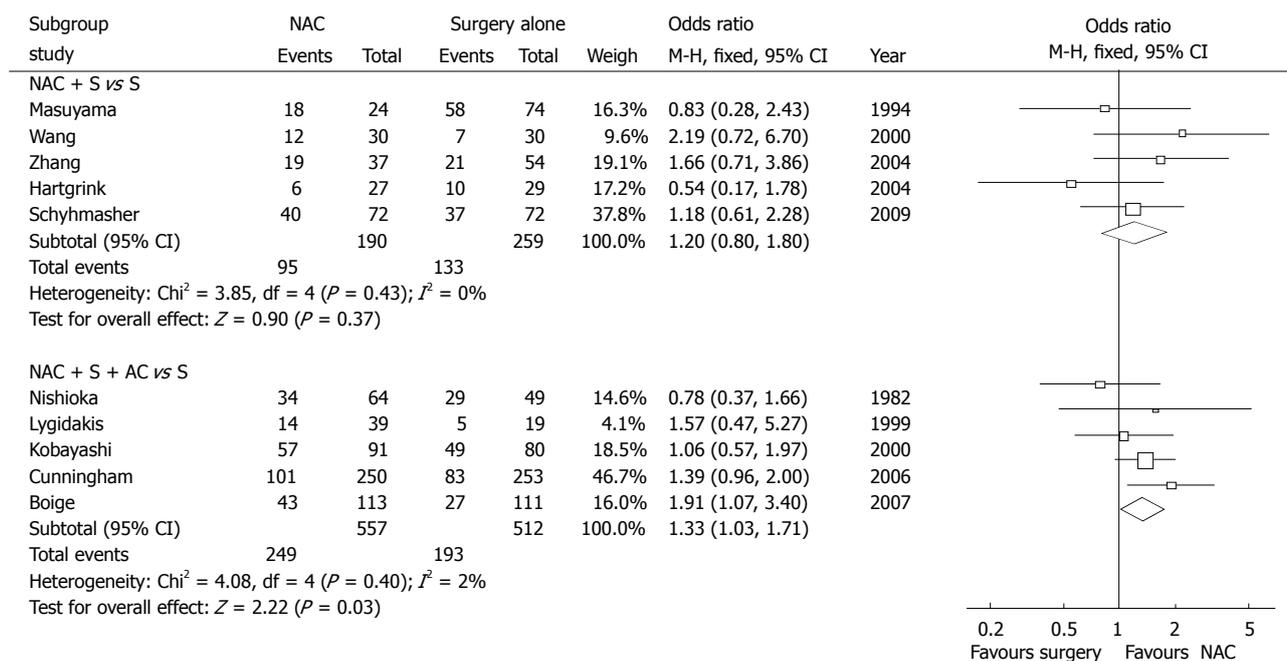


Figure 1 Subgroup comparison of neoadjuvant chemotherapy or neoadjuvant chemotherapy plus adjuvant chemotherapy vs surgery alone for locally advanced gastric cancer (based on the published meta-analysis and excluding the trials contaminated with adjuvant chemotherapy in control arm)^[1]. M-H: Mantel-Haenszel test. NAC: Neoadjuvant chemotherapy; AC: Adjuvant chemotherapy.

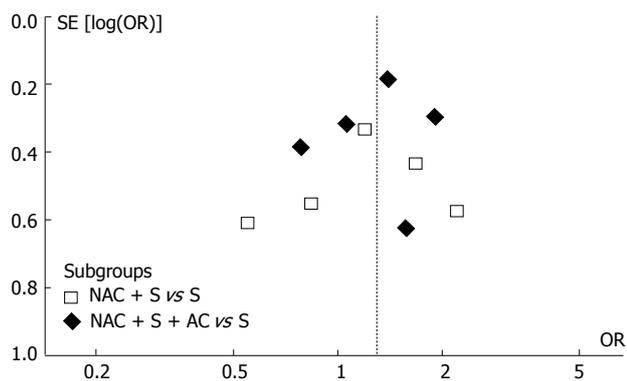


Figure 2 Funnel plot analysis of publication bias. OR: Odds ratio; NAC: Neoadjuvant chemotherapy; AC: Adjuvant chemotherapy.

trials, compared the patients treated with NAC and those without NAC, showed an R0 resection rate of 75.2% *vs* 66.9% [odds ratio (OR) = 1.51, $P = 0.0006$, fixed model]. Therefore, this made us well understand the substantial effectiveness of NAC for locally AGC.

Based on the original meta-analysis, we performed another subgroup analysis by classifying the intervention arms into NAC plus surgery or NAC plus both surgery and AC, while the trials contaminated with AC in the control arm were excluded^[1]. In each subgroup, 5 prospective trials were included for repooled analysis (1518 patients). The meta-analysis showed that the NAC plus surgery did not bring overall survival benefit to the patients with locally AGC (OR = 1.20, 95% CI 0.80-1.80, $P = 0.37$) and the number needed to treat (NNT) was 74 (Figure 1). However, NAC plus both surgery and AC

had a slight overall survival benefit (OR = 1.33, 95% CI 1.03-1.71, $P = 0.03$) and NNT was 14, which was superior to the NAC plus surgery (Figure 1). It implies that one out of 14 locally advanced patients treated by NAC plus both surgery and AC was benefited in survival, while the remainings might be at risk of recurrence or even death.

In addition, funnel plot observation did not indicate obvious publication bias in the two subgroups (Figure 2). The sensitivity analysis showed similar results by excluding the trials with Jadad score less than 3^[1]. The original meta-analysis noted that the effect of NAC is more pronounced in Western countries and in doublet or triplet chemotherapy regimens^[1]. In our subgroup analysis, the subgroups involved both Western and Asian studies as well as both single-agent and multi-agent regimens. These factors might not confound the present analysis.

Interestingly, EORTC trial 40 954 did not show any survival benefit by NAC for the locally advanced diseases (UICC stage III and IV-cM0)^[4], but the meta-analysis by Li *et al*^[1] found that more advanced diseases (pT3-4) were benefited by NAC (OR = 1.91, $P < 0.05$) than pT1-2 diseases ($P > 0.05$). Based on the original data from Li *et al*^[1], it is impossible to perform subgroup analysis of stage III and IV-cM0 diseases, but it would be meaningful to figure out which sub-population might actually benefit from NAC plus both surgery and AC.

In summary, we think NAC is an effective therapy to increase the R0 resection rate for locally AGC, however, combined NAC and AC would improve the overall survival of the patients. Whether the increased R0 resection rate is associated with the improved overall survival and which sub-population might benefit from the combined

treatment requires further studies.

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S- Editor Tian L L- Editor Ma JY E- Editor Li JY

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.

Meenakshisundaram Ananthanarayanan, Associated Professor, Department of Pediatrics, Annenberg Bldg, Rm14-24A, Box 1664, The Mount Sinai Medical Center, One Gustave L Levy Place, New York, NY 10029, United States

Yeun-Jun Chung, MD, PhD, Professor, Director, Department of Microbiology, Integrated Research Center for Genome Polymorphism, The Catholic University Medical College, 505 Banpo-dong, Socho-gu, Seoul 137-701, Korea

Adrian G Cummins, Dr., Department of Gastroenterology and Hepatology, (DX 465384), 28 Woodville Road, Woodville South, 5011 South Australia, Australia

Gianpiero Gravante, MD, BsC, MBBS, Department of Hepatobiliary and Pancreatic Surgery, Leicester General Hospital, Flat 38, Room 8, Hospital Close, Leicester, LE5 4WU, United Kingdom

Alexander G Heriot, MA, MD, FRCS, FRACS, Associate Professor, Department of Surgical Oncology, Peter MacCallum Cancer Centre, 1 St Andrews Place, Melbourne, VIC 3002, Australia

Kevin Cheng-Wen Hsiao, MD, Assistant Professor, Colon and Rectal Surgery, Tri-Service General Hospital, No. 325, Sec 2, Cheng-Kung Rd, Nei-Hu district, 114 Taipei, Taiwan

Takashi Kojima, DVM, PhD, Department of Pathology, Sapporo Medical University School of Medicine, S1, W17, Chuo-ku, Sapporo 060-8556, Japan

Richard A Kozarek, MD, Department of Gastroenterology, Virginia Mason Medical Center, 1100 Ninth Avenue, PO Box 900, Seattle 98111-0900, United States

Michael Leitman, MD, FACS, Chief of General Surgery, Beth Israel Medical Center, 10 Union Square East, Suite 2M, New York, NY 10003, United States

Flavio Maina, PhD, Developmental Biology Institute of Marseille-Luminy, CNRS UMR 6216, Campus de Luminy-case 907, Marseille cedex 09, 13288, France

Satoshi Mamori, MD, PhD, Department of Gastroenterology and Hepatology, Shinko Hospital, 1-4-47 Wakihama-cho, Chuo-ku, Kobe, Hyogo 651-0072, Japan

Giulio Marchesini, Professor, Department of Internal Medicine and Gastroenterology, "Alma Mater Studiorum" University of Bologna, Policlinico S Orsola, Via Massarenti 9, Bologna 40138, Italy

Espen Melum, MD, Medical Department, Rikshospitalet University hospital, Sognsvannsven 20, Oslo, 0027, Norway

Patrick O'Dwyer, MB, BCh, BAO, FRCS (I), MCh, FRCS (Glasg), University Department of Surgery, Western Infirmary, Glasgow, G11 6NT, United Kingdom

Alberto Piperno, Professor, Department of Clinical Medicine and Prevention, Clinical Medicine, San Gerardo Hospital, Via Pergolesi 33, 20052, Monza, Italy

John Plukker, MD, PhD, Professor of Surgical Oncology, Department of Surgery, University Medical Center groningen, Hanzeplein 1, Groningen, 9700 RB, The Netherlands

Adrian Saftoiu, MD, PhD, Professor, Research Center of Gastroenterology and Hepatology Craiova, University of Medicine and Pharmacy Craiova, 2 Petru Rares str, Craiova 200349, Romania

Henning Schulze-Bergkamen, MD, Henning Schulze-Bergkamen, First Medical Department, University of Mainz, Langenbeckstr, 1, 55101 Mainz, Germany

Bronislaw L Slomiany, PhD, Professor, Research Center, C-875, UMDNJ-NJ Dental School, 110 Bergen Street, PO Box 1709, Newark, NJ 07103-2400, United States

Hitoshi Tsuda, MD, PhD, Diagnostic Pathology Section, Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

Vamsi R Velchuru, MRCS, FRCSEd, FRCS (Gen Surg), James Paget University Hospital, Great Yarmouth, 6 Pickwick Drive, Off Market Lane, Blundeston, NR32 5BX, United Kingdom

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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

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APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver Bangkok, Thailand

February 22, 2011-March 04, 2011
 Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011

Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011

2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011

International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011

Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011

Childhood & Adolescent Obesity:

A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011

42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011

Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011

British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011

41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011

Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011

UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011

MedicRes IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011

26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011

IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

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International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

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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

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The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

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Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

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May 21-24, 2011

22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venice, Italy

May 25-28, 2011

4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011

The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011

Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011

International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011

ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011

XI Congreso Interamericano de Pediatría "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011

Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011

New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011

ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011

Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011

Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011

19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011

ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011

Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011

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

GENERAL INFORMATION

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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 Identifier, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 Gastroenterology and Hepatology).

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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 Pixu-diarrhoea. *Shijie Huaren Xiaobua 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 PMID: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 Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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 biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek 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 \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as ν (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
<http://www.wjgnet.com>
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Fax: +86-10-8538-1893

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