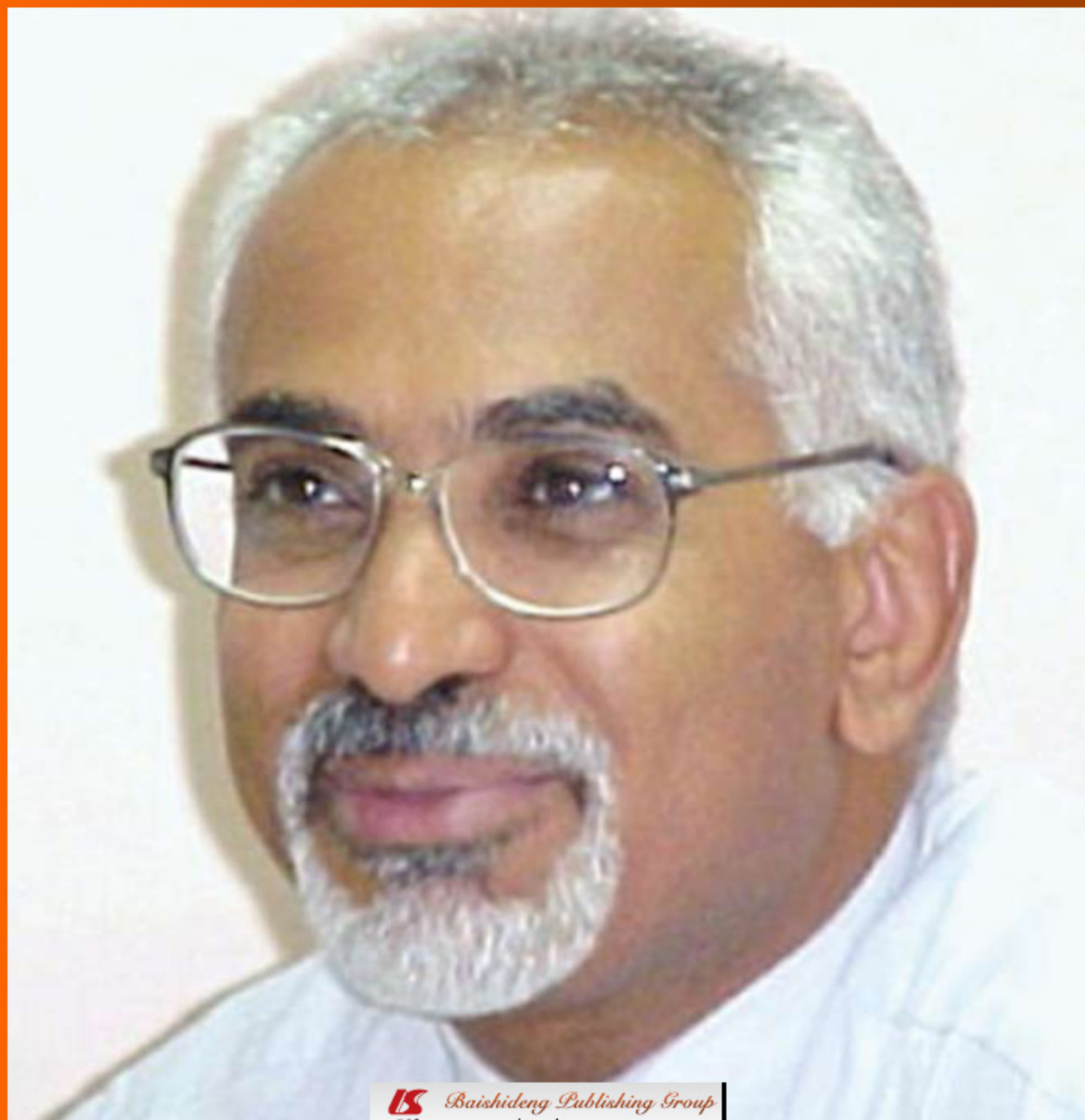


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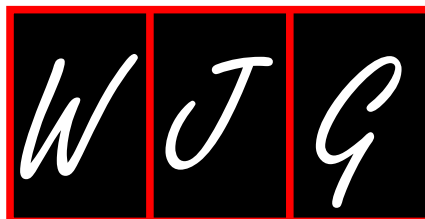
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Time to detoxify medical literature from guideline overdose

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overtreatment, inefficiency, and patient inconvenience. There is an urgent need to restrict articles with Guidelines and develop some strategy like have an intermediate stage of pre-guidelines and after 5-10 years of trials, a systematic launch of the Guidelines. There can be better ways than this for putting together guidelines as has been suggested by multiple authors and researchers.

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Abstract

The current financial turmoil in the United States has been attributed to multiple reasons including health-care expenditure. Health care spending has increased from 5.7 percent of the gross domestic product (GDP) in 1965 to 16 percent of the GDP in 2004. Healthcare is driven with a goal to provide best possible care available at that period of time. Guidelines are generally assumed to have the high level of certainty and security as conclusions generated by the conventional scientific method leading many clinicians to use guidelines as the final arbiters of care. To provide the standard of care, physicians follow guidelines, proposed by either groups of physicians or various medical societies or government organizations like National Comprehensive Cancer Network. This has lead to multiple tests for the patient and has not survived the test of time. This independence leads to lacunae in the standardization of guidelines, hence flooding of literature with multiple guidelines and confusion to patients and physicians and eventually

INTRODUCTION

A well researched guideline will reduce financial burden in United States healthcare. Increasing and excessive health care budget is affecting major industries in the United States. We know it is not the sole cause, but is one of the major reasons for the crisis. A jaw dropping fact is that health care spending has increased from 5.7 percent of the gross domestic product (GDP) in 1965 to 16 percent of the GDP in 2004^[1]. It has been estimated that in 2015, we will be spending close to 20 percent of the GDP, or an estimated \$4 trillion, up from \$1.9 trillion in 2004, on health care^[2]. There is an obvious role of guidelines in reducing healthcare expenditure.

The initial step in systemic review was in 1970 with a perinatal trial^[3]. Although guidelines are intended to assist physicians, this functionality seems frequently lost among the sea of guidelines that are available. Indeed, at any

given point there are frequently multiple guidelines available with regard to a particular disease process, forcing physicians who wish to avoid accusations of undertreatment to order more than one diagnostic study or treatment (polypharmacy) referencing various guidelines. A well researched and tested guideline reduces healthcare cost and mismanaged malpractice lawsuits, and allows for better physician and patient understanding and better parameters for quality control in medical care. Most guidelines indicate that they are not a rule and should not be construed as establishing a legal standard of care.

MULTIPLE TESTING DUE TO MULTIPLE GUIDELINES

Guidelines are generally assumed to have the high level of certainty and security as conclusions generated by the conventional scientific method leading many clinicians to use guidelines as the final arbiters of care^[4] and sense of a shield to malpractice litigation. This has led to multiple tests and it has not survived the test of the time. Fuchs suggested greater expenditures do not necessarily result in better health outcomes^[5]. Guidelines may cause higher utilization of medical tests and procedures and, in some cases, have negative consequences for patients, as in the case of false-positive screening test results^[6].

GUIDELINES AND ITS USE IN MEDICAL MALPRACTICE

Another very compelling reason for its standardization is the use of guidelines in the malpractice lawsuit. Guidelines are frequently used as evidence by expert witness against a physician. It is very hard for judge and jury to understand the quality of evidence, but it is easy for them to concur with evidence coming from an expert with a guidelines document.

GUIDELINES AS A TOOL FOR QUALITY MEASURE FOR HOSPITAL AND HEALTHCARE PROVIDER

Guidelines are used as a tool for quality measures. It seems immature to use consensus statements as performance measures or other similar tools to critique the quality of a physician's care. It could be better addressed if it is derived from high-quality guidelines based on the highest level of evidence and derived in a more thoughtful manner. Protocols for quality must include guidelines, but it must have safety and patient care at its core with efficiency also as an outcome parameter. Any project without safety and efficiency is going to fail; hence we must have safer guidelines.

ROLE OF GUIDELINES IN PATIENT AND PHYSICIAN EDUCATION

Guidelines are the basis of evidence based medicine

(EBM). Guidelines are widely perceived as evidence based, not authority based, and therefore viewed as unbiased and valid. A systematic review uses preplanned scientific protocols and methods to summarize similar but separate studies to perform a scientific investigation for a specific question^[7]. Druss *et al*^[8] are of the opinion to rely more on guidelines due to increase in the quantity of relevant information. Evidence is a very strong word, but it's important to understand how the facts are studied, and realize the biases of studies and researchers. A major responsibility of an educator is to be meticulous with the data collection and near perfect interpretation, but it can, at times, be challenging due to the size of data and variables involved. This is primarily due to an unprecedented explosion of medical knowledge in the past 50 years secondary to breakthroughs in numerous areas of medical science and information technology.

The Institute of Medicine stresses on use of guidelines for medical care to ensure high quality patient care^[9]. It is needed as even the most sophisticated health care consumer has difficulty in deciding appropriate care for his or her condition^[10,11]. Guidelines will assure the patient of adequate and appropriate treatment. Institute of Medicine stresses a need for a strong pertinent guideline for any particular disease. The providers and patients are faced not only with incongruous but also conflicting research findings.

A major reason to stress their implementation is to reduce undesirable practice variation and reduce the use of services that are of minimal or questionable value. National Guidelines Clearinghouse (NGC, established in 1998) is a national body in the United States whose responsibility is to compile a bank of guidelines either by submission or active retrieval thru bodies like National Comprehensive Cancer Network (NCCN) and American Heart Association (AHA). It has established its own submission standards and substandard guidelines are not incorporated. Even then there are at least 500 new guidelines submitted or reviewed every year (Figure 1). Similarly, National Institute of Clinical Excellence (NICE) is established in the United Kingdom, with an objective to standardize the health care by issuing its own guidelines, after studying relevant literature and guidelines available at a given time in point. NICE uses term guidance in place of guidelines.

OVERUSE OF TERM GUIDELINES AND OVERLAPPING GUIDELINES

The term guideline has been drifting far from the original intent of the Institute of Medicine. Unfortunately a major share of current articles called "guidelines" is actually expert consensus reports^[12]. There are interestingly too many guidelines, often on the same topic. For example, clinicians do not need 10 different adult pharyngitis guidelines^[13]. Our information of total guidelines, compiled by NGC, shows there are close to 25% guidelines renewed every year and a disease as localized as breast has an average of more than 30 new guidelines

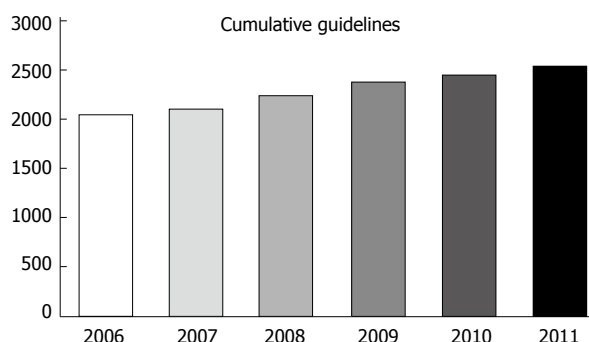


Figure 1 Cumulative number of all guideline summaries published on National Guidelines Clearing House. (Includes prior years) (Information taken from National Guidelines Clearing Annual Reports to Agency for Healthcare Research and Quality) (Source: NGC House, AHQR.com).

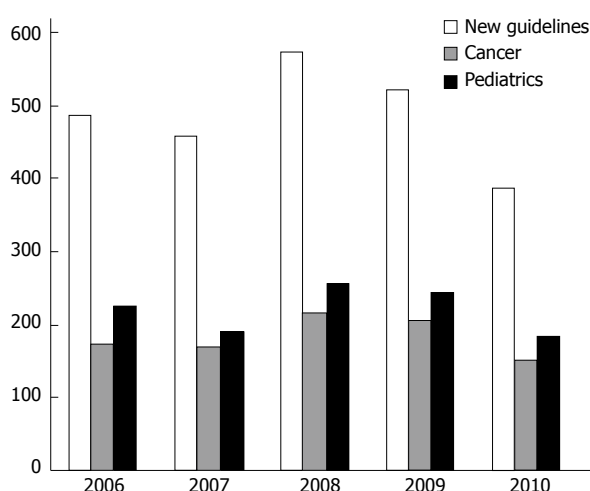


Figure 2 Total number of all new/revised, cancer and pediatrics guideline summaries organized by year of publication/revision. (Includes only that year) (Source: National Guidelines Clearing House, AHQR.com).

every year for last 5 years (Figures 1-3) (source: NGC). In 1999, after 1 year of NGC incorporation, there were total of 290 guidelines, with 82 cancers, 86 pediatrics and 21 and 7 breast and colon guidelines. The total number of guidelines, when this article is published has gone up to 2573 from 2060 in 2006 (source: NGC). This is according to the available information, the best compiled database of guidelines, by any organization internationally. According to their sources, it is possible a large number of guidelines are not even part of this pool.

CONFLICT OF INTEREST AND FACTORS INFLUENCING GUIDELINES

Personal bias has been studied by multiple researchers. In Choudhary's article, eighty-seven percent of authors had some form of interaction with the pharmaceutical industry with only seven percent admitting its influence on their inferences. Fifty-eight percent received financial support to perform research and fifty-five percent of respondents indicated that the guideline process had

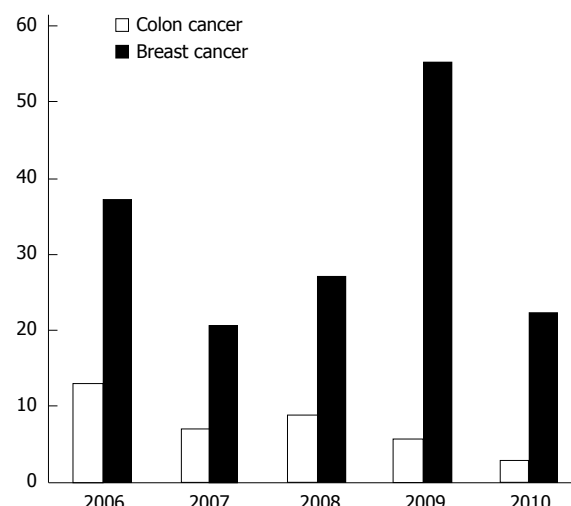


Figure 3 Total number of colon and breast cancer guideline summaries organized by year of publication/revision. (Includes only that year) (Source: National Guidelines Clearing House, AHQR.com).

no formal process for declaring these relationships^[14]. Unfortunately, all guideline committees begin with implicit biases and values affecting their worth^[15]. A very relevant question arises: affect of sponsorship by organizations, personal biases, and absence of restriction by journals, organizations and publishing bodies in using term guideline.

Another reason for the shortcomings in the guidelines is the need for resources to conduct a rigorous systematic review. The resource crunch might push guideline developers to revert to short-cuts or processes centered on expert opinion^[16]. On the contrary, a substantial investment in evidence gathering does not guarantee a sound guideline/evidence for a disease^[17]. Guideline authors often rely on research that are non rigorous, and yield conflicting results^[18]. This leads to pressure to rely more heavily on professional opinion which is problematic.

MULTIPLE GUIDELINES WITH CONFLICTING RECOMMENDATIONS AND QUALITY ISSUES

The exponential increase in guidelines is due to increasing number of research groups, societies, and associations performing analysis of a section of innumerable publications. It constitutes 40%-50% of indexed articles average annual volume. A large section of this growth is in articles on randomized trials and other types of clinical research used to guide evidence-based practice. Unfortunately, we are not seeing consistency in the guidelines issued on major areas of medicine. Contrast the guidelines with the decisions of any court of appeal in which some judgments are issued unanimously but most are not. Although unanimity is the rule in individual guidelines, it can be strikingly absent when different guidelines are compared.

Table 1 A simplistic presentation of conflicting statements in mammography and clinical breast examination by multiple authorities in last few years

Yr	Age for first mammogram	Recommending authority	Frequency	Clinical breast exam
2001	50-69 yr	Canadian Task Force	1-2 yr	1-3 yr
2003	40-49 yr (1-2 yr) > 50 yr (annually)	ACOG	As in column 2	Annually after 20 yr
2007	40-49 yr (individualize) 50-74 yr (1-2 yr)	ACP	As in column 2	No statement
2009	40-49 yr (individualize) 50-74 yr (2 yr)	USPSTF	2 yr	Insufficient evidence
2010	40 yr and above	American Cancer Society	Annually	20-40 yr every 3 yr and then annually
2011		NCCN		
2010	40 yr	NCI	1-2 yr	No statement
2011	47-73 yr	NHS, United Kingdom	3 yr	No statement

ACOG: American College of Obstetrics and Gynecology; ACP: American College of Physicians; USPSTF: United States Preventive Services Task Force; NCCN: National Comprehensive Cancer Network; NCI: National Cancer Institute; NHS: National Health Services.

EXAMPLES OF MEDICAL PRACTICE WITHOUT VALIDATION

It seems like we have come a full circle. The role of evidence-based practice, medical textbooks and editorials is to filter the literature filled with thousands of statements (based solely on the belief of the author or at best a consensus of experts)^[19].

In an article on guidelines on management of pharyngitis, review of 4 North American and 6 European guidelines were included, and recommendations were found to go tangential to each other with regard to the use of a rapid antigen test and throat culture and the indication for antibiotics^[13]. The reason for conflict is use of different data sets by different organizations. It has been suggested that guidelines are not necessary for every disease but are needed for diseases having significant practice variability and for which a valid evidence base can guide recommendations. They also suggested prioritizing individual recommendations^[12].

A simple example is commonly used guidelines issued by cardiology organization, American College of Cardiology (ACC)/AHA guidelines. It is largely developed from lower levels of evidence or expert opinion^[20]. It is flawed as it does not appear to follow the Institute of Medicine recommendations to separate the systematic review process from guideline formulation. According to the author, less than twenty percent of recommendations advocating a particular procedure or treatment in ACC/AHA practice guidelines are based on level A evidence. In another study of quality of recommendation, less than 45% recommendations in guidelines are based on high quality evidence^[21]. These studies bring a very valid question to the rational of application of evidence based medicine, when evidence itself is weak.

Another example of rapid turnover and too early update is due to the pressure of incorporating the development in technology to clinical arena without due research is inclusion of immunohistochemistry results in a sentinel lymph node specimen in cancer. This was used in tumor node metastasis staging and was withdrawn after

a short time. Situations like this make us wonder if we should make a pre-guideline of the results for a period of time and evaluate the data for its implication before incorporating the results for mass application. A similar example is withdrawal of hormone replacement therapy (HRT) in menopausal females. HRT was initially thought (1995) to have more benefits and no absolute contraindications or major risks, but after its aggressive introduction and urgency for compliance, increased incidence of hormone related side effects like lethal breast cancer, ovarian cancer and stroke persuaded clinical bodies to withdraw the major recommendations of guidelines for HRT therapy. Another example is a simplistic tabulation of guidelines on mammography. There are five guidelines from the leading medical organizations in last two years with highly conflicting recommendations, except for almost identical recommendations from NCCN and American Cancer Society. Similarly, the guidelines for colonoscopy have huge conflicts, except the frequency of every 10 years. This has not been challenged for last 40 years, even though this is based on a very flimsy theory. Clinicians and researchers are still struggling to identify appropriate intervals for colonoscopy in low risk patients, although there is some progress in identifying patients needing early colonoscopy (Table 1).

SOME OPTIONS TO MAKE GUIDELINES MORE ROBUST

Differentiating effective and ineffective health resource utilization is an important health policy objective. There are multiple articles suggesting their opinion of the subject. This awkward situation can be partially resolved by training physicians in biostatistics, a necessary tool to interpret the findings of published clinical research^[22]. At this point in time, the processes underlying guideline development is often vulnerable to bias and conflict of interest leading to sometimes poor quality. Grimshaw and group suggested a standardized and more diverse panel of physicians with competing interest with minimal bias^[23]. Another group suggested two separate grading

systems: (1) Quality of the evidence; and (2) Recommendations^[24]. We don't believe this will fix the state of affairs, but can definitely be one of the constructive steps. Another reason for streamlining the process is health quality measure reliance on guidelines. The NGC has thousands of guidelines produced by close to 300 organizations^[25] underlining the need for urgent streamlining.

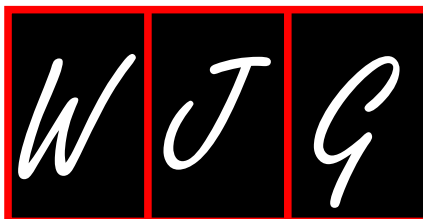
A more radical thought is to reject them unless there is evidence of appropriate changes in the guideline process. Physicians will make better clinical decisions based on valid primary data^[12]. Another option is to update guideline after every 7-10 years or more if not much new data present and in areas of active research supplementing literature with pre-guidelines every 3-4 years. Also there can be a journal on guidelines where researchers can submit their opinion at the time of issues due date. And all the facts are incorporated and a final suggestion is given by the panel selected from the contributors and other established experts.

In summary, EBM is complex and incomplete. Guidelines must be directed only to the interests of patients and not to those who profit from them^[4].

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Recent advances in small bowel diseases: Part I

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Abstract

As is the case in all parts of gastroenterology and hepatology, there have been many advances in our knowledge and understanding of small intestinal diseases. Over 1000 publications were reviewed for 2008 and 2009, and the important advances in basic science as well as clinical applications were considered. In Part I of this Editorial Review, seven topics are considered: intestinal development; proliferation and repair; intestinal permeability; microbionics, infectious diarrhea and probiotics; diarrhea; salt and water absorption; necrotizing enterocolitis; and immunology/allergy. These topics were chosen because of their importance to the practicing physician.

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Key words: Diarrhea; Infectious diarrhea; Intestinal de-

velopment; Intestinal proliferation and repair; Intestinal permeability; Microbionics; Necrotizing enterocolitis; Probiotics

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INTESTINAL DEVELOPMENT

The development of the small intestine and its implications for nutritional support has been reviewed previously^[1]. The mucosa of the small intestine has many functions other than nutrient absorption and fluid secretion. These functions include host defense and immune responses. All of these small intestinal functions may be influenced by diet as well as by the intestinal microbionics, and may also be developmentally regulated.

At birth, there is rapid colonization of the intestinal lumen of the neonate, both with bacteria from the mother's milk, such as Gram-positive lactobacilli and bifidobacteria, and colonization with Gram-negative anaerobic bacteria. These bacteria are essential for growth and development of the gastrointestinal (GI) tract. In response to luminal microorganisms, intestinal Paneth cells produce microbicidal peptides, such as α -defensin. Paneth cells differentiate while migrating towards the crypt base, where these cells are eventually cleared by phagocytosis. In addition to the importance of Paneth cells in the innate immune defense against microorganisms, they are also involved in intestinal angiogenesis.

There is a complex interaction between bacteria in the intestinal lumen, the epithelial surface, and the underlying matrix, immune cells and enteric nervous system.

Enterocytes sense pathogenic bacteria, occupy the Toll-like receptors (TLRs) in response to their activation *via* pathogen-associated molecular patterns (PAMPs), leading to nuclear factor (NF)- κ B activation. Pattern recognition molecules (PRMs) detect any conserved bacterial products. PRMs, such as TLRs and nucleotide-binding oligomerization domain containing (NOD) receptors, are further important regulators of the intestinal barrier. TLRs are pattern-recognition receptors (PRRs) expressed by immune and nonimmune cells that signal in response to PAMPs expressed by bacteria in the intestinal lumen. Most TLR molecules signal through the adapter molecule MyD88 to interleukin (IL)-1-receptor-associated kinase. MyD88 activates the NF- κ B pathway, which is a critical regulator of tumor necrosis factor (TNF)- α and IL-1 β . Deficiency in MyD88 increases susceptibility to bowel inflammation, presumably by increasing brush border membrane (BBM) permeability. TLR ligands alter the properties of the intestinal barrier by redistributing zonula occludens (ZO)-1 and β -defensin 2. Cyclooxygenase-2-derived prostaglandin (PG)E₂ is important in TLR mucosal repair^[2].

When a child is born prematurely, its intestine is also premature, and is potentially ill-prepared for the extrauterine environment and contact with food bacteria. Microbial manipulation with antibiotic treatment or exposure of the pregnant rat dam to *Escherichia coli* (*E. coli*) results in long-lasting and potentially adverse effects on postnatal microbiota of the offspring^[3]. PAMPs produce an intestinal immune response through interaction with PRRs, including the TLRs. Transmembrane signaling may occur through TLRs^[4]. TLRs trigger signaling pathways which lead to NF- κ B activation and propagation of inflammatory responses. In addition to exogenous inflammatory mediators, TNF- α as well as IL-1 β trigger cytokine production through activation of this NF- κ B pathway. The NF- κ B proteins activate transcription, resulting in immune and inflammatory responses.

Intestinal alkaline phosphatase (AP) detoxifies lipopolysaccharide (LPS) by hydrolyzing it so that it can no longer activate TLR-dependent inflammatory responses^[5,6]. AP is involved in maintenance of homeostasis between the luminal bacterial microbiota and the body^[5,6]. About two-thirds of persons taking short or long courses of nonsteroidal anti-inflammatory drugs (NSAIDs) develop small intestinal damage. The NSAID diclofenac may induce enteropathy by way of the c-Jun-N-terminal kinase (JNK) pathway^[7]. This concept of stress-related changes in intestinal barrier and secretory function has therapeutic implications, as does the potential of JNK inhibitors to protect the GI tract from the damage of NSAIDs. For example, the NSAID indomethacin may enter the small intestine through a TLR4/MyD88-dependent pathway^[8]. In animals with stress as a result of immobilization, there will be an increase in TNF- α along with an increase in tight junction (TJ) permeability. These permeability changes are associated with alteration in the TJ proteins ZO-1, claudin-2, claudin-4, claudin-5, and β -catenin^[9].

IL-8 secretion in response to bacteria, IL-1 β and TNF- α are increased more in human fetal small intestinal epithelial cells than in older children or adults. This increased activation of NF- κ B DNA binding and transcriptional activity is due to reduced inhibition of signaling, resulting in increased phosphorylation, ubiquitination and degradation of the inhibitor of NF- κ B, in conjunction with decreased baseline expression and delayed resynthesis of this inhibitor^[10]. This represents a mechanism explaining the enhanced inflammatory response occurring in immature intestinal tissue.

The intestinal epithelial stem cells (ISCs) reside in the crypts of Lieberkuhn, giving rise to five daughter cell lines: Paneth cells, enterocytes, goblet cells, enteroendocrine cells, and M cells. In the upper third of the intestinal crypt, daughter cells differentiate into enterocytes, goblet cells, enterochromaffin cells, and Paneth cells. The Hedgehog platelet-derived growth factor and bone morphogenetic protein (BMP) signaling pathway are key mediators in morphogenesis of the intestinal villi and crypts, and maintain tenancy of ISCs. The ISCs are of two types: the fast cycling "crypt base columnar" (CBC) cells residing among the Paneth cells at the base of the crypt, and the slow cycling ISCs above the Paneth cells (+4ISC). Only one clonogenic cell is needed to regenerate a crypt and to maintain the appropriate number of crypts after injury. It is the +4ISCs that are highly sensitive to modulation and to chemotherapeutic agents such as doxorubicin^[11]. The ISCs produce progenitor cells, which form the enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. Paneth cells are a major component of the innate immunity in the intestine, releasing granules containing β -defensins, matrix metalloproteinase-7, synovial phospholipase A2, and lysozyme. Colony stimulating factor (CSF)1 acts in a direct juxtacrine/paracrine fashion, or acts indirectly through macrophages in the lamina propria, to regulate Paneth cell development^[12]. Fibroblast growth factor receptor (FGFR)-3 acts on the ISCs in the lower portion of the crypts to signal through both the β -catenin/Tcf-4 dependent and independent pathways, the expansion of the ISCs, expanding ISCs and crypt macrophages, including Paneth cells^[13]. The behavior of ISCs is regulated by canonical Wnt signals. Inhibition of Wnt signaling reduces epithelial proliferation in adult mouse intestine. In the developing intestine, the relationships among Wnt signaling, epithelial proliferation, and tissue differentiation are reversed^[14].

The intestinal epithelium is renewed through a series of programmed developmental transitions in the form and function of the intestine. For example, lactase-phlorizin hydrolase (LPH), fatty acid binding protein (Fabp1), and sucrase-isomaltase (SI) are intestinal proteins that are important for nutrition during different stages of development. LPH hydrolyzes milk lactose, SI hydrolyzes α -saccharidases, and Fabp1 is a cytoplasmic protein important for intracellular lipid transport. A member of the zinc finger transcription factor family (Gata4) and the hepatocyte nuclear factor α 1 (Hnf1 α) are essential for

LPH and Fabp1 gene expression but they do not mediate the glucocorticoid-induced precocious maturation of the intestine. Rather, specific intestinal genes have differential requirements for Gata4 and Hnf1 α , and these genes are dependent on the developmental time-frame in which each gene is expressed^[15].

Protective factors for the intestinal epithelium include secretion of mucins, as well as IgA and defensin peptides that are produced from epithelial cells. Epithelial cells also produce cytoprotective stress proteins, such as heat shock proteins (HSPs). N-formylmethionyl-leucyl-phenylalanine (fMLP) is a tripeptide produced by many enterobacteria as a by-product of protein synthesis. fMLP is a chemotactic agent from neutrophils and may be proinflammatory in nature. fMLP induces expression of HSPs and inhibits activation of the proinflammatory transcription factor NF- κ B^[16]. This finding needs to be translated to a clinical application.

Amniotic fluid and breast milk contain intestinal trophic factors that stimulate enterocyte development, and enhance various intestinal defense processes. Hydrocortisone is a trophic factor that is present in large amounts in human breast milk. Hydrocortisone reduces the cholera toxin (CT)-induced secretory response observed in fetal human small intestinal epithelial T84 cells, by inducing a pathway change from a clathrin-mediated to a caveolae-mediated endocytic process^[17]. Hydrocortisone also increases ganglioside/lipid raft association, which may also reduce intestinal secretion.

Dietary fat in mother's milk is a major modifiable environmental factor that influences intestinal growth and development of the baby. The n-6 fatty acid, linoleic acid, is the metabolic precursor for synthesis of arachidonic acid (ARA). α -linolenic acid is a precursor for synthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Eicosanoids derived from ARA are proinflammatory, whereas the n-3 fatty-acid-derived metabolites are anti-inflammatory. EPA and DHA have direct effects on intestinal cells through gene expression and ion channels, as well as on G-coupled protein receptor activities. As a result of the potential for dietary substances to affect normal growth and development of neonatal intestine, the consumption of early-life non-maternal milk products must be undertaken with the greatest of care.

In adult animals, glucagon-like peptide (GLP)-2 has a trophic effect on the intestine, and enhances nutrient absorption. In contrast, in young animals, there is malabsorption of several lipids with GLP-2 or GLP-2 plus dexamethasone, but not with dexamethasone itself^[18]. The loss of body weight and jejunal atrophy induced by dexamethasone is prevented by giving GLP-2. Giving dexamethasone or GLP-2 and dexamethasone to lactating rat dams enhances glucose and lipid uptake in their suckling offspring^[19].

The premature human neonate has higher fasting levels of GLP-1 and GLP-2 compared to adults, and feeding increases these levels even further^[20]. GLP-2 levels are correlated with residual small intestinal length in the

infant with nutrient malabsorption following intestinal surgery^[21]. GLP-2 levels are directly correlated with absorption of fat, carbohydrate, and protein.

In suckling rats, GLP-2 plus dexamethasone increased the maximal transport rate (V_{MAX}) for jejunal glucose uptake, and the combination of these may be useful in stimulating glucose uptake in the developing intestine^[22]. In suckling animals, dexamethasone reduces jejunal lipid uptake to levels similar to those seen in weanling animals. Giving dams GLP-2 or dexamethasone during pregnancy and lactation reduces lipid uptake in the offspring, and this reduction persists for at least 1 mo^[23]. The impact this may have on the nutritional wellbeing of the animal in later life is unknown. This important consideration needs to be explored in terms of its long-term implications if nursing mother or child were to be exposed to steroids or GLP-2.

PROLIFERATION AND REPAIR

Homeostasis of the intestine is balanced between the process of proliferation, migration, differentiation, mitosis and apoptosis. Remodeling of the epithelium results from cell-matrix and cell-cell contacts. The proliferating regions are usually maintained by Wnt/ β -catenin signaling, whereas differentiation is regulated by BMP, transforming growth factor (TGF)- β , and Hedgehog signaling. These interact within the stem and progenitor cells in the lower regions of the intestinal crypts, and regulate the expansion and cell fate decision of the interstitial epithelial cells (IECs). Several proteins involved in the contact between cells act as the source (i.e., Notch signaling, which regulates the development of enterocytes)^[24] or the downstream effectors of signaling pathways such as E-cadherin/ β -catenin. Notch signaling regulates the formation of the Ephrin-B2-positive proliferating crypt cells, as well as the Ephrin-B1-positive differentiated cells^[25].

Homeostasis of the intestinal epithelium is a dynamic process created by a balance between proliferation, differentiation and apoptosis. This process is regulated by luminal nutrients, hormones, growth factors and cytokines. When acute damage is done to the GI tract, the usual repair process does not involve proliferation, but rather involves mucosal restitution. Restitution occurs by epithelial cell migration to reseal the superficial wound. Polyamines are involved in stimulation of intestinal epithelial cell migration during restitution. These polyamines (including spermidine, spermine, and precursor, putrescine) regulate calcium homeostasis by inducing alterations in cytosolic free Ca^{2+} concentration. Phospholipase C (PLC)- γ_1 modulates calcium store mobilization and calcium influx, thereby promoting intestinal epithelial restitution after wounding^[26].

Especially early in life, the BBM of the enterocyte is involved in the process of endocytosis, in which a tiny portion of membrane forms a bud around a luminal constituent, and breaks away from the rest of the BBM. This

hole in the remaining BBM must be quickly repaired. The subapical terminal web cytoskeleton inhibits movement of endosomes, and thereby helps to maintain permeability characteristics of the epithelium by keeping the endocytic activity occurring at the BBM under regulated control^[27].

Differential display and microarray analyses have been used to identify genes that may be involved in molecular mechanisms regulating proliferation and differentiation of the intestinal epithelium^[28]. Examples of transcription factors include the BMP expressed in the mesenchyme, Wnt which is essential for regulation of crypt stem cell proliferation and maintenance, and Notch signaling within the epithelium influencing differentiation of enterocytes, goblet cells, Paneth cells and enteroendocrine cells.

Glutamine is an amino acid that is the preferred fuel source for enterocytes. Glutamine activates extracellular signal-regulated kinase (ERK) and inhibits phosphorylated Akt, suggesting that ERK has an important role in the glutamine-mediated intestinal homeostasis^[29]. Activation of phosphoinositide 3-kinase (PI3K)/Akt during periods of glutamine deprivation limits apoptosis, and thereby may have a protective role. Glutamine-response genes influence the expression of a number of signaling pathways such as NF- κ B^[30] and peroxisome proliferator-activated receptor (PPAR) α ^[31]. This potential basis for protection with glutamine, for example, in necrotizing enterocolitis, remains to be tested.

The Notch signaling pathway is essential for the development of enterocytes, and the Wnt cascade is required for the development of Paneth, goblet and enteroendocrine cells (secretory lineage). The commitment of crypt stem cells to differentiate into enterocytes reduces the differentiation of these secretory cells. This interaction by Notch to activate the Hes1 transcription factor, which then blocks the MATH1 transcription factor, influences the Ets-domain transcription factor Spdef, thereby blocking steps in the differentiation of the stem cells into the secretory lineage^[32].

Notch, Wnt and Hedgehog signaling pathways are involved in epithelial cell proliferation and differentiation. Indian hedgehog (Ihh) is a signal sent by mature Paneth cells to their stem cell precursors, negatively regulating their differentiation. PPAR β is a nuclear hormone receptor activated by fatty acids in the intestinal lumen. PPAR β acts on Paneth cell homeostasis by downregulating expression of Ihh^[33]. Hedgehog proteins are members of a family of secreted signaling factors that orchestrate development of many organs and tissues, including those of the GI tract^[34]. In addition to epithelial differentiation being influenced by this variety of signaling cascades, amino acids and carbohydrates regulate gene expression and cellular function. For example, coenzyme A (CoA)-thioester derivatives contribute to cellular renewal along the crypt-villus axis in human small intestine^[35].

The Wnt/ β -catenin pathway involves molecules secreted by the mesenchyme, and controls proliferation and migration of enterocytes and Paneth cells. Transcription

regulators such as the CDX2 and CDX3 homeoproteins, members of the homeobox gene family of transcription factors, activate several intestine-specific genes such as SI and LPH, as well as the adhesion proteins L1-cadherin and claudin-2. Furin is a calcium-dependent serine proteinase which processes many proteins such as BMP-4, the insulin receptor, the Notch1 receptor, and the cell adhesion protein E-cadherin. CDX2 regulates furin expression in intestinal epithelial cells^[36]. Hepatocyte growth factor-1 α cooperates with CDX2, and regulates transcription of SI, LPH, claudin-2, calbindin 3 and liver Fabp1 gene^[37]. The crosstalk between lamina propria lymphocytes and intestinal epithelial cells may also be mediated by keratinocyte growth factor (KGF), such that lymphoepithelial interactions may both promote barrier function as well as regulate mucosal immune responses^[38].

Mitogen-activated protein kinases (MAPKs) and PI3K are activated in a cascade-like fashion by various growth factors. Three of the MAPKs that have been identified include ERK, JNK and p38 MAPK. Rac1 also mediates apoptosis irrespective of ERK1/2 and Akt activation^[39]. Signaling cascades such as the NF- κ B and MAPK pathways provide transcriptional activation of inflammatory mediators such as chemokines, adhesion molecules and antibacterial peptides. In addition, reactive oxygen species (ROS) are important mediators in cellular signal transduction cascades regulating proliferation, apoptosis and migration. TNF- α is an inducer of apoptosis that requires the small GTPase Rac1, which has a role in the control of ROS production^[40]. Protein-tyrosine kinase 6 (PTK6) is a stress-reduced kinase that promotes differentiation, and is a target of the serine threonine kinase AKT. After DNA damage, PTK6 is induced to promote apoptosis^[41].

The intestinal mucosa is constantly being deformed by peristalsis, movement of the villi, and passage of chyme along the surface of the intestine. This strain by a variety of signals stimulates the migration of epithelial cells across fibronectin [such as Scn, focal adhesion kinase (FAK), and ERKs]^[41].

INTESTINAL PERMEABILITY

The intestinal BBM of enterocytes constitutes the small intestinal epithelial barrier, which helps to protect the body against invasion by millions of luminal bacteria. This barrier function is a balance between the bacterial microbiota, and homeostasis provided by the preservation of the integrity of the BBM. TJs, innate immune cell function, and microbial peptides are important in establishing and maintaining this barrier integrity.

Modifications in NOD2 alter intestinal homeostasis, and may be associated in some individuals with chronic intestinal inflammation. PRMs, TLRs and NOD receptors are regulators of the intestinal epithelial barrier, which is essential along with innate immune cells and antimicrobial peptides, to maintain normal intestinal permeability^[42].

TJs between the intestinal epithelial cells provide a dynamic structure, altering cellular permeability. TJs are

formed by organization of a number of specific proteins including occludin, ZO-1, ZO-2, ZO-3, claudins, and junctional adhesion molecules. Mucosal hydration occurs through activation of ion channels and transporters, as well as through secretion of mucous and antibacterial protein. These are additional defense pathways against pathogens^[43]. Under some circumstances such as intestinal ischemia, there may be increased mucosal penetration and translocation of bacteria, as well as transcytotic movement of bacteria through intestinal epithelium by way of lipid rafts. This movement of bacteria across the disrupted intestinal barrier may precede cytokine-induced disruption of TJs^[44].

Adherens junctions (AJs) are a major part of the junctional complexes. The main transmembrane protein of AJ is E-cadherin. E-cadherin mediates adhesion or anchorage of the enterocytes to the matrix by a process that is Ca^{2+} -dependent. Epidermal growth factor receptor (EGFR) is induced in the process by which there is deprivation of the E-cadherin-dependent junctions, and enterocytes lose their interactions with the basal lamina. This leads to anoikis, a form of apoptosis caused by the loss of cell anchorage^[45]. Symplekin is also a component of the TJ cytoskeletal plaques. Symplekin co-operates with the transcription factor ZONAB to regulate negatively the differentiation of goblet cells in the intestine^[46].

Mast cells (MCs) in the lamina propria contain both preformed bioactive mediators such as tryptases and histamine, as well as synthesized mediators like prostanoids and leukotrienes. MCs express corticotrophin-releasing factor receptor 1 (CRFr1) and CRF receptor 2 (CRFr2), G-protein-coupled corticotrophin-releasing factor receptors^[47]. In pigs, when CRFr1 is activated during early weaning stress in pigs, the epithelial barrier becomes leaky, whereas CRFr2 may be protective.

When the serotonin reuptake transporter (SERT) is lost, there is enhanced intestinal translocation of bacterial endotoxin in a murine model of chronic increased intake of fructose^[48].

The permeability of the intestinal mucosa is determined by the relative balance of uptake from the intestinal lumen, across the BBM and into the cytosol of the enterocyte, and efflux from the cytosol across the BBM and into the lumen, through transporters such as P-glycoprotein (P-gp; ABCB1), multidrug resistance-associated protein 2 (MRP2; ABCC2), and breast cancer resistance protein (BCRP; ABCG2). Changes in the activity of these efflux transporters will modify net drug absorption. For example, sulfasalazine uptake across the BBM is adequate, but sulfasalazine may be transported out of the enterocytes (efflux) by MRP2 and BCRP, contributing to the apparent low permeability of the small intestine to this drug^[49].

The transport and barrier properties of the intestinal epithelium are regulated, influenced by food and the microbionics that colonize mucus overlying the epithelium. Epithelial transport mechanisms are present to regulate the long-term adaptation of intestinal function. Specific

molecular components of TJs decrease or increase trans-epithelial resistance^[50]. In enterocytes in the upper portion of intestinal crypts, there are cell cycle promoting and inhibiting genes, as well as transcription/translation related genes. In the enterocytes, in the middle of the villi, there are metabolism-related genes, and vesicle/transport-related genes^[51].

Stressful events will increase intestinal permeability to macromolecules, deplete intestinal mucus, and alter interaction of bacteria in the intestinal lumen with the intestinal epithelial cells. Stress may even lead to inflammation, bacterial invasion of the mucosa, as well as T-cell activation. The disturbance of epithelial cell kinetics depends on the duration of the stress. The stress-induced increase of intestinal permeability may be related to an increased number of apoptotic cells in the epithelium^[52]. Perfusion of a jejunal segment in persons with low *vs* moderate background stress (called “pain stress”), results in an increase in chloride-related peak secretory response, as well as enhanced permeability to albumin^[53].

In addition to glucocorticoid-induced precocious maturation of the intestine, “stress” may also play a role in disease susceptibility through its actions on CRF, and subsequent activation of CRF receptors expressed locally in the intestine. It is predominantly the CRF-r1 activation that alters intestinal function in times of stress. One such type of stress in the developing animal is weaning, when the increased intestinal permeability and secretory activity are mediated by the intestinal CRF receptors. This is possibly through activation of enteric neural and PG synthesis pathways in weaned intestinal tissues^[54].

In humans, the increased intestinal permeability due to the acute effects of radiotherapy may be reflected by increased fecal excretion of DHA and calprotectin^[55]. From 50% to 70% of NSAID users have damage to the GI tract beyond the stomach. When capsule endoscopy, fecal calprotectin measurement, and urinary recovery of orally administered lactulose and mannitol are used, about half of NSAID users will have defects in the small bowel^[56].

The TJ (also known as the zonula occludens) has both a gate function (regulating the passage of molecules through the paracellular pathway), and a fence function. In some intestinal disorders, the permeability of the intestine may increase (“leaky” TJs), and endogenous PPAR β ligands may reduce the degree of TJ permeability, thereby reducing the degree of leakiness^[56]. This raises the therapeutic potential of using PPAR β ligands to correct the “leaky gut”. Adrenomedullin (AM) may also prove to be useful to reduce intestinal permeability. AM is a 52-amino-acid peptide formed in the mucosa. AM increases cAMP, activates protein kinase (PK)A, and stabilizes the barrier function of the intestine (i.e., prevents increased permeability and decreases leakiness^[57]). EGF binds to its receptor, and improves epithelial barrier function (i.e., decreases intestinal permeability). During sepsis there is barrier dysfunction, increased apoptosis, and higher levels of cytokines. Potentially all of these may lead to multiple

organ failure. Systemic EGF prevents peritonitis-induced intestinal barrier dysfunction^[58], and may thereby reduce mortality from noninfectious inflammation and intestinal injury. It remains to be established whether this modification of intestinal permeability will have any therapeutic benefits in humans.

MICROBIOTICA, INFECTIOUS DIARRHEA AND PROBIOTICS

Probiotics

The World Health Organization defines probiotics as “live microorganisms which, when consumed in adequate amounts as part of food, confer a health benefit on the host”. Probiotic therapy has been used for traveler’s diarrhea, to prevent antibiotic-associated diarrhea, and to prevent relapse of pouchitis (after colectomy and ileoanal pouch formation). *Lactobacillus rhamnosus* GG (LGG) is a probiotic bacteria that prevents cytokine-induced apoptosis by way of two LGG-produced viable proteins p75 and p40. These activate Akt and regulate intestinal epithelial cells as well as antiapoptotic responses by a PKC-MAPK-dependent mechanism^[59]. The clinically tested VSL#3 probiotic formula and its secreted components may augment the protective mucus layer through MUC2 gene expression^[60]. Treating infectious diarrhea in children with *L. rhamnosus* shortens the duration of diarrhea, and also reduces the duration of the need for parental rehydration^[61].

A meta-analysis of five randomized controlled trials of *Saccharomyces boulardii* has shown moderate effectiveness in preventing antibiotic-associated diarrhea (AAD) in children and adults. *S. boulardii* reduced the risk of AAD from 17% to 7%, with the number needed to treat to prevent one case of AAD being only 10^[62].

The probiotic bacterium *Lactobacillus casei* strain Shirota (LCS) suppresses the LPS/TLR4 signaling pathway and prevents indomethacin-induced intestinal injury, possibly by way of the production of lactic acid^[63]. The nonpathogenic yeast *S. boulardii* inhibits EGFR and thereby also inhibits the downstream ERK1/2 MAPK pathway^[64]. This raises the possibility that *S. boulardii* may have both anti-inflammatory and antineoplastic therapeutic potential.

It will soon be possible to target the human microbiome with antibiotics, probiotics and prebiotics. “Culture-independent strategies such as high-throughput parallel sequencing and comparative genomes, metabolic proliferating and functional genomes, fluorescence *in situ* hybridization, and phylogenetic microarrays will provide new insights into the composition, architecture and functional roles of the human microbiota”^[65].

A therapeutic approach to enteric infections is to express molecular mimics of toxin receptors on the surface of harmless bacteria, so that the toxin binds to the bacterium rather than to its natural target, the intestinal mucosa. This strategy using molecular mimicry has been used to develop recombinant probiotics for treatment and prevention of diarrheal disease caused by enterotoxigenic

E. coli^[66], and for prophylaxis and treatment of cholera^[67].

Infections

The protozoon *Giardia lamblia* is a human GI parasite that is common in Western Europe and in North America. Some 15% of infected individuals develop chronic symptomatic disease. *G. lamblia* infection increases intestinal permeability due to downregulation of TJ occludin 1, reduced sodium-dependant glucose absorption, and by increased epithelial apoptosis, as well as increased active electrogenic anion secretion^[68]. Exposure to DDT, organochlorine, reduces the efficacy of treatment of mice with experimental giardiasis^[69].

Cyclospora responds variably to albendazole^[70]. Nitazoxanide is effective in treating diarrhea and enteritis caused by *Cryptosporidium* in immunodeficient as well as non-immunodeficient patients aged ≥ 12 years^[71].

The synthesis of NO is increased in infectious diarrhea, but its physiological role is unknown. Inducible NO synthase (iNOS) is constitutively expressed by the intestinal villous cells. After acute injury of the intestinal epithelium rapidly induces iNOS and synthesizes NO. The induction of iNOS in response to neonatal piglet *Cryptosporidium parvum* is a nonspecific response^[72].

Campylobacter spp. are a common cause of diarrhea in developing countries. They causes mild to severe bloody diarrhea, chronic relapsing infection, and invasion of the mucosa. This may be associated with extraintestinal complications such as neuropathy in the peripheral nervous system, (e.g., Guillian Barré syndrome). Norepinephrine (NE) is present in high concentrations in the noradrenergic neurons in the intestine. Exposure to NE increases the virulence-associated properties of *Campylobacter*^[73]. This suggests that microorganisms may have evolved a “sense and respond” system. The evidence linking inflammatory bowel disease (IBD) and *Campylobacter jejuni* infection has been reviewed^[74].

Diarrhea in the traveler may be termed “traveler’s diarrhea,” therefore, detection of bacterial enteropathogens is not uniform. When diarrhea occurs in travelers, ciprofloxacin (500 mg *bid* for 3 d), levofloxacin (500 mg *qid* for 3 d), rifaximin (200 mg *qid* for 3 d), or azithromycin (1000 mg, single dose) should be taken in conjunction with an antidiarrheal agent^[75]. Indeed, there is an advantage of adding loperamide to fluoroquinolone, rifaximin, and azithromycin treatment of traveler’s diarrhea. Two new antisecretory drugs that have been shown to be useful for reducing stool frequency of traveler’s diarrhea including crofelemer, a chloride channel blocker^[76], and zaldaride, a calmodulin inhibitor^[77]. Racecadotil, an enkephalinase inhibitor, may also be useful for the treatment of acute diarrhea^[78-80].

Vibrio cholerae secretes CT, which binds to mucosal enterochromaffin cells, and release large amounts of 5-hydroxytryptamine (5-HT). The sustained hyperexcitability of CT on the submucosal secretomotor neuronal pathway depends on 5-HT₃, and nicotinic receptors^[81]. The small GTP-binding proteins increase the CT-catalyzed ADP-ribosylation of the heterotrimeric guanine nucleo-

tide-binding (G) protein Gs β . There is increased susceptibility of neonates to infectious diarrhea. For example, there is increased uptake of CT by a developmentally regulated clathrin-endocytic pathway. Immature human enterocytes, DRF1, play a role in clathrin-mediated CT trafficking through the endoplasmic reticulum and Golgi, and ARF6 enhances Gs β activation by CT^[82].

Shiga toxin (Stx) 1 and 2 are virulence factors for noninvasive enterohemorrhagic *E. coli* (EHEC) infection, such as by *E. coli* 0157:H7 infection. Stx is taken up by microendocytosis in cells that lack the Stx1 receptor, mediated by Gc3 on the BBM^[83].

Clostridium difficile accounts for 15%-25% of episodes of AAD. Methicillin-resistant *Staphylococcus aureus* (MRSA), and particularly the enterotoxin-producing strains, may also cause nosocomial AAD^[84].

Rotavirus is the major cause of life-threatening diarrheal disease in infants and young children. Rotavirus-induced diarrhea can be prevented by use of a live-attenuated vaccine^[85]. Following acute mucosal injury such as from rotavirus infection, there is enterocyte migration that is proliferation-independent. This restitution or migration of cells requires NO, PGs and polyamines. Acute regulation of protein synthesis is achieved through changes in the rates of translation of mRNA, by way of alterations in peptide chain initiation. This chain initiation process is influenced by mammalian target of rapamycin (mTOR). mTOR is an amino acid-sensing mechanism that targets ribosomal p70 S6 kinase (p70^{S6K}). p70^{S6K} is activated during an active state of mucosal regeneration produced by a piglet rotavirus enteritis^[86].

The etiologic agent of Whipple's disease (WD) is *Tropheryma whippiei*, a PAS-positive, rod-shaped actinomycete. Some healthy persons harbor *T. whippiei* in their bowel, and there is no correlation between *T. whippiei* genotypes and clinical manifestations. The cumulative odds ratio (OR) for disease are 2.23 for the HLA-DRB1 X 13 allele, and 2.25 for the DBQ1*06 allele^[87]. A randomized controlled trial of 40 persons with previously untreated WD showed that daily infusions of ceftriaxone 1 $\times$ 2 g or meropenem 3 $\times$ 1 g for 14 d, followed by 12 mg oral trimethoprim-sulfamethoxazole, both gave an OR of 0.95 for remission for at least 3 years^[88]. One patient with asymptomatic cerebrospinal fluid infection with WD was resistant to ceftriaxone and to meropenem, but responded to chloroquine and minocycline. This compares favorably to retrospective case series with treatment of *T. whippiei* with tetracycline, penicillin, streptomycin and low trimethoprim-sulfamethoxazole, in which only about three quarters of WB patients had an initial response, and approximately a quarter of those who had an initial response subsequently relapsed.

Both Crohn's disease (CD) and intestinal tuberculosis (TB) (caused mainly by *Mycobacterium tuberculosis* or *Mycobacterium bovis*) have a predilection for the tumor ileum. An algorithm has been developed for the investigation of persons in whom the diagnosis includes CD and TB^[89].

Diarrhea is the most common GI symptom in persons

with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), affecting 50%-90% of those with a low CD4 lymphocyte count. However, in some 30%-40% of cases of HIV/AIDS, enteric pathogens cannot be identified. Conceptually, HIV enteropathy has been supported by the detection of HIV proteins in the intestinal epithelial cells as well in gut lymphocytes and macrophages. In a case report, a 33-year-old woman with HIV enteropathy was described with structural changes in the duodenal mucosa, regressing after triple antiviral therapy^[90].

About half of the HIV-infected persons in the United States have diarrhea. The intestinal immune system is central to the pathogenesis of AIDS (transmission, viral complication, CD4⁺ T-cell destruction), with the progression of AIDS due to a decrease in the expression of genes that maintain the mucosal barrier, and HIV translocation^[91]. Endoscopy and biopsy of the upper and lower GI tract has identified pathogens in about half of those HIV-infected persons who were initially thought to have an idiopathic disorder or AIDS enteropathy. Viral replication occurs early in HIV in the gut-associated lymphoid tissue CD4⁺ T cells and macrophages. AIDS enteropathy is "an idiopathic, pathogen-negative diarrhea, and treatment includes highly active antiretroviral therapy (HAART), fluid and electrolyte balance, and nutritional support^[92]". The opportunistic infections can still occur in HIV-positive persons taking HAART, especially in those with a high viral load and a low T-cell count. Also note that these opportunistic disorders occur even if the HIV load is low and the CD4⁺ T-cell count is near normal.

In a young patient with chronic diarrhea, bacterial rDNA (16S rDNA) has been extracted from the duodenal biopsy, and was thought to be due to *Stenotrophomonas maltophilia*^[93]. Such genomic techniques may be used for the diagnosis of chronic infections, including those with HIV enteropathy.

Matrix metalloproteinases (MMPs) are a group of enzymes that are capable of degrading all components of the extracellular matrix. Fibroblasts are the major source of MMPs in the human intestine. Fibroblasts are a potential target of IL-21, a T cell-derived cytokine that acts on the IL-21 receptor, and then interacts with the common γ chain receptor IL-21 that controls MMP secretion by fibroblasts^[94].

Enteropathogenic *E. coli* (EPEC) is a leading cause of infantile diarrhea in developing countries. The EPEC-induced inflammation is a balance between pro- and anti-inflammatory proteins^[95]. Nosocomial infections with *Pseudomonas aeruginosa* are associated with a high fatality rate. A virulence circuitry of *P. aeruginosa* is activated by both soluble and contact-mediated elements of the intestinal epithelium^[96]. *P. aeruginosa* metabolizes adenosine to inosine, which enhances virulence of the organism^[97].

Malnutrition may be associated with prolonged episodes of EPEC infection. In the human epithelial Caco-2 cell model, EPEC inhibits uptake of thiamine^[98]. EPEC also inhibits SERT, the serotonin inhibitor; reduction in

SERT by EPEC may represent an additional and new mechanism of EPEC-mediated diarrhea^[99].

Severe acute respiratory syndrome (SARS) is characterized clinically by fever, nonproductive cough, dyspnea, lymphopenia, and rapidly progressing changes on chest X-ray, however, it is not uncommon for SARS patients to have diarrhea, nausea and vomiting, as well as abdominal pain. This may be associated with atrophy of the mucosal lymphoid tissue, staining of intestinal epithelial cells and lymphocytes for corona virus (*via in situ* hybridization), and demonstration by electron microscopy of SARS-corona virus-like particles in the mucosal epithelial cells^[100].

The total microbial population within the GI tract exceeds the total number of mammalian cells in the body by at least an order of magnitude. There is a complex environment of microbes that interfaces with the mucosal lining of the GI tract. This coexistence between eukaryotes and prokaryotes has both beneficial and unfortunately harmful interactions^[101]. The intestinal mucosa downregulates proinflammatory signaling pathways, expresses antimicrobial proteins, and initiates epithelial repair after mucosal injury. Indeed, commensal bacteria are actively involved in shaping the very barriers that confine them to the gut lumen^[102].

Lipid rafts are cholesterol-sphingolipid-rich microdomains in the enterocyte BBM. These lipid rafts contain digestion and transport enzymes that are important in trafficking and signaling of various proteins. For example, Cx43 is present on enterocyte lipid rafts, and interferon (IFN) inhibits enterocyte migration by displacing Cx43 from these lipid rafts in enterocytes^[103]. Some pathogens recognize these lipid-rich specialized areas, and modify function of the nutrient digestive and transport proteins. Anti-glycosyl antibodies are induced by a glycosyl antigen, and are associated with lipid rafts. These anti-glycosyl antibodies act as competitors to pathogens, having a protective role on the lipid rafts by preventing lactic-like pathogens from gaining access to the intestinal BBM^[104].

SMALL INTESTINAL BACTERIAL OVERGROWTH

Small intestinal bacterial overgrowth (SIBO) occurs as a result of a reduction in gastric acid secretion, a failure of mucosal and systemic barrier functions, as well as a failure of immune responses. SIBO also occurs in the presence of anatomical abnormalities such as intestinal fistulae or multiple small intestinal (not colonic) diverticulae. Gram-positive and Gram-negative bacteria, including both aerobes and anaerobes, contribute to the diarrhea and malabsorption of nutrients. While the prevalence of SIBO is increased in persons with irritable bowel syndrome (IBS), especially those with diarrhea-predominant IBS, the exact rates depend upon the method used to diagnose SIBO, with low prevalence rates of 4% using the gold standard test of a positive jejunal aspirate and culture, and a prevalence of 54% using the lactulose or glucose hydrogen breath test^[105]. The crux of this association of IBS and

SIBO is whether treatment of the positive test for SIBO will improve the patient's symptoms.

SIBO may recur after it has been successfully treated with appropriate antibiotics (e.g., metronidazole or ciprofloxacin). The recurrence rates after initially successfully treated SIBO are about 13% at 3 mo, 28% at 6 mo and 44% at 9 mo^[106]. SIBO recurrence is more likely in older persons, those treated chronically with proton pump inhibitors, and curiously, those with a previous history of appendectomy. Retreatment with the same or different antibiotics will be necessary, and some persons with recurrent SIBO require maintenance use of antibiotics.

The absorbable antibiotic metronidazole is more effective than the nonabsorbable antibiotic rifaximin for treatment of SIBO in persons with blind loop syndrome^[107]. Rifaximin may safely prevent traveler's diarrhea: in a study in travelers to Mexico, rifaximin reduced the risk of diarrhea from 54% in those taking placebo to 15% in those taking rifaximin^[108].

In persons with chronic rosacea, 46% had SIBO, vs 5% of healthy controls. After eradication of the SIBO, the cutaneous rosacea lesions cleared in 71% and greatly improved in 21%, whereas those treated with placebo remained unchanged in 90% or worsened in 10%^[109].

DIARRHEA, SALT AND WATER ABSORPTION

The topic of diagnosis and treatment of acute or persistent diarrhea has been reviewed^[110]. Host genetic polymorphisms may influence susceptibility to traveler's diarrhea. The topic of chronic diarrhea has been reviewed and can be accessed for a tutorial (BMJ Learning <http://gut.dnjj.com/tutorials/collection.dtl>)^[111].

Na^+/H^+ exchange (NHE) is the principal exchanger isoform responsible for transepithelial Na^+ absorption at the BBM of the intestinal epithelium. Coupled activity includes both Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchangers. The $\text{Cl}^-/\text{HCO}_3^-$ exchanger, SLC26A3 [also known as down-regulated in adenoma (DRA)], also exchanges sulfate and formate. Electroneutral NaCl absorption is mediated through NHE3 as well as DRA as the major $\text{Cl}^-/\text{HCO}_3^-$ exchanger^[112]. NHE is highly regulated by a number of second messenger systems including cAMP-dependent protein kinase (PKA), cGMP dependent kinase, Ca^{2+} , PKC, PI3K, and Akt. The phosphorylation of NHE by PKA alters the clathrin-mediated cAMP-stimulated endocytosis of NHE3, thereby reducing Na^+ absorption. Synaptotagmin 1 (Syt1) directs NHE3 to the adaptor protein 2-clathrin complex^[113].

There are two Cl^-/OH^- exchangers, DRA (SLC26A3) and PAT-1 (putative anion transporter-1; SLC26A6). DRA is in the colon, whereas PAT-1 is in the human jejunum and ileum. Cytokines such as IL-1 β , TNF- α and IFN- γ increase secretion in models of inflammatory diarrhea. IL-1 β decreases DRA mRNA, as also does IFN- γ by means of the STAT1/JAK (Janus kinase) pathway^[114]. This opens the way for the development of potential antidiarrheal

agents for the treatment of persons with inflammatory bowel disease. For example, *Lactobacillus acidophilus* prevents enteroinvasive *E. coli*-mediated disruption of the BBM barrier in Caco 2 cells, and increases Cl^-/OH^- exchange by enhancing the mRNA and activity of $\text{DRA}^{[115]}$.

Lysophosphatidic acid (LPA) is a glycerophospholipid present in abundance in foods such as soybeans and egg yolk. There are seven LPA receptors, and LPA^[116] acts through these receptors to inhibit the activity of the cystic fibrosis transmembrane conductance regulator (CFTR)-dependent Cl^- channel. LPA also stimulates BBM Cl^-/OH^- exchange and levels of $\text{DRA}^{[117]}$. This combined mechanistic effect of LPA may explain its antidiarrheal effect. This raises the possibility of using LPA for the treatment of diarrhea.

Serotonin (5-HT) is a neurotransmitter and hormone that influences many physiological functions. Over 97% of the serotonin in the body is in the enterochromaffin cells. SERT is present in the human intestine^[118], and the enterochromaffin cells have apical microvilli that protrude into the intestinal lumen. Serotonin is secreted from granules at the basolateral pole of the enterochromaffin cells. Abnormalities in the release, reuptake and catabolism of serotonin may be responsible for altered secretion, motility and pain sensation in patients with IBS, inflammatory bowel disease, or in persons with diarrhea or constipation. Release of serotonin may be mediated through central and enteric nervous system regulation, or by way of the enterochromaffin cells acting as sensors in the gut luminal milieu, responding to tastants such as (caffeine, tyramine and octopamine), olfactants (thymol and eugenol), as well as glutamine, deoxycolate, 2-deoxyglucose, and the artificial sweetener, sucralose. There are 11 secretory-associated genes, including the vesicle docking inhibitor STXBP3, which may be upregulated in response to luminal glutamine or bile acids, whereas anti-sense knockdown reduces serotonin secretion^[119].

NHE_3 is regulated by cAMP, cGMP, intracellular calcium, PI3K , glucocorticoid kinase-1 and PKC. The acute regulation involves changes in both functional activity and membrane trafficking. This acute effect on NHE_3 may be mediated by the glucocorticoid receptor, which stimulates SGK-1. Membrane insertion of NHE_3 may be caused by Na^+/K^+ -ATPase-induced changes in A^+ transporter Na^+ flux^[120].

NHE_3 help to maintain intracellular pH homeostasis, cell volume regulation, and electroneutral sodium chloride (NaCl) absorption. NHE_2 , NHE_3 and NHE_8 are present in the apical membrane of enterocytes and colonocytes. Sp3 is the major activator of NHE_2 gene transcription in the intestinal epithelial cells^[121]. NHE_3 is also regulated by certain growth factors, protein kinase, and $\text{IFN-}\gamma$. The Sp transcription factor is involved in cell cycle regulation, hormonal activation, and development patterning. NHE_3 facilitates intestinal neutral NaCl absorption, and thereby increases the net transmucosal absorption of bicarbonate in rat duodenal mucosa. This mechanism is similar to the proximal renal tubular bicarbonate absorption^[122].

The epithelial sodium channels (ENaCs) are a rate-limiting component for electrogenic sodium absorption in the distal colon. ENaC function is regulated by mineralocorticoid and glucocorticoid hormones. Steroid hormones act through transcriptional induction of β - and γ -ENaC subunits. In macroscopically noninflamed colon in persons with active CD, there is impaired ENaC-mediated Na^+ absorption, which contributes to the diarrhea arising from the inflamed mucosa^[123].

Water movement across the intestinal epithelium is driven osmotically, and is therefore influenced by the balance between sodium absorption and chloride secretion. These processes, absorption and secretion, are regulated. Intestinal motor and sensory function is altered by the action of transmitters and by changes in ion channel function. CFTR is a cAMP-dependent Cl^- channel. CFTR is located in the intestinal epithelium, and is activated by PKA-mediated phosphorylation. A minor component of Cl^- transport occurs by way of intestinal and colonic epithelial cell transport, the type 2 Cl^- channel (ClC-2) volume-regulated channel. CFTR acts as an entry pathway for Cl^- into epithelial cells, in parallel with Na^+ entry through apical ENaCs. ClC-2 promotes anion secretion, with little anion reabsorption. The properties of ClC-2 differ depending upon the conditions in the tissue in which the ClC-2 channels are studied^[124].

Diarrhea is common after pelvic radiotherapy, and this may be associated with modification in the diversity of luminal microflora, as reflected by a drop in the similarity index of the enteric bacteria. Cluster analysis of the microbial profile 5 wk after radiotherapy displayed a dentogram which was different in patients who presented with or without diarrhea^[125]. It is not clear why there is this differential effect of radiation on the intestinal organisms.

Newer and even more effective antidiarrheal agents are needed. In the presence of malabsorption of Na^+ and water in the small intestine, the ENaCs in the distal colon are upregulated in order to absorb more Na^+ /water, and hopefully thereby reduce diarrhea. ENaCs are responsive to mineral corticoids as well as to glucocorticoids. Glucocorticoids act through p38 MAPK to enhance the transcription of the B- and J- subunits of ENaCs, thereby rapidly increasing water absorption^[126].

DRA is a $\text{Cl}^-/\text{HCO}_3^-$ exchanger. When DRA is eliminated in mice, there is an approximately 50% reduction of basal and cAMP-stimulated HCO_3^- secretion^[127]. Microfluorimetry of villi shows that DRA is the major $\text{Cl}^-/\text{HCO}_3^-$ exchanger in the lower villous epithelium. In humans, loss-of-function mutations cause congenital Cl^- losing diarrhea^[128].

The development and control of the inflammatory reactions, which occur in food allergies, are mediated by T helper (Th)2 or regulatory responses, in which IL-4 is a factor. Allergic responses include IL-4 induction of the differentiation of committed effector Th2 lymphocytes for mRNA for $\text{TNF}\beta$, Th1, $\text{IFN}\beta$, IL-12p40, regulatory cytokines or Foxp3 (forkhead box P3). These may serve

as “rescue channels” for all Cl⁻ secretion, but lubiprostone activation of Cl⁻ secretion requires CFTR. The PPAR- β agonist rosiglitazone reduces cAMP-dependent Cl⁻ secretion as a result of reduced expression of the CFTR Cl⁻ channel, KCNQ, K⁺ channel, and Na⁺/K⁺/2Cl cotransporter proteins^[129].

IBS

About half of diarrhea-predominant IBS patients report an exacerbation of their symptoms within 90 min of eating. Normally, the duodenal and ileal breaks slow the passage of chyme along the intestine, and this helps to prevent exceeding the approximately 2-L composition of the colon to absorb water. Indeed, the small bowel water content initially falls, and then rises after a meal of liquids and solids^[130]. This could possibly be due to MC products being released from the small bowel mucosa, causing rapid transit of mill as intestinal fluid secretion.

IBS may aggregate in families, and twin studies demonstrate both a genetic as well as an environmental basis for IBS. Human intestinal smooth muscle cells and ICCs express SCN5 (gene encoded Na⁺ channel, expressed in interstitial cells). There may be an association between genetic defects in SCN5A and GI symptoms, and SCN5A may be a candidate gene in the pathophysiology of IBS^[131].

In a group of 62 patients with presumed idiopathic chronic watery diarrhea, HLA-DQ2/DQ8 genotyping, eHCAT abdominal retention test, small bowel follow-through, and hydrogen breath test were undertaken. In 45% of these patients, bile acid was presumed to be the cause of diarrhea. Sugar malabsorption was seen in 16%, gluten sensitive enteropathy in 16%, and both bile acid and sugar absorption in 3% of patients. It is important to note that not only was the cause of the chronic watery diarrhea not idiopathic in 81% of the patients, but it responded to specific treatment^[132].

NECROTIZING ENTEROCOLITIS

Intestinal mucus is one of the important factors protecting the intestinal epithelium. Without this well-formed gel layer, the underlying mucosa is more susceptible to attack by bacteria. Much of the oral intake of threonine is used for mucin production. A threonine-deficient diet will lead to a reduced number of acidic mucin-producing goblet cells in the small intestine. The abnormalities in mucus resulting from the impaired oral intake of threonine can be prevented through parenteral administration of this amino acid^[133].

The mucus layer over the intestinal epithelium acts as a barrier, retarding the access of bacteria and bacterial products to the BBM. Goblet cells secrete mucin 2 (MUC2), a glycoprotein with high density and viscoelasticity. MUC2 contains large amounts of threonine and proline. Studies determining the fractional synthetic rate of MUC2 in human neonates have shown rapid incorporation of systemic threonine into MUC2^[134].

Premature infants are at increased risk of developing necrotizing enterocolitis (NEC). The initial colonization of the intestine by bacteria, and the use of enteral feeds, contribute to the development of NEC. The maldigestion of carbohydrate may also be a risk factor^[135]. This is possibly because maternal diets are high in n-6/n-3 fatty acids. These dietary lipids are epigenetic factors that contribute to the increased incidence of ischemia by altering the composition of amniotic fluid and intestinal membrane structural lipid essential fatty acids^[136].

There is a wide range of mortality rates of infants with NEC, depending on the number of risk factors (e.g., prematurity, use of feeding formula, bacterial colonization of the GI tract), as well as the extent of the damage to the bowel (inflammation, necrosis, perforation, peritonitis, sepsis, and systemic inflammatory response). In the healthy child, there is high blood flow and low resting vascular resistance. One mechanism of development of NEC is reduced blood flow in the arteries penetrating the wall of the intestine, as well as the submucosal arterial plexus.

NEC is a major cause of death from GI disease in neonates. The mucosal damage and increased intestinal permeability lead to translocation of enterobacteria, activation of macrophages in the lamina propria, and initiation of a systemic inflammatory response. NO is released in a response to cytokine induction of the enzyme iNOS. Restitution is regulated in part by the Rho family of small-molecular-weight GTPases. NO inhibits enterocyte migration through protein tyrosine phosphatase (SHP-2)^[137].

Intestinal barrier function can also be altered as a result of impaired intestinal restitution. This restitution is decreased in, for example, hemorrhagic shock, sepsis, intestinal ischemia and NEC. The increase in intestinal permeability that occurs with stress and the presence of viable nonpathogenic *E. coli* is exaggerated by TNF- α release from macrophages^[138]. The proinflammatory mediators IL-18 and TNF- α are involved in the development of NEC. As noted by these authors, this raises the possibility that “...anti-TNF- α could be used as a potential therapy for human NEC”^[139]. The use of TNF- α modifiers may also be effective to normalize *E. coli*-associated increased intestinal permeability. It is unknown whether the inhibition of membrane or soluble TNF- α may be used clinically to prevent or to treat NEC.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is produced by epithelial cells, where it decreases apoptosis, preserves the integrity of the cytoskeleton, and rebuilds depleted intracellular stores of ATP. In newborn rat pups, HB-EGF maintains the blood flow of the microcirculation that normally would be decreased in NEC^[140]. Histological abnormalities correlate with changes in blood flow, and less histological damage occurs with the use of HB-EGF. This may prove to be a useful preventive or therapeutic agent in newborn human infants who are at risk of developing NEC.

The probiotics lactobacilli and bifidobacteria may help to prevent the development of NEC. TLRs on the

intestinal BBM are PRRs that identify components of the usual intestinal microbiota. TLR4 recognizes the Gram-negative LPS, which is increased in NEC. The TLR changes occur before the histological changes, suggesting that the activator of the inflammatory response through TLRs may be causative in the development of NEC^[141].

As a result of the importance of the formation of the microbiota in the development of NEC, the possibility has been raised for the therapeutic potential of altering the bacterial contents by the use of probiotics^[142]. Feeding premature rats the probiotic *Bifidobacterium bifidum* reduced the prevalence of NEC from 57% to 17%. This dramatic protective effect was associated with reduced and indeed normalized levels of proinflammatory IL-6, trefoil factor 3 and mucin-3, as well as TJ and AJ proteins^[143].

The release of bacterial LPS from enterobacteria associated with NEC increases the enterocyte expression of integrin. Integrin leads to adhesion of the enterocyte to the underlying matrix and healing of the damaged mucosa by the migration process of restitution. Dystroglycans (DGs) are a component of the dystrophin-glycoprotein complex that are involved in integrin activation in the intestinal epithelium^[144]. With restitution, the migrating cells transiently adhere to the underlying matrix, reducing fibrillar adhesions containing a structural core of transmembrane integrins. These integrins provide anchors (adhesion complexes) and also sensors for mechanical events, as well as signal transducers. Removal of neurocytes requires disassembly of the integrin-based adhesions structures. β 1-Integrin is endocytosed *via* a dynamin-dependent lipid-raft-mediated pathway that helps to regulate intestinal epithelial cell migration and wound closure^[145].

The bacterial ligand activated TLRs signal intracellularly to upregulate both pro- and anti-inflammatory cytokines. There are several genes induced in the inflammatory process such as cytokines, chemokines and adhesion molecules. A factor that is central to the regulation of these various genes is NF- κ B. For example, NF- κ B is induced in NEC. Platelet activating factor (PAF) is an endogenous proinflammatory phospholipid that rapidly activates NF- κ B. PAF also stimulates neutrophils to produce the chemokine that used to be known as macrophage inflammatory protein (MIP)-2, now called CXCL₂. The bowel injury produced by PAF is mediated through the processing of the NF- κ B p105 into NF- κ B p50^[146].

PAF induces Cl⁻ channel activation associated with intracellular acidosis. The Cl⁻ channel activation occurs through caspase-3 activation and DNA fragmentation, as well as by apoptosis^[147]. In NEC, PAF and accelerated apoptosis of enterocytes results in tissue necrosis. Inhibition of caspase activation prevents the progression of disease in a neonatal rat model of NEC. Palmitoylation is a post-translational modification that is necessary for efficient signal transduction by many G protein-coupled receptors (GPCRs). Nutrients in the intestinal lumen may be sensed. For example, free fatty acids lead to release

of peptide YY. This affects GPCRs, thereby modifying cell proliferation and differentiation as well as hormonal secretion^[148]. Inhibition of the PI3K/Akt signaling pathway is the main mechanism of PAF-induced apoptosis in enterocytes. Polyunsaturated fatty acids block this mechanism early in the signaling cascade, probably by way of an effect on protein palmitoylation^[149].

After a period of bowel ischemia, the reperfusion of blood actually increases damage to the tissue. As oxygenation of the reperfused tissue occurs, free radicals are produced, MCs degranulate, leukocytes adhere to the endothelial wall, and thus ischemia/reperfusion (I/R) damages especially the mucosal cell layer and villous structure, as well as mucosal permeability and mitochondrial activity. 3,4-Dihydroxy-phenyl lactic acid reduces the production of peroxide and the expression of adhesion molecules in neutrophils and thereby minimizes the disturbance in the microcirculation^[150]. If the initial period of ischemia is short (about 15 min in mice), these adverse effects may reverse, but in longer periods of ischemia, the changes in structure and function are irreversible^[151].

During hypoxia-associated inflammation, endogenous adenosine is protective. In hypoxia, the levels of equilibrative nucleoside transporter-2 (ENT2) falls in human epithelial cells^[148], whereas hypoxia-inducible-factor-12-dependent repression of ENT2 increases mucosal adenosine signaling, thereby reducing the hypoxia-associated inflammation of the intestine. During hypoxia, the actin cytoskeleton is aggregated in endothelial, epithelial and vascular smooth muscle cells. This aggregation results from the disposition of IgM and complement (C3), causing further tissue injury^[152].

IMMUNOLOGY/ALLERGY

Immune-associated disease is of epidemic nature in postindustrial society. For example, adverse reactions to food are common, and true food allergies are believed to affect about 7% of children under the age of 10 years, and 2% of the adult population. These immune reactions are mediated by IgE-dependent or -independent mechanisms involving MCs, eosinophils, and other immune cells^[153].

The intestinal immune system includes Peyer's patches, isolated lymphoid follicles, mesentery lymph nodes, lamina propria mononuclear cells, and intraepithelial lymphocytes. The human intestine has unique natural killer (NK) cells derived from hemopoietic stem cells in the bone marrow. These NK cells contribute to maintenance of immune homeostasis in the intestine^[154].

Chemokines and their receptors control the influx of T and B cells into the Peyer's patches of the intestine. There is different expression of chemokines along the length of the small intestine^[155].

The M (microfold) cells are a clonal population derived from a single stem cell in the small intestinal crypts, within the follicle-associated epithelium of the Peyer's patches. M cells transport antigens and microorganisms

into the underlying lymphoid tissues. Mature enterocytes may convert to the M cell phenotype under the influence of either T and B lymphocytes or microorganisms^[156].

Macrophages in the intestinal tract are important to host defense by way of recognizing, phagocytizing and killing microorganisms. Classically, activated Th1 immune response macrophages are major effector cells in the Th1 immune responses. These mediate the production of NO by inducing NOS-2. Alternatively, activated macrophages are induced by T helper (Th)2 cytokines, including IL-4 and IL-13, as well as signal transducer and activator of transcription 6^[157].

Under normal circumstances, the gut immune system is poorly responsive to food allergens and to commensal bacteria. This hyporesponsiveness occurs through a process of immune tolerance. The Th2 phenotype is involved in development of allergic disease through release of IL-4, IL-5 and IL-13. These cytokines play a role in antigen-specific IgE production by B cells. IL-4, IL-5 and IL-13 are also important in mucous secretion, muscle contraction and eosinophilia.

IL-4 is the key Th2 cytokine involved in switching antibody responses to IgE. IL-4 induces the expression of IgE receptors on MCs, as well as on other cell types. IL-4 gene transfer to the small bowel serosa leads both to intestinal inflammation and smooth muscle hyperresponsiveness^[158]. Sensitization induces expression of CD23 on intestinal epithelial cells. This CD23 enhances IgE-dependant transepithelial antigen uptake by encoding a functional IgE receptor on human intestinal epithelial cells^[159].

A localized Th2 milieu has been observed in the intestine of subjects with food allergic disorders. A gut-homing phenotype is induced by mesenteric lymph node, with selective upregulation of the Th2 chemoattractants^[160]. TGF β is an immunoregulatory cytokine that is constitutively expressed in the intestine by epithelial cells, fibroblasts and lamina propria mononuclear cells. As noted by Di Sabatino *et al.*^[161], "one of the paradoxes of human mucosal T-cell responses in health is the dominant Th1 profile and interferon γ (IFN γ) secretion of normal human gut T cells".

It is of interest that there is a link between intestinal immunology and motility. The Th2 cytokines are implicated in intestinal muscle dysfunction following tissue injury. Products of the innate and adaptive immune responses contribute to the activation and sensitization of primary sensory neurons. T lymphocytes are an important determinant of visceral nociception, and may provide an important opioid-mediated antinociceptive influence in the gut^[162].

Cell surface molecules are required for development of intestinal allergy^[163]. Disruption of the enteric glial cell (EGC) network is associated with intestinal and mesenteric T-cell infiltration, leading to Th1 cytokine-associated bowel inflammation. EGCs express major histocompatibility complex (MHC) class I, and regulate MHC class II in persons with bowel inflammation. CD8 and CD4 T

cells trigger autoimmunity. Direct presentation of antigen by lymph node stromal cells protects against CD8 T-cell-mediated intestinal autoimmunity^[164].

CD23 is a tight II intercoil membrane glycoprotein with a carboxy terminal C-type lectin head that binds its ligand, IgE, in a calcium-dependent manner. CD23a is expressed constitutively on mature activated B cells, whereas CD23b is induced by IL-4 or IL-23. CD23a is increased in food-allergic patients, and CD23a is expressed by primary human IECs. The increase in antigen uptake by specific IgE is through the process of diverting antigen from delivery to lysosomes. Delivery of antigen-IgE complexes across the intestinal epithelial barrier may induce deactivation of MCs, and contribute to food-induced pathophysiology of the GI tract^[165].

The *in vitro* treatment of smooth muscle tissue with IL-1 β decreases expression of CPI-17. Smooth muscle serine-threonine protein phosphorylases are endogenous inhibitory proteins. This TNF- α -mediated process inhibits muscle contraction in the small intestine^[166]. It is not yet known whether intestinal motility may be modulated for therapeutic purposes by immune alterations.

The vagus nerve regulates GI motor and digestive functions through the release of acetylcholine (ACh). This parasympathetic neurotransmitter activates mAChRs, the nicotinic ACh receptors. The afferent vagal nerve, working through the hypothalamic pituitary adrenal axis, acts to inhibit the activation of NF- κ B. This reduces proinflammatory cytokine secretion by macrophages, as well as modifying macrophage endocytosis and phagocytosis. Thus, "vagus nerve efferent activity may stimulate surveillance in the intestinal mucosa and peritoneal compartment^[167]". This role of the vagus nerve awaits study as a potential therapeutic target in persons with allergies.

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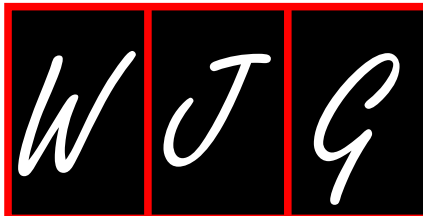
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Recent advances in small bowel diseases: Part II

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Abstract

As is the case in all areas of gastroenterology and hepatology, in 2009 and 2010 there were many advances in our knowledge and understanding of small intestinal diseases. Over 1000 publications were reviewed, and the important advances in basic science as well as clinical applications were considered. In Part II we review six topics: absorption, short bowel syndrome, smooth muscle function and intestinal motility, tumors, diagnostic imaging, and cystic fibrosis.

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Key words: Absorption; Cystic fibrosis; Diagnostic imaging; Intestinal motility; Short bowel syndrome; Smooth muscle function; Tumors

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ABSORPTION

Triglycerides

For triglycerides (TG), it has been traditionally considered that lipid uptake is by way of passive permeation through the lipophilic portion of the intestinal brush border membrane (BBM). However, enterocyte-binding or transport proteins have been identified as also being important in this process^[1]. Gata4 is a zinc-containing transcription factor, expressed in the epithelium of the upper small intestine, and functions to assist in fat and cholesterol absorption^[2]. Lipid micelles at the BBM modulate a large number of genes, and this transcriptome responding to dietary lipids has an impact on cell architecture, signaling and metabolism genes^[3]. Most lipids are in the enterocyte, and may be bound to the liver and the intestinal fatty acid (FA) binding proteins (L-FABP and I-FABP). L-/I-FABP function to translocate long chain FAs and monoacylglycerol from the BBM to the endoplasmic reticulum (ER). These long FAs are then used in the resynthesis of diacylglycerol and then triacylglycerol.

The absorption of dietary TG in the small intestine is accompanied by a rise of intestinal alkaline phosphatase (IAP) in the serum, and by the secretion of IAP-containing surfactant-like particles (SLPs) from the enterocytes. IAP is a membrane-bound protein that hydrolyses monophosphate esters at high pH optimum, and limits fat absorption by enterocytes by way of its action as a SLP^[4]. Translocation of IAP across the enterocyte BBM occurs within 5 min of lipid intake by way

of induction of endocytosis *via* clathrin-coated pits^[5]. After fat has been taken up into the enterocyte, IAP is incorporated into membranes surrounding intracellular lipid droplets, and is also incorporated into basolaterally secreted SLPs. IAP is not associated with chylomicron formation, but rather with chylomicron secretion. Serum IAP levels are correlated with levels of apolipoprotein B-48 (apoB48), a protein exclusive to intestinal chylomicrons in humans^[6].

After ingestion of a meal rich in TG, the small intestine continues to form very low density lipoprotein (VLDL), but the predominant TG-rich lipoprotein particles secreted in this postprandial condition are the larger chylomicron particles^[7]. In the liver, TG is synthesized and packaged with apoB100 to form VLDL particles, whereas chylomicrons produced by the human gut contain apoB48. ApoB48 provides efficient chylomicron formation and lipid absorption. Apolipoprotein A-IV synthesis in the small intestine is regulated by lipid absorption, and plays a role in the regulation of chylomicron assembly and secretion.

Hepatocyte nuclear factor-4 α (HNF-4 α) is a nuclear receptor that regulates gene expression during enterocyte differentiation. HNF-4 α is also involved with the transcription of genes involved in lipid metabolism, such as *Apo-IV*^[8]. In newborn swine intestine, dietary lipid causes ligand-independent transactivation of HNF-4 α to induce Apo-A IV and microsomal triglyceride transfer protein (MTP).

The uptake of FAs across the BBM may be partially passive and partially facilitated, mediated by the multiligand scavenger protein CD36. CD36 also participates in the orosensory detection of lipids and the production of the sensation of satiety. Thus, CD36 may play a role in lipid preferences and feeding behaviour^[9]. Monoglyceride and free FAs in the cytoplasm reform TG by the successive actions of monoacylglycerol acyltransferase and diacylglycerol acyltransferase at the membrane of the smooth ER. After transfer in the ER lumen, TG droplets associate with primordial lipoprotein comprising apoB48 and phospholipids through the actions of MTP, to form TG-rich lipoproteins (TRL). The lipid droplets fuse with apoB48 plus a resident ER chaperone, MTP. MTP-dependent fusion of lipid droplets with apoB48 in the ER is the crucial restriction point in the formation of chylomicrons. The lipoprotein particle enlarges as more TG is added to the droplet. The maturing lipoprotein particles (prechylomicrons) undergo vectorial vesicular transport through the Golgi membranes. Chylomicrons cross the basolateral membrane (BLM) and into the lacteals.

Glucagon-like peptide-2 (GLP-2) increases lipid absorption, but how does this occur, when enterocytes have no GLP-2 receptors? Perhaps the GLP-2 acts on the enteroendocrine L cells, releasing insulin-like growth factor (IGF-1). GLP-2 increases the glycosylation of CD36 and increases the number of chylomicrons.

After 1 wk of feeding with a high fat diet (HFD) in mice, there is repression of genes involved in FA syn-

thesis, and an increased expression of genes involved in lipoprotein assembly (*apoB*, *MTP*, *apoA-IV*). This process may be coordinated by an increase in the transcription factor SREBP-IC^[10]. The number of secreted chylomicrons falls, but there are larger chylomicrons containing increased associated TG, as well as increased amounts and activity of MTP. These changes result in postprandial hypertriglyceridemia, but normal fasting levels of TGs. This postprandial hypertriglyceridemia in the absence of changes in fasting levels may explain some of the risk factors for the development atherosclerosis and cardiovascular diseases.

Cholesterol

Dietary and biliary cholesterol are solubilized by bile acid micelles in the upper intestinal lumen. These are large negatively-charged unilamellar vesicles, smaller mixed micelles or monomeric bile acids. Bile acids promote cholesterol absorption and reduce cholesterol synthesis^[11].

It is now recognized that intestinal absorption of cholesterol is a complex process, involving both BBM permeation and cotransporters^[12,13]. Uptake of cholesterol from the intestinal lumen across the enterocyte BBM is also mediated by at least five proteins: Niemann-Pick C1-like 1 (NPC1L1), the scavenger receptor B-1 (SR-B1), CD36, the ATP-binding cassette protein 5 (ABCG5) and ATP-binding cassette protein 8 (ABCG8) ATP-binding cassette transporters^[14,15]. NPC1L1 protein is predominantly expressed in the liver and in the proximal intestine^[16]. Modulation of NPC1L1 expression is by cholesterol, as well as by the involvement of several nuclear receptors, such as liver X receptor (LXR), peroxisome proliferator-activated receptor (PPAR)- α , and by sterol regulatory element (SRE) binding proteins (SREBPs). SREBPs are transcription factors which regulate cholesterol synthesis and metabolism^[17]. SREBP-2 activates the NPC1L1 promoter, which has two sterol regulatory elements.

The ATP-binding cassette transporter ABCG1 promotes cholesterol efflux across the BLM and out of the enterocyte. In contrast, ABCG5/G8 facilitates cholesterol efflux back across the enterocyte BBM and into the intestinal lumen^[18]. The ATP-binding cassette transporters are target genes of the nuclear receptor LXR. Mice on a high-fat cholesterol free diet have reduced or downregulated NPC1L1, ABCA1, ABCG5, and ABC8, reduced fractional cholesterol absorption, and a posttranslational increase in 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity. Downregulation of cholesterol transporters is independent of LXR A^[19].

NPC1L1 also occurs in intracellular compartments, and is involved as well in the absorption of dietary saturated FAs such as steric and palmitic acids^[20]. The drug ezetimibe binds NPC1L1, reduces intestinal absorption of cholesterol as well as saturated FAs, and reduces weight gain in animals fed a diabetogenic diet. In this way, the drug may protect against diet-induced hyperglycemia and insulin resistance^[20]. NPC1L1 and the FA translocase (FAT/CD36), as well as scavenger receptor class B type 1 (SR/B1)

transporter protein, have been shown to be influenced by 5 mmol/L glucose in the intestinal lumen; enhancing protein expression of NPC1L1 and CD36, decreasing SR/B1 protein, but having no effect on the protein expression of ABCA1 and ABCG8^[21]. Higher intraluminal glucose concentration increases 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity, increasing the transcription factors LXR- α and LXR- β , PPAR- β and PPAR- γ , as well as SREBP2. Thus, reducing the luminal concentration of glucose will also reduce uptake of cholesterol.

Aging enhances cholesterol absorption by suppressing expression of the sterol efflux transporters ABCG5/G8. In contrast, estrogen enhances cholesterol absorption due to upregulated expression of NPC1L1, ABCG5 and ABCG8^[22]. Cholesterol absorption is also enhanced in diabetes; medium levels of glucose concentration in Caco-2 cells in culture increase cholesterol uptake as well as the expression of NPC1L1 and CD36 proteins^[21].

Liver FA binding protein (L-FABP) increases FA uptake, intracellular transport, esterification, and oxidation in transfected transformed cells, and gene-ablated mice with no L-FABP show reductions in these steps of FA metabolism^[23]. L-FABP may also play a role in hepatic cholesterol metabolism^[24].

Phytosterols are cholesterol-like compounds found in plants, which reduce cholesterol absorption and plasma concentrations of low density lipoprotein cholesterol. Natural phytosterol glycosides purified from lecithin are bioactive in humans^[25].

Two Na⁺-coupled (SMIT1 and SMIT2) and one H⁺-coupled (HMIT) secondary active intestinal transporters for myo-inositol have been identified^[26].

One hypothesis suggests that cholesterol is absorbed by an energy independent passive diffusion process regulated *via* a concentration gradient^[14]. The second hypothesis proposes that cholesterol is absorbed through an energy-dependent, protein-mediated process^[27].

NPC1L1: NPC1L1 is the main cholesterol transporter in the jejunal BBM^[16]. NPC1L1 shares 42% amino acid homology with Niemann-Pick type C1 protein (NPC1), a protein involved in the intracellular transport of cholesterol^[28]. Post-translationally, NPC1L1 moves from internal membranes to the mucosal membrane during cellular cholesterol depletion, facilitating absorption^[29]. Other studies suggest that NPC1L1 is located at the BBM of enterocytes^[30]. NPC1L1 mRNA expression appears to be positively correlated with plasma apoB48 and chylomicron cholesterol content^[31].

Scavenger receptor B1: Scavenger receptor B1 (SRB1) is highly expressed in the BBM of the proximal small intestine^[32]. Intestinal SRB1 overexpression in transgenic mice has been associated with increased cholesterol absorption^[33]. Moreover, antibodies against SRB1 demonstrate abolishment of high affinity binding of cholesterol to BBM vesicles that would normally be observed in *NPC111/-* mice^[32]. SRB1 may play a role in the initial step of cho-

lesterol absorption by facilitating high affinity cholesterol binding to the mucosal BBM, but alternative cholesterol transporters may compensate for the absence of SRB1 in mediating cholesterol absorption in KO models^[32].

FAT/CD36: FAT/CD36 (translocase), a human analogue of SRB1, is expressed along the BBM of the duodenum and jejunum. CD36 deficiency correlates with abnormal lipid processing in enterocytes^[14].

ABCG5/8: ABCG5 and ABCG8 are located at the enterocyte BBM^[14]. Their expression is greatest in the duodenum and jejunum, where they work in tandem to efflux cholesterol (mainly plant sterols) from the enterocyte back into the lumen for excretion^[34]. A negative correlation exists between ABCG5/8 and chylomicron cholesterol content^[31]. Mutations of *ABCG5* and *ABCG8* in humans enhance intestinal cholesterol absorption, and predisposes these individuals to atherosclerosis^[35].

ATP-binding cassette protein 1: ATP-binding cassette protein 1 (ABCA1) not only mediates cholesterol efflux from the basolateral surface of enterocytes to high-density lipoprotein^[36], but it also contributes to the efflux of cholesterol out of the enterocyte and back into the intestinal lumen^[37].

Bile acids

Bile acids are synthesized from cholesterol in the liver, secreted into the bile ducts, stored in the gallbladder, and intermittently released into the duodenum in response to a meal, where bile acids solubilize lipids in the intestinal lumen by formation of micelles^[38]. Bile acids dissociate from the lipids which they stabilize prior to the uptake of the lipids across the BBM of the proximal intestine. The bile acids are absorbed passively along the length of the small intestine. In the ileum, enterocyte BBM sodium-dependent bile acid transporters (ASBTs) also mediate bile acid uptake and bile acids are returned to the portal circulation. This is known as the “enterohepatic” circulation of bile acids. ASBT is, in effect, a salvage mechanism for luminal bile acids, providing for maintenance of cholesterol homeostasis, as well as for efficient lipid absorption.

The apical ASBT in the lipid rafts of the ileal BBM functions in concert with hepatic bile acid efflux transporters to regulate hepatic bile acid synthesis from cholesterol. One of the green tea catechins decreases the maximal transport rate (V_{max}) of ASBT, without altering its content in the BBM. This reduction in V_{max} is achieved by moving the transporter out of the lipid rafts^[39]. This suggests a role for lipid rafts in the modulation of the function of this transporter, reducing the size of the bile acid pool, stimulating the hepatic synthesis of bile acids from cholesterol, and thereby reducing the serum concentration of cholesterol.

Initially, ASBT in the enterocyte cytosol undergoes vesicular trafficking to microdomains in the BBM. These

ASBT lipid rafts are enriched with sphingolipids and cholesterol. Alterations in cholesterol content of the BBM lead to rapid modulation of the activity of ASBT^[40]. Obstructive cholestasis leads to downregulation of ASBT mRNA expression. Thus, luminal bile acid levels may be involved in regulation of ASBT gene regulation^[41]. In patients with ileal inflammation, such as Crohn's disease, reduced bile acid transport may be due to diminished ASBT protein expression, as the result of ASBT inhibition by inflammatory cytokines *via* direct interactions of c-fos with the ASBT promoter^[42].

Once bile acids are in the ileal enterocytes, they bind to ileal bile acid-binding protein (I-BABP). Organic solute transporters (Ost) α and β are located in the BLM of the ileocytes. Ost α /Ost β expression is induced by bile acids through ligand-dependent transactivation of both Ost genes by the nuclear bile acid receptor/farnesoid X receptor (FXR)^[43]. "By coordinated control of Ost α /Ost β expression, bile acids adjust the rate of efflux from enterocytes in response to changes in intracellular bile acid levels". Ost α is a seven transmembrane domain protein, and Ost β is a single transmembrane domain polypeptide. Ost α -Ost β is the major BLM transporter of bile acids and conjugated steroids in the intestine, as well as in the kidney and liver^[44]. Ost α and Ost β promoters harbor both FXR and liver receptor homolog-1 (LRH-1) elements. FXR and LRH-1 mediate positive- and negative-feedback regulation, respectively^[45].

When the BBM uptake of bile acids is impaired, excess bile acids spill into the large intestine, where bile acids stimulate cAMP and cause a secretory diarrhea. The locally-acting steroid budesonide is beneficial for the symptoms of collagenous colitis, which in turn is associated with bile acid malabsorption. This clinical benefit may be due in part to stimulation of bile acid absorption, with decreased bile acids entering the colon, less stimulation of cAMP, and less secretory diarrhea^[46].

Glucose-dependent insulintropic polypeptide (GIP) is a potent insulin secretagogue. GIP is an incretin, a gut factor released after intestinal transport of hexoses, long-chain FAs and TG, and GIP stimulates insulin secretion at physiological concentrations. GIP is secreted by enteroendocrine K cells in the proximal small intestine. Intestinal lymph contains high concentrations of GIP that respond to both enteral carbohydrate and to fat absorption. The combination of glucose and lipid has a potentiating effect on stimulation of GIP secretion in lymph fistula rats^[47].

Approximately 25% of individuals with irritable bowel syndrome (IBS) have a previous history of enteric infection, such as with *Campylobacter* or *Salmonella*. Persistent chronic diarrhea is more frequently associated with infectious IBS, and bile acid malabsorption may be observed in as many as a third of patients with diarrhea-predominant IBS. In a mouse model of IBS, it was shown that ileal uptake of bile acids was reduced. Surprisingly, this was associated not with a decrease but rather with an unexpected increase in expression of the

BBM Na⁺-dependent bile acid transporter (ASBT)^[48].

Bile acids act as detergents to solubilize lipids, but also act as signaling hormones: bile acids activate the G-protein-coupled receptor TGR5, resulting in changes in energy expenditure and glucose homeostasis, as well as having an anti-inflammatory role. Novel patent and selective bile acid derivatives are being developed as TGR5 agonists for possible therapeutic enhancers^[49].

Bile acids are synthesized from cholesterol. In the neutral pathway, the rate-limiting enzyme β hydroxylase (Cyp7a7) converts cholesterol to 7-hydroxycholesterol. In the attenuated acidic pathway in mitochondria, sterol 25-hydroxylase or 27-hydroxylase hydroxylates the cholesterol, and a β hydroxyl group is added from catalysis by oxysterol β hydroxylase (Cyp7b1). The ring structure is then modified, and the side chain is oxidized and shortened, and further hydroxylation occurs to form the primary bile acids, cholic and chenodeoxycholic (chenic) acid. Bile acids regulate their own synthesis by way of negative feedback on the transcription of the rate-limiting enzyme, Cyp7a1. When bile acid concentrations are high, there is activation of the nuclear FXR, which leads to increased transcription of short heterodimeric partner (SHP). Cyp7a1 is activated by the SHP-dependent as well as by the SHP-independent pathway.

The small size of the bile acid pool in neonates is increased as the result of elevated mRNA levels of FXR and SHP, and later by an increase in mRNA and protein levels of Cyp7a1^[50]. The increase in Cyp7a1 levels and therefore the increased synthesis of bile acids occurs independently of FXR and SHP, and is not influenced by the administration of sterols^[50].

Gangliosides

Gangliosides are sialic acid-containing glycosphingolipids which are found in lipid rafts in outer plasma membranes, such as the BBM of the small intestine. The oligosaccharide portion of the ganglioside faces the cell surface, whereas the lymphatic ceramide portion is anchored into the inner (cytosolic side) layer of the BBM. In the rat intestine, 34% of the glycosphingolipids are gangliosides. The amount of ganglioside in the membranes varies along the intestine, being higher distally than proximally. Gangliosides differ depending upon whether ingested in micelles or unilamellar vesicles. GM3 is localized on the BBM whereas GD3 is mainly localized on the BLM. GD3 uptake into Caco-2 cells is greater across BLM than BBM, and gangliosides taken up by the BLM are largely metabolized by these enterocyte-like cells^[51]. In contrast, GD3 uptake across the BBM is time- and concentration-dependent, reaches a plateau, and the GD3 is metabolized, stored, or transported out of the cell across the BLM. GD3 is found in milk and colostrum, and feeding GD3 increases its content in the intestinal lipid rafts, and in the blood membrane: the main ganglioside in the BBM is GM3, whereas GD3 is the main ganglioside in the BLM. This raises the possibility of the oral use of gangliosides to modify or to enhance some of their functions,

such as regulating cell signaling, protein functions, as well as the recognition of microbes and macromolecules.

Sugars

SGLT1, the Na⁺-glucose cotransporter in the enterocyte BBM, is a secondary active transport process which requires a favorable intracellular Na⁺ gradient. This gradient is provided by Na⁺-K⁺-ATPase on the BLM of enterocytes. Constitutive nitric oxide (cNO) has opposite effects on the two primary Na⁺-absorptive pathways in the mammalian small intestine: reducing cNO inhibits SGLT1 and stimulates the Na⁺/H⁺ hydrogen exchanger NHE3^[52]. cNO also regulates mucosal blood flow, mucous secretion, and intestinal motility. The glucocorticoid-inducible kinase-1 (SGK1) stimulates SGLT1 as well as NHE3. The effects of glucocorticoid on SGLT1 are fully dependant on SGK1, whereas for NHE3 the effects of glucocorticoids also involve some additional processes^[53].

During chronic intestinal inflammation, there is a transcription-mediated decrease in the number of glucose transporters. This is possibly due to altered binding of Sp1 and Hnf1, transcription binding sites for the SGLT1 promoter regions^[54].

When glucose is taken by mouth, there is a fast rise in expression of SGLT1. Intestinal sugar uptake is increased in diabetes and in obesity. Roux-en-Y gastric bypass (RYGB) is a successful form of bariatric surgery. RYGB reduces glucose absorption in the Roux limb, as well as in the remaining intestine^[55].

Fructose is prevalent in the diet either as a free hexose, as the disaccharide sucrose, and in the polymerized form, fructans. About 50% of adults are unable to absorb a 25 g load of fructose. Fructans are neither hydrolyzed nor absorbed in the small intestine. This osmotic load may alter intestinal motility and change the microbiota by producing a mucosal biofilm. Restricting dietary intake free of fructose and/or fructan has symptomatic benefits in some persons with diarrhea and bloating^[56].

The revised SLC Transporter Gene Tables are available online at <http://www.bioparadigms.org/slc/intro.htm>.

Carbohydrate malabsorption, as assessed by hydrogen breath testing, is common in persons with Crohn's disease (CD) and celiac disease (CeD)^[57]. The absolute increase in the rate of fructose malabsorption is about 20% higher in Crohn's disease, and lactose malabsorption is 30% higher.

The BBM hydrolysis of carbohydrates takes place by the BBM-bound glycoproteins sucrase-isomaltase (SI), maltase-glucoamylase, and lactase-phlorizin hydrolase (LPH). The pro-SI passes from the ER to the Golgi apparatus. With glycosylation it becomes targeted to the BBM, where it is cleaved by trypsin to form sucrase and isomaltase. Compound heterozygous mutation defects in the protein folding, the direct interaction between sucrase and isomaltase, and an intermolecular chaperone included in the intracellular transport of SI, all have a role in the development of congenital sucrase-isomaltase

deficiency^[58]. Congenital lactase deficiency results from mutations in the coding region of LPH, with misfolding of LPH which prevents the mutant protein from exiting the ER^[59].

Amino acids and proteins

The numerous BBM transporters for amino acids are differentiated functionally by their substrate specificity and driving forces. Neutral amino acids are transported by the system B0+ (Na⁺-dependent transporter for neutral and cationic amino acids), as well as by the ASC system (Na⁺-dependent transporter for mid-size neutral amino acids).

Glutamine comprises approximately 20% of the total amino acid content in the human blood stream, and as such is an important amino acid. Glutamine is the preferred substrate for enterocytes, and is also important for mucosal integrity and the intestinal permeability barrier. The Na⁺-glutamine cotransporter in the BBM of the enterocyte is B0AT1 (SLC6A19)^[60]. Glutamine is converted to citrulline in the enterocytes. A citrulline generation test has been developed to assess enterocyte function, and the value of the slope from baseline to peak plasma citrulline concentrations is reduced in persons with celiac disease^[61].

Under the influence of cholecystokinin (CCK), bile and pancreatic enzymes are secreted into the duodenal lumen where the pancreatic proteolytic enzymes (trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase D) digest proteins and polypeptides into peptides, which are usually 2-6 residues in length. Conjugated bile acids accelerate protein hydrolysis by pancreatic proteases^[62].

During chronic intestinal inflammation, there is a decrease in the activity of several transporters such as the short-chain FA-bicarbonate exchanger, H⁺-dipeptide cotransporter, Na⁺-amino acid transporter, Na⁺-glucose cotransporter 1 (SGLT-1), and Na⁺-bile acid transporter. There may be a decreased number of SGLT -1 transporters in villus cells (lowering the value of the maximal transport rate, V_{max}), and decreased affinity of the cotransporter for Na⁺-neutral amino acid transport (increasing the value of the affinity constant, K_m).

For amino acids, the reduction in transport during chronic inflammation arises from a decrease in the affinity of the transport systems, and may be mediated through an increase in leukotriene D4 (an eicosanoid pathway product), which is released in chronic inflammation^[63].

The proton-amino acid transporter 1 (PEPT1) transports small neutral amino acids as well as small peptides, through mediation of an inwardly directed H⁺ gradient across the enterocyte BBM. PEPT1 also transports drugs such as β-lactam and angiotensin-converting enzyme inhibitors. PEPT1 is under diurnal variation, relating to food intake. It is also influenced by transcription factors, such as Sp1, Cdx2, and PPAR-α. Leptin treatment increases enterocyte uptake of di- and tripeptides *via* the PepT1 transporter, through transcription activation of

the MAPK pathway as well as translational activation *via* ribosomal protein S6^[64].

The albumin D site-binding protein (DBP) expression is regulated in a circadian manner by oscillators called “circadian clocks”. These circadian clocks reside in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. This clock system consists of single-cell circadian oscillators that are composed of several clock genes. The expression of DBP is in phase with that of *PEPT1*. DBP binds to the DBP binding site in the distal promoter region of the *PEPT1* gene, and thereby induces transcriptional activity^[65].

The *SLC6A19* gene encodes the main sodium-independent BBM transporter for neutral amino acids, B0AT1. The expression of B0AT1 requires angiotensin-converting enzyme 2 (ACE2)^[66]. It is unknown whether the use of inhibitors of ACE2 in humans alters the protein homeostasis of the body by way of inhibiting the intestinal uptake of neutral amino acids in the small intestine (as well as in the proximal tubule of the kidney).

Small peptides are absorbed predominantly in the proximal small intestine, and free amino acids in the distal intestine. The uptake of sugars is increased by a high carbohydrate diet by upregulation of the Na⁺-dependent BBM glucose transporter SGLT, and the amino acid alanine also controls its own absorption, through capsaicin-sensitive primary afferent neuronal fibers as well as by the peptide calcitonin gene-related peptide, CGRP^[67].

Glutamines become conditionally essential during metabolic stress. Glutamine prevents apoptosis and also plays a role in regulating glucose metabolism. For example, during fasting as well as in diabetes, the intestine by way of its glucose-6 phosphatase (G6Pase) contributes about a quarter of endogenous glucose production through gluconeogenesis.

After a RYGB, used to treat severe obesity, the glucose sensing vagal afferents in the portal vein influence glucose homeostasis. After RYGB, the absorption of glutamine by B0AT is increased in both the biliopancreatic (3.8-fold increase) and the Roux limbs (1.4-fold increase), but not in the common channel. The levels of glutaminase are also increased, but the levels of GEPase (intestinal gluconeogenesis) and PEPCK-C (cytosolic phosphoenolpyruvate carboxykinase, a measure of glutamine metabolism) were not seen to be affected^[68].

Biotin

Biotin is a coenzyme for the “carboxylases” which catalyze essential steps in FA biosynthesis, gluconeogenesis, and catabolism of several amino acids and FAs. Biotin is essential for cellular functions including growth and development. The human intestine utilizes the sodium-dependent multivitamin transporter (hSMVT) for biotin uptake across the enterocyte BBM^[69]. The uptake process is adaptively regulated during biotin deficiency, by induction of protein and mRNA levels of hSMVT, mediated by transcriptional regulatory mechanisms.

Two other functionally unrelated nutrients, the water-

soluble vitamin pantothenic acid and the metabolically important antioxidant lipoate, share the biotin transport system (hSMVT).

Iron

Dietary non-heme ferric (Fe³⁺) iron in the intestinal lumen is reduced to the ferrous (Fe²⁺) form by cytochrome b reductase1 (Cybrd1) in the BBM of the mature villus enterocytes of the proximal small intestine. Iron is transported across the BBM by the divalent-metal ion transporter 1 (DMT1). DMT1 is also known as solute carrier family 11, member 2, (SLC11A2). Fe²⁺ is transported through the cytoplasm of the enterocyte, and is then transferred across the BLM of the enterocyte and into the body by the BLM exporter ferroportin (solute carrier family 40, 1, Slc40a1). There is coordinated expression of ferroportin in enterocytes as well as in tissue macrophages^[70].

Ferroxidase (hephaestin) in the BLM promotes the conversion of Fe²⁺ to Fe³⁺. Once the Fe³⁺ is in the vascular system, it binds to transferrin (Tf). There are two mechanisms by which mRNA levels of iron homeostasis-related genes are regulated; firstly, by post-transcriptional mechanisms mediated by the iron response element/iron regulatory protein system, and secondly, by mechanisms related to transcriptional regulation. In peripheral tissues, the Tf-Tf receptor (TfR) system delivers iron in the Tf-Fe loaded TfR by way of endocytosis. Unlike TfR1, TfR2 mRNA does not contain an Fe-responsive element, and TfR2 mRNA expression is not regulated by intracellular Fe levels. Instead, hepatic TfR2 protein is regulated post-translationally by diferric (Fe²⁺) transferrin. In this way, TfR2 is a sensor of body iron status, and regulates duodenal Fe²⁺ absorption and liver Fe³⁺ uptake^[71]. Hephaestin expression also occurs in gastric antrum, enteric nervous system and pancreatic β -cells^[72].

Fe²⁺ uptake across the enterocyte BBM responds to body iron stores, whereas transport across the BLM is regulated by the enterocyte iron status. When the enterocyte intracellular ferritin level is increased, iron will be transferred across the BLM and into the portal blood. In addition to a potential cytoplasmic route for iron across the enterocyte, there is evidence of vesicular transport or transcytosis of apotransferrin (apoTf). Approximately half of iron transfer across the enterocyte BLM is by way of apoTf and non-apoTf-dependent vesicular pathways^[73].

Factors that affect hepcidin have recently been reviewed and include body iron stores, rate of erythropoiesis, hypoxia and inflammation^[74]. The amount of iron absorbed is regulated by the hepatic synthesis of hepcidin. The most common inheritable form of iron overload is an autosomal recessive disorder caused by mutation in the *HFE* gene, HFE-associated hereditary hemochromatosis (HH). *HFE* codes for a major histocompatibility complex class I (MHC-I)-like molecule. HFE also needs to be associated with a β 2 microglobulin for its appropriate expression of the cell surface.

HFE modulates the expression of hepcidin in the liver. HFE may influence iron status by acting on hepatocytes and/or Kupffer cells, as well as on duodenocytes^[75]. Hepcidin inhibits cellular efflux of iron by binding to and inducing degradation of ferroportin^[76]. Hepcidin causes ferroportin on the BLM to be internalized and degraded^[77]. In macrophages, hepcidin inhibits iron export by inducing ferroportin degradation, whereas in enterocytes hepcidin inhibits DMT1 transcription and thereby reduces BBM iron uptake^[76,78]. Other critical regulators of systemic iron homeostasis are intestinal hypoxia-inducible transcription factors (HIFs)^[79]. HIFs (HIF-1 and HIF-2) are critical mediators of cellular adaptation to hypoxia. HIF-2 α , but not HIF-1 α , promotes iron absorption in mice^[80].

The normal decline in intestinal iron absorption which occurs from neonatal to adult animals is due to loss of the iron transporters (particularly ferroportin) from the distal small intestine and colon^[81]. Curiously, in iron deficiency there is altered intestinal lipid metabolism resulting in production of biologically active lipid molecules (12-HETE, 13-HODE and 13-HOTE), arising as a result of changes in arachidonate 12-lipoxygenase (Alox15)^[82]. It is unknown if this has any clinical significance.

The cytochrome b reductase in the BBM of the duodenal enterocytes (Dcyt6) reduces dietary iron from Fe³⁺ to Fe²⁺. Fe²⁺ is transported across the BBM by divalent metal transporter (DMT). The Fe²⁺ is transported into vesicles containing either ferroportin (FPN1) or hephaestin (Heph). These Fe²⁺-containing FPN1 and Heph-containing vesicles cross the enterocyte cytoplasm to the BLM. The Heph oxidizes the Fe²⁺ to Fe³⁺. Fe³⁺ binds to transferrin and is released into the circulation^[83]. Hepcidin is secreted from the liver in response to the body iron stores: increased body iron stores result in increased hepcidin, decreased FPN1 mRNA expression and increased FPN1 internalization and degradation. The end result of this repositioning of the FPN1 from the BLM is to reduce iron efflux from across the BLM of the duodenocyte, and thereby decrease iron absorption.

Heph, therefore, is a protein in the BLM of the duodenum which has ferroxidase activity to oxidize dietary Fe²⁺ to Fe³⁺. Heph is also found by immunocytochemistry to extend from the gastric antrum along the length of the entire GI tract, and to be present in both the submucosa and the myenteric plexi of the entire nervous system^[72].

In HH, the variable phenotypic expression of the homozygous HFE C282Y genotype has been attributed to possible disease-modifying genes which affect the iron transporters. In HH “expressors” and “nonexpressors”, there is a significant difference in the expression of DMT1 and DMT1 (IRE), such that HFE C282Y homozygotes without phenotypic expression do not have significantly decreased duodenal gene expression of non-transport genes compared with HH subjects with iron overload^[84]. Also, regardless of phenotype, “...there is coordinated regulation between duodenal expression of

FPN1 [ferroportin] and DMT1 [divalent metal transporter 1], FPN1 and DCYTB [ferriductase duodenal cytochrome b] and FPN1 and HEPH [ferroxidase hephaestin] and also DCYTB and HEPH...”.

Calcium

Canonical transient receptor potential (TRPC)1 acts as a calcium channel, with the total calcium effect being mediated by calcium influx through calcium-permeable channels in the plasma membrane, as well as calcium release from intracellular stores such as the ER and cytoplasmic reticulum^[85].

Much of our understanding of calcium (Ca²⁺) absorption has come from studies in animals. Ca²⁺ enters the enterocyte across the BBM using TRPV₆ (aka CAT, or ECAC₂), a Ca²⁺ channel. Intracellular Ca²⁺ is bound to calbindin-D9K, maintaining a low intracellular concentration of free Ca²⁺. PMCA (a Ca²⁺ ATPase) pumps Ca²⁺ across the BLM. The major storage form of vitamin D is 25-hydroxy vitamin D (25OHD). In humans, 25OHD is metabolized by the gene product of CYP27B1 [25-hydroxy vitamin D 1 α -hydroxylase (1 α OHase)] to the biologically active 1 α , 25-dihydroxycholecalciferol [1,25(OH)₂O₃]. 1 α OHase forms 25OHD, which increases the transcription of TRPV₆, PMCA, and CYP₂₄, thereby enhancing Ca²⁺ absorption^[86].

The active hormonal form of vitamin D is 1,25 dihydroxyvitamin D₃ [1,25(OH)₂D]. 1,25(OH)₂D activates the vitamin D receptor (VDR) which heterodimerizes with the retinoid X receptor to interact on response units such as the apical membrane Ca²⁺ channel, TRPV₆ (the transient receptor potential cation channel, subfamily V, member 6), and the Ca²⁺ binding protein calcium binding protein D9k (calbindin D9k). VDR and 1,25(OH)₂D, acting on TRPV₆ and calbindin D9k, maintain high rates of Ca²⁺ absorption^[87].

Copper

Copper is a mineral essential for normal growth and development. The level of copper in the body is regulated, because excessive amounts may be toxic. The copper transporter (Ctr1) is copper-specific; its transport function is energy-independent and saturable. Copper efflux from enterocytes across the BLM is mediated by ATP7A. The ability of suckling rat pups to tolerate varying amounts of dietary copper may be due to changes in copper transporters, Ctr1 and ATP7A, facilitated by transcriptional and post translational mechanisms^[88].

The Steap proteins on the BBM reduce copper to the cuprous form, which is then transported by Ctr1 across the BBM. In the enterocyte, copper is bound to chaperone Atox1, and reaches ATP7A for export across the BLM^[89]. When copper intake is high, Ctr1 is endocytosed into the enterocyte, where there is induction of the copper-binding protein metallothionein, and ATP7A moves to a more basal lateral location. Maturation of small intestinal copper transport occurs by way of increased abundance and local alteration of Ctr1, ATP7A

and ATP7B.

Zinc

The intestinal absorption of zinc is regulated to meet zinc requirements in the body. ZIP4 is a major intestinal zinc transporter; absorptive upregulation of ZIP4 enhances the uptake of zinc from the intestinal lumen, replenishing any deficiency. When the zinc content of the diet is low, there is induction of the transcription factor Kruppel-like factor 4 (KLF4)^[90], which leads to increased intestinal zinc uptake, thereby preventing disease manifestations of zinc deficiency such as acrodermatitis enteropathica.

SHORT BOWEL SYNDROME AND TRANSPLANTATION

“Intestinal failure” refers to a condition in which inadequate digestion and/or absorption of nutrients leads to malnutrition and/or dehydration. The most common condition resulting in intestinal failure is the short bowel syndrome (SBS). The SBS occurs following massive resection of the small intestine^[91]. SBS may be defined anatomically as less than 30% of the normal intestinal length (less than 200 cm in adults). In the United States, the estimated annual prevalence of SBS in patients who have non-malignant intestinal disease, and who require home parenteral nutrition, is at least 4 per 10⁵. The point prevalence is reported to be between 0.6 and 12.7 per 10⁵. The commonest cause of SBS is surgical resection of small intestine for Crohn’s disease. Other common causes include mesenteric infarction, congenital abnormalities, and multiple strictures due to adhesions or abdominal irradiation.

Early in the adaptive response after an intestinal resection, there is an increase in proliferation of intestinal epithelium, with increased depth of crypts, increased villous height, and increased microvillous surface area. The process of adaptation involves the presence of luminal nutrients, gastrointestinal secretions, the mesenchyme, as well as neuronal and hormonal factors. Expansion of the number of intestinal stem cells (ISC) occurs following intestinal resection. This increases the number of intestinal crypts, through the process of intestinal dilation^[92]. These ISCs are located deep in the crypts of Lieberkuhn. Isolation of ISCs has been simplified by the use of side population sorting of viable fractions of cell progenitor characteristics^[93].

Wnt proteins are regulators of cell proliferation, differentiation and adhesion. Mutation in mice of the adenomatous polyposis coli (*APC*) gene, together with augmented Wnt signaling in the intestine, results in an enhanced adaptive response to extensive small bowel resection^[94]. The increased mucosal surface area occurring following resection is due to sustained increases in crypt depth and villus height. This arises from resetting of proliferative responses, with increases in expression of mRNAs associated with proliferation (c-MYC) and dif-

ferentiation of goblet cells and Paneth cells^[95]. This raises the possibility that early expansion of intestinal secretory lineages within the epithelium may serve to amplify the signal(s) for initiating and sustaining intestinal adaptation. Further proof of concept studies are needed.

The Hedgehog (Hh) signaling pathway plays an important role in epithelial-mesenchymal interactions in gut morphogenesis and in epithelial cell proliferation. Hh proteins are produced in epithelial cells, and interact with underlying mesenchymal/stromal cell receptors. Blocking Hh signaling in the fetus or neonate leads to increased crypt cell proliferation, crypt-villus axis structural abnormalities, and alterations in enterocyte morphology. In Hh antibody-treated mice following intestinal resection, enterocyte migration from the crypt to the villus tip is increased, and apoptosis is also increased^[96].

The epidermal growth factor receptor (EGFR) is important in the pathogenesis of intestinal adaptation. This EGFR-mediated induction of enterocyte proliferation requires induced expression of the cyclin-dependent kinase inhibitor p21 to transcribe *waf1/cip1*, as well as mitogen-activated protein kinase (MAPK)^[97]. The cyclin-dependent kinase inhibitor (CDK1) p21*waf1/cip1* may be necessary for induction of enterocyte proliferation following initiation of intestinal adaptation^[98]. To maintain the new homeostasis achieved with adaptation, the high cell production rate must be matched by an equivalent rate of cell loss. EGFR signaling regulates specific Dcl-2 (Dax and Dcl-w) in the intestinal crypts, and this regulation of Dcl-2 influences apoptosis following extensive small bowel resection^[99].

The vascular endothelial growth factor (VEGF) enhances angiogenesis (the growth of new blood vessels from pre-existing blood channels). Angiogenesis is a requirement for successful healing or adaptation. As expected, VEGF is important in the intestinal adaptive response^[100].

The bcl-2 family of intracellular proteins has apoptotic properties. An increase in the ratio between pro- and anti-apoptotic members of these pathways occurs after massive small bowel resection, with upregulation of inducers of apoptosis including Fas and TNF- α by way of the death receptor pathway. Angiotensin converting enzyme (ACE) also promotes apoptosis in association with a reduced bax-bcl-2 protein ratio^[101]. Thus, ACE may play an important role in epithelial cell adaptive responses.

GLP-2 is released from the ileum and colon in response to nutrients in the intestinal lumen. GLP-2 enhances morphologic and proliferative indices of intestinal adaptation, and this adaptation is inhibited by GLP-2 immunoneutralization^[102]. GLP-2 administration enhances intestinal crypt cell proliferation and villus height, and increases expression of glucose transporters. Basal and postprandial GLP-2 levels are correlated with the magnitude of intestinal resection in experimental SBS^[103].

A number of hormones and peptides act on the intestinal tract^[104]. For example, glucagon-like peptide-1 (GLP-1) stimulates glucose-dependent insulin secretion, pancreatic B-cell proliferation, and reduces lipid absorp-

tion, food intake and the rate of gastric emptying^[105]. GLP-2 may reduce Ach release from the enteric nervous system, and thereby reduce neuronally evoked intestinal crypt epithelial Cl⁻ secretion^[106]. GLP-2 enhances the absorption of sugars and lipids^[107] and has a therapeutic potential in patients with the SBS^[108].

SBS patients with an end jejunostomy and no colon have limited meal-stimulated GLP-2 secretion. This is due to the removal of GLP-2 secreting L cells which are located primarily in the terminal ileum and colon. Teduglutide (ALX-0600), a dipeptidyl peptidase IV resistant GLP-2 analog, has been administered to enhance the adaptive process in patients with SBS, and to aid intestinal absorption^[109]. Thus, there is a therapeutic role for GLP-2 analogs in SBS.

Long-term parenteral nutritional (PN) support may be necessary in persons with SBS. Because of the potential complications of PN, such as infection or cholestatic liver disease, efforts have been undertaken to understand and to improve the intestinal adaptive process, and to thereby enhance nutrient absorption and to possibly reduce the need for PN^[110]. The success of surgical procedures designed to optimize intestinal absorptive function, such as bowel tapering or lengthening, has only been modest.

Small intestinal transplantation is an accepted treatment for severe intestinal failure. Over the past 50 years, more than a thousand children have received small bowel transplantation (SBT), alone or with liver and other organs. The one- and five-year graft survival routinely exceeds 90% and 80%, respectively^[111]. However, transplantation is usually used only in those persons who have SBS with complications from home PN. With good control of acute rejection and infections, patient and graft survival after small intestinal transplantation is approximately 77% and 65%, respectively. Patient and graft survivals of 60% and 59% are seen in those with combined liver and small bowel transplantation. Unfortunately, almost half of these transplanted patients require enteral nutrition again within two years after transplantation^[112]. Thus, small bowel transplantation has its risks and limitations for the SBS patient.

NOD2 is an intercellular microbial sensor present in macrophages, dendritic cells and Paneth cells. *NOD2* polymorphisms may be associated with impaired expression of certain Paneth cell-derived antimicrobial peptides. The likelihood of allograft failure is about one hundred-fold higher in small bowel transplantation recipients with mutant *NOD2* alleles, as compared with recipients with wild-type *NOD2* loci^[113].

While intestinal stem cell transplantation may play a role in refractory patients with Crohn's disease (CD) or celiac disease, the role of stem cells in treatment of other intestinal disorders remains at an early stage of consideration^[114,115].

Patients with short bowel syndrome from other non-CD causes were recently reported to develop CD in the residual intestine. The authors suggested that this shortened intestine may be a predisposing factor because of

alterations in the motility of the intestine as well as alterations in the intestinal flora^[116]. In persons with a short bowel syndrome, continuous tube feeding alone or with oral feeding enhances nutrient absorption, as compared with oral intake alone^[117]. "SBS results from surgical resection, congenital defect, a disease-associated loss of absorption and is characterized by the inability to maintain protein-energy, fluid, electrolyte, or micronutrient balances when on a conveniently accepted, normal diet"^[118].

SMOOTH MUSCLE FUNCTION AND INTESTINAL MOTILITY

Segmentation in the intestine consists of rhythmic contractions of the inner circular muscle and occurs after meals. These rhythmic contractions are regulated by slow waves, with the enteric nervous system (ENS) having a permissive role. These stationary contractions are independent of slow-wave activity, while simultaneously activating surrounding inhibitory motor neurons^[119].

The ENS "...coordinates the peristaltic and secretory activity of the gut and is also involved in the regulation of blood flow and modulation of the immune system"^[120]. ENS and enteric glial cells (EGCs) are in the submucosal plexus and the myenteric plexus. The ENS is composed of two ganglionated plexuses, the submucosal and the myenteric plexus, as well as the mucosal plexus. This extends within the lamina muscularis mucosae and the lamina propria mucosae beneath the epithelial lining of the intestine. Activation of human submucosal neurons decreases cellular permeability, and also decreases intestinal epithelial cell proliferation. Neurons respond to changes in intracellular calcium levels or to the expression of activation markers such as c-FOS. TTF- β 1 mRNA is expressed and TGF- β 1 is secreted by EGCs and they have anti-proliferative effects on intestinal epithelial cells^[121]. EGCs promote neuronal survival by regulating substrate supply, and thereby help to maintain neuronal homeostasis. EGCs also synthesize cytokines, and in inflammatory conditions may modulate glia proliferation. Glial disruption alters neurochemical coding of the enteric neurons, and leads to dysmotility^[122].

A method has been described for isolation and culture of primary enteric neurons. These cell lines have neuronal characteristics similar to those of primary enteric neurons^[123]. It is possible to isolate and expand enteric progenitor cells from human adult tissue^[120], thereby providing a potential future role for cell-based therapies for disorders of the ENS.

Patients with IBS may have abnormal colonic transit, as well as increased or decreased rectal sensation. The β 3-adrenoceptor (β 3-AR) is a member of the family of G-protein-coupled receptors. β 3-ARs are co-localized with choline acetyltransferase in human neurons in the cholinergic myenteric and submucosal plexus. Selective β 3-AR agonists inhibit cholinergic contractions, and enhance the release of somatostatin without altering carbachol-induced contractions. β 3-ARs inhibit cholinergic

contractions and inhibit spontaneous contraction of the human colon, as well as relaxing pre-contracted colonic longitudinal and circular muscle. Somatostatin may act as an endogenous analgesic substance, and a β -adrenergic agonist may modify visceral sensitivity. Solabegron is a β 3-AR agonist which has been studied in healthy human volunteers, where it is well tolerated^[124]. This raises the possibility that β 3-ARs may be useful for the pain suffered by persons with IBS.

The IGF system influences cell development, growth, and apoptosis. IGF-binding proteins (IGFBP-1 to -6) transport IGFs in the blood. IGFBP-3 to -5 are present in human smooth muscle, and modify the interaction of IGF-I with its receptor, IGF-IR. IGFBP-5 has a role in regulating smooth muscle growth independent of IGF-1, by activating the G protein G_{i3} ^[125].

Acetylcholine (ACh) is released from cholinergic neurons in the myenteric plexus, and activates M2 and M3 receptors on the smooth muscles of the gastrointestinal tract. This results in smooth muscle contraction. The M2 and M3 receptors are expressed in the ratio of approximately 75%/25%, respectively, and are coupled to TRPC4 and TRPC6 (transient receptor potential channels), which depolarize the intestinal smooth muscle cells^[126]. Cholinergic nerves contain substance P, and there are also nitrergic nerves and nerves which release ATP as well as other mediators in the deep muscular plexus.

Substance P (SP)-mediated sustained contraction of the small intestine is negatively regulated at a pre-synaptic level by the M2 receptor, whereas the atropine-sensitive phasic contraction is positively regulated at the M2 receptor^[127]. Cytokines exert differential effects on the muscarinic receptors of intestinal longitudinal smooth muscle^[128]. Corticotropin-releasing-factor (CRF) acts through both central and peripheral mechanisms. CRF induces Ca^{2+} transients in myenteric neurons *via* a CRF-1 receptor-dependent mechanism^[129]. Calcium influx through voltage-operated Ca^{2+} channels, and in particular the R-type channels, causes the calcium transients necessary for muscle contraction.

Nonselective cationic channels in the smooth muscle cells generate muscarinic receptor-induced nonselective cation currents (MICAT), with increased Ca^{2+} influx by way of voltage-dependent Ca^{2+} channels. The Ca^{2+} leads to smooth muscle contraction, and peristalsis.

The gastrointestinal tract contains most of the serotonin (5-hydroxytryptamine, 5-HT) in the body, where it acts as a neurotransmitter, neuromodulator, and a paracrine factor. 5-HT is produced and released by enterochromaffin cells and by enteric nerves of the intestine. In addition to 5-HT having an effect on motility, it also regulates cell survival and proliferation. The 5-HT_{2D} receptors are expressed on the interstitial cells of Cajal (ICC). Exogenous 5-HT regulates the number of ICC through the 5-HT_{2B} receptor, in part by increasing ICC proliferation^[130].

The sensory intrinsic primary afferent neurons (IPANs) from an ENS network modify enteric reflexes,

which in turn alter gastrointestinal functions. The sensory terminals of IPANs are close to the enterochromaffin cells, which contain serotonin (5-HT). Extrinsic afferents (vagus and spinal afferents) have similar innervation territories, and both respond to chemical and to mechanical stimulation. In response to mechanical stimuli, extrinsic afferent neurons do not crosstalk with the IPANs. 5-HT activates the extrinsic afferents by a Ca^{2+} -dependent pathway which is different from the N-type Ca^{2+} channels in IPANs^[131].

The topic of the role of the ICC in gastrointestinal motility has been reviewed^[132]. It has become controversial with regard to the way nerves transmit their signals to regulate activity of intestinal smooth muscle^[133]: the c-kit receptor may be of importance^[134]. ICC help in maintaining the gradient of resting membrane potential, rather than by pacing the slow waves or assisting in their propagation^[135]. It is by volume transmission rather than wire transmission *via* the ICC that there is communication between the enteric neuronal varicosities and muscle cells. While the ICC may be impaired in numerous motility disorders, "...a cause-and-effect relationship between ICC impairment and motility dysfunction is not established". In the small intestine, ICC in the deep muscular plexus mediate neurotransmission, whereas ICC surrounding the myenteric plexus generate slow waves. The slow waves are transferred to the adjacent smooth muscles, and can be recorded as straight "...spontaneous, rhythmic, electrical oscillations of the resting membrane potential"^[136].

The ICC occur in the myenteric plexus, within either the circular (CM) or longitudinal (LM) muscles, contributing to pacing these muscle slow waves. ICC also line the septa (ICC-SEP) separating circular muscle bundles, and ICC-SEP form an important conduction pathway for spreading excitation deep into muscle bundles in the human jejunum^[137]. The $Na^{+}/K^{+}/2Cl^{-}$ cotransporter (NKCC1) is involved in generation of slow waves in the jejunal musculature. ICC at the deep muscular plexus (ICC-DMP) are closely associated with the enteric nerve endings. Varicosities of nitrergic and other nerves are found on ICC-DMP or on CD34-positive, c-kit-negative fibroblast-like cells. The gap junction coupling is necessary for pacing or nerve transmission to the circuit or muscle of the mouse intestine^[138].

The ICC act as pacemakers, producing slow waves of depolarization along the intestinal muscle. A transient outward K^{+} current may moderate the uptake of the pacemaker potential, resulting in motility arising from the waves of depolarization^[139].

Bone morphogenetic protein 2 (BMP-2) is a regulatory molecule which induces the phosphorylation of the Smad1 signaling cascade, and thereby increases the differentiation of the neurons of the ENS^[140].

Endogenous adenosine (eADO) is a metabolite of ATP that acts on A₁, A_{2A} and A_{2B} receptors on enteric neurons to suppress coordinated responses triggered by immune-histamine H₂ receptor activation^[141].

Extracellular adenosine levels control adenosine re-

ceptor signaling. Enzymes that produce CD73 or degrade adenosine deaminase (adenosine), and thereby alter activity of transport systems in the plasma membrane, influence these extracellular adenosine levels, thereby affecting adenosine receptor signaling, which in turn alters intestinal motility^[142]. β -Adrenoceptors are G-protein-coupled receptors which, when activated by an agonist, stimulate adenylyl cyclase to produce the second messenger, cAMP. cAMP activates cAMP-dependent protein kinase (PKA). There is compartmentalization of the process by which these proteins form an interaction.

Caveolae are non-clathrin-coded plasma membrane invaginations which are present in a variety of cells, including monocytes. These caveoli are present in microdomains, also known as lipid rafts, an area of the plasma membrane which is rich in cholesterol and sphingolipid. The caveolae are coated on their cytoplasmic side by caveolins, a family of integral membrane proteins including adenylyl cyclase, which are involved in signal transduction. In knockout mice depleted of caveolin-1, there is reduced PKA activity and thereby reduced function of the β -adrenoceptors^[143].

Adenosine is generally accepted to be the ligand for the P1 receptor, and ATP is the ligand for P2 receptors. Adenosine A2A receptors on neuronal cells are excitatory in nature, but A1 receptors in the submucosal plexus have inhibitory actions^[144]. In the murine enteric nervous system, adenosine "... suppresses synaptic transmission, efferent function of extrinsic capsaicin-sensitive sensory nerves, mucosal reflexes, neuroeffector transmission, and morphine withdrawal diarrhea". Purinergic signaling pathways are important in sensory signaling in enterochromaffin cells and secretomotor reflexes in the intestinal tract; purinergic modulation of synaptic transmission also occurs in human intestine^[145].

Intestinal motor activity and secretion are linked, and are changed cyclically in a rhythm called the migrating motor complex (MMC). Submucous neurons are both directly and indirectly mechanosensitive, and myenteric neurons can be activated by stretch. There are both rapid and slow components to the potential difference (PD) response to intestinal stretching or distension. The rapid component operates *via* nicotinic transmission and NK1 receptors; the slow component operates by way of VIP-ergic transmission and involves both NK1 and NK3 receptors^[146].

Intestinal inflammation causes hyperplasia of smooth muscle, and this thickening of the smooth muscle layer results in dysmotility. IL-1 β is a proinflammatory cytokine which results in production of PGE2 and NO from macrophages within the ileal smooth muscle tissue, and IL-1 β acts as an anti-proliferative mediator^[147]. Nematode infection in the small intestine induces a smooth muscle hypercontractility that depends on IL-4 and IL-3 (Th-2 cytokines) activation of the signal transducer and activator of transcription (STAT) 6. 5-HT_{2A} is one of the molecules downstream from STAT6 activation that mediates changes in smooth muscle function^[148].

Integrins are a family of transmembrane proteins, and the expression of integrins and their preferred ligands is tissue specific. In the small intestine, occupancy of a specific integrin receptor acts in concert with IGF-I-stimulated receptor tyrosine kinase activity on muscle cell growth^[149]. Both insulin and IGF-I prevent apoptosis through the activation of phosphatidylinositol 3-kinase (PIK3-kinase). Through the subsequent activation of the downstream protein serine/3 kinase, Akt IGF-I stimulates proliferation and inhibits apoptosis in intestinal smooth muscle^[150].

Mechanisms underlying the sustained tonic contraction of the intestinal smooth muscle include prolonged myosin-like chain phosphorylation, phosphorylation of cytoskeleton filaments and associated proteins, alterations in Ca²⁺ influx, and increased sensitivity of contractile elements to Ca²⁺^[151]. Muscarinic agonists acting through M3 receptors contract gastrointestinal smooth muscle by a protein kinase C (PKC)-dependent mechanism in the guinea pig ileum; this is thought to be through a novel PKC, PKC- δ ^[151].

Electrical stimulation may be synchronized with the intrinsic intestinal smooth muscle slow waves [synchronized intestinal electrical stimulation (SIES)]. SIES induces small intestinal contractions during phase I of the MMC in the fed state, and improves postprandial small intestinal hypomotility^[152]. SIES remains to be of proven clinical use.

There are olfactory receptors in human mucosal enterochromaffin cells. Odorants present in the luminal environment of the gut may stimulate serotonin release by way of olfactory receptors present in these EC cells^[153].

The "ileal break" describes the process by which high concentrations of lipids in the terminal ileum will slow gastric emptying and intestinal transit. High intra-ileal carbohydrate and lipid loads induce phase III motility, probably through release of neurohormonal mediators, glucagon-like peptide (GLP-1) and peptideYY (PYY). Physiological postprandial ileal lipid concentrations inhibit human digestive pancreatic protease and bile acid output, but do not influence intestinal motor activity^[154].

Acute radiation exposure of the abdomen is associated with accelerated small intestinal transit through involvement of cholinergic receptors. This raises the possibility that M3 receptors "...may provide specific therapeutic targets in acute radiation enteritis"^[155]. The mucosal damage in the small intestine produced by abdominal radiation may occur independently of intestinal dysmotility, and may result in diarrhea and nutrient malabsorption. Interestingly, the high molecular weight polyethylene glycol-based copolymer PEG 15-20 prevents radiation-induced intestinal injury in rats, prevents apoptosis and lethal sepsis due to *Pseudomonas aeruginosa* in mice, and protects cultured intestinal epithelial cells from apoptosis and microbial adherence, possibly by binding characteristic lipid raft coalescence during the development of intestinal radiation damage^[156].

The mechanisms of drug-associated changes in intes-

tinal motility have been reviewed^[157]. Chronic intestinal dysmotility or chronic intestinal pseudo-obstruction may be primary, or secondary to disorders such as diabetes mellitus or scleroderma. These disorders may affect isolated components of the GI tract, or the entire GI tract. There may be absence of the phase III component of the MMC, postprandial low amplitude contractions, bursts of sustained uncoordinated phasic contractions, and clusters of contractions. All of these mechanisms contribute to the pathophysiology and the high morbidity of these dysmotility syndromes^[158].

Cannabis (CB) and cannabinoid receptors inhibit intestinal motility. The CB1 receptor is present in the central and peripheral nervous system, including the enteric nervous system, as well as in non-neural tissues such as liver and adipose tissue. CB2 receptor expression is present in cells of the immune system as well as in the brain. Lipopolysaccharide (LPS) enhances intestinal transit, and this effect is reversed by cannabinoid CB2 receptor agonists^[159].

Lubiprostone is a bicyclic FA compound, a prostone derived from a metabolite of prostaglandin E1. Prostones have highly selective activity on ClC-2 chloride channels, enhancing intestinal fluid secretion, but also accelerating small intestinal and colonic transit^[160]. These compounds may be used clinically for the treatment of constipation.

Bowel inflammation may lead to abnormalities in intestinal motor and secretory pattern, through changes in enterochromaffin cell activity, as well as through changes in the excitability of primary afferent neurons of the enteric nervous system. Inflammation at one site of the intestine also alters the cellular components of enteric reflex circuits in non-inflamed regions^[161]. Intestinal inflammation is a key event in the pathogenesis of post-operative ileus, and in rats the degree of intestinal paralysis is directly proportional to the degree of intestinal handling and inflammation which occurs at the time of surgery. Intestinal handling triggers mast cell activation and prolongs post-operative ileus^[162]. The therapeutic role of this observation in preventing or treating post-operative ileus remains to be proven.

There are three endogenously-produced biologically-active gases, carbon monoxide, hydrogen sulfide and nitric oxide. Methane is also produced by enteric bacteria in one- to two-thirds of humans, and may slow intestinal transit by augmenting small bowel contractile activity^[163]. Excess methane production has been recognized in a proportion of persons with constipation-predominant IBS.

Substance P and neurokinin A are the main endogenous tachykinins in the enteric neurons. Stimulation of the NK3R receptor in the GI tract activates protein kinase C, then protein kinase D, leading to noncholinergic slow excitatory postsynaptic potentials in the myenteric intrinsic primary afferent neurons of the guinea pig ileum^[164].

The ICC generate pacemaker potentials which drive the electric slow waves that contribute to neuromuscular

signaling leading to motor neurotransmission in the GI tract. ICC express receptor tyrosine kinase c-kit. *Kit* gain-of-function mutations lead to hyperplasia of ICC, with maintenance of both pacemaker function and normal enteric neural responses^[165]. This is in contrast to the association between fewer ICC and the development of disturbances in GI motility. *Ano1* is part of a family of 10 gene products, and labels ICC around ganglia in the deep muscular plexus^[166]. Because *Ano1* does not label mast cells, it may prove to be a better marker than c-kit for ICC and for mesenchymal tumors.

C-kit immunohistochemistry is used to diagnose gastrointestinal stromal tumors (GIST), since about 94% of mesenchymal tumors are positive for c-kit receptors. Between 80% and 90% of GIST tumors have gain-of-function mutations in *Kit*.

Stimulation of the myenteric plexus of the ENS by food in the intestinal lumen or by stretching of the intestine activates mucosal pathways to produce three different types of slow excitatory post-synaptic potentials (EPSPs) which are mediated by tachykinin or purine nucleotide neurons^[167]. The predominant cell type in the ENS, the glial cells, provide functional purinergic neuron-glia communication in the ENS^[168].

Myofibroblasts are an intermediate cell type between smooth muscle cells and fibroblasts. In persons with Crohn's disease, there is disruption of the subepithelial myofibroblasts of the epithelial sheath, adding to the suggestion that myofibroblasts may be involved in the pathogenesis of inflammatory bowel disease^[169]. This role of myofibroblasts is likely because they serve as a component of the innate immune system, and respond to luminal bacterial adjuvants such as LPS^[170].

Manipulation of the intestine rapidly causes activation of the p38 nitrogen-activated protein kinase (MAPK), a stress-activated protein kinase. There is liberation of NO and prostaglandins from the macrophages in the muscularis external to the intestine, and the extravasation of immunocompetent white blood cells^[171]. In turn, this leads to postoperative ileus, which can be prevented by giving mice a single preoperative dose of semapimod, which inhibits p38-MAPK and NO production in macrophages. This is an exciting development for its possible future application to humans undergoing abdominal surgery.

An increase in the intracellular concentration of Ca^{2+} in the smooth muscle of the intestine results from the release of Ca^{2+} from intracellular stores, as well as from the entry of Ca^{2+} into the cell through L-type Ca^{2+} channels. With stretching of the wall of the intestine there develops tension in the plasmalemmal membrane. This tension is transmitted to the mechanosensitive L-type Ca^{2+} channels, thereby leading to increased intracellular Ca^{2+} and the possibility for smooth muscle contraction^[172].

The peristaltic reflex is mediated by IPANs (intestine sensory efferent neurons), interneurons, as well as excitatory and inhibitory motor neurons. The antipropulsive effect of cannabinoids on the small and large intestine result from the inhibition of the calcitonin gene-related

peptide (CGRP)-containing neurons that begin the peristaltic reflex, as well as the inhibition of both the excitatory cholinergic/tachykinergic and inhibitory VIPergic motor neurons responsible for ascending contraction and descending relaxation, respectively^[173].

The balance between myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) controls the overall phosphorylation of the 20-kDa regulatory light chains of myosin III. Ca^{2+} -independent contraction of longitudinal ileal smooth muscle is potentiated by a zipper-interacting protein kinase pseudosubstrate peptide^[174]. This raises the possibility of developing synthetic peptides from the autoinhibitory region of the smooth muscle myosin light chain kinase to treat hypomotility disorders of the GI tract.

TUMORS

Small bowel cancers represent a heterogeneous group of rare tumors, and the prognosis depends upon the cell type. The standardized incidence rate for primary malignant small bowel cancer is 1.2/10⁵ men and 0.8/10⁵ women^[175]. The four main histological types are adenocarcinoma (40%), carcinoid (31%), lymphoma (20%) and sarcoma (9%). The five-year survival rate is about 37%, and varies between 57% for neuroendocrine tumors and 18% for sarcomas.

The gastrointestinal tract is the most common extranodal site for non-Hodgkin's lymphoma (NHL). GI NHL may be primary or secondary, the latter usually representing involvement from diffuse nodal disease. GI NHL used to be increased in the HIV-positive population, but with HAART therapy, GI NHL has virtually disappeared^[176]. One intestinal nuclear receptor map has been developed, which "...indicates that the localization pattern of a receptor in normal intestine (signature) predicts the modulation of its expression in tumors"^[177].

DIAGNOSTIC IMAGING

Imaging techniques for the small intestine include the classic small bowel series, enteroclysis, CT enterography, MR enterography, and more recently capsule endoscopy (CE), push enteroscopy (PE), and double balloon enteroscopy (DBE)^[178]. The topic of recent developments in CE has been reviewed^[179-182]. Optimal bowel preparation for CE is a PEG solution plus simethicone^[183]. Using duodenal histology as the gold standard, the performance characteristics of CE for the diagnosis of celiac disease are: sensitivity 88% (95% CI: 76%-99%), specificity 91% (95% CI: 81%-100%), positive predictive value 97% (95% CI: 90%-100%), negative predictive value 71% (95% CI: 56%-87%), as well as positive and negative likelihood ratios of 9.6 and 0.14, respectively^[184]. Of 43 celiac patients, 42% had mucosal changes extending beyond the duodenum, and in 7% the alterations involved the entire small intestine. Interobserver agreement for the diagnosis of celiac disease by CE ranges between 79% and 94%; and κ

values range between 0.6 and 0.9. Murray *et al.*^[185] reported a sensitivity of CE for the detection of small intestinal mucosal atrophy, as compared with upper endoscopy, to be 92% *vs* 55% ($P = 0.0005$), with a specificity of 100%. Other authors have agreed with this high sensitivity of CE (over 90%), but reported a much lower specificity of approximately 64%^[186].

The topic of small bowel enteroscopy has been reviewed^[187]. CE is contraindicated under a number of circumstances^[188]: (1) Swallowing disorder; (2) Known or suspected intestinal obstruction, strictures, or fistulae; (3) Pregnancy; (4) Children less than 10 years old; and (5) Persons with implanted electromedical devices.

In 120 persons on long-term NSAIDs or COX-2 selective agents, CE demonstrated that the 62% with abnormal CE had denuded areas (39%), mucosal breaks (29%), or reddened folds (13%)^[189]. CE demonstrated small intestinal polyps in 60% of subjects with familial adenomatous polyposis (FAP) and in 75% of subjects with Peutz-Jeghers Syndrome^[190].

A meta-analysis of nine studies compared CE *vs* other diagnostic methods for Crohn's disease. The diagnostic yield for CE *vs* barium radiography was 63% and 23%, respectively. The yield for CE *vs* ileoscopy was 61% and 46%, respectively, and the yield of CE compared to computed tomography (CT) enterography/CT enteroclysis was 69% and 30%, respectively. A meta-analysis of the yield of CE *vs* other modality examinations of the small intestine in patients with non-stricturing Crohn's disease showed that CE was superior to small bowel barium radiography, ileoscopy, CT enterography, CT enteroclysis and PE, as well as small bowel magnetic resonance imaging (MRI)^[191]. In patients with previous surgical resection for Crohn's disease, CE is inferior to ileocolonoscopy, but does detect about two-thirds of lesions outside the reach of the colonoscope^[192]. These authors suggest that CE "...cannot systematically replace ileocolonoscopy in the regular management of patients after surgery" (for Crohn's disease). The CE findings have an impact on patient management: physicians change post-CE diagnostic strategy in 61% of patients^[193]. Clearly, CE has proven its diagnostic potential.

There is an incremental diagnostic yield (yield of CE minus yield of comparative modality) of 38% comparing CE to PE, and 22% compared to small bowel MRI. As compared with PE, CE provides superior identification of obscure bleeding sites in the small intestine (50% *vs* 24%). While CE missed 8% of lesions, these sites were accessible to standard endoscopy; in contrast, PE missed lesions in 26% of patients^[194].

DBE may be used from the oral or anal route, or from both. The overall detection rate of small bowel diseases using CE is superior to that with DBE (72% *vs* 41%, respectively), and is also superior for the detection of small bowel diseases in patients with obscure gastrointestinal bleeding (88% *vs* 60%, respectively)^[195]. In another study, for detection of causes of obscure gastrointestinal bleeding, 80% of small bowel abnormalities were detected by

CE vs 60% with DBE^[196]. PE may be inferior to push-and-pull enteroscopy to find lesions in patients with suspected small bowel bleeding^[197]. DBE performance has also been evaluated in patients with refractory CD who had circumferential, discreet, or confluent ulcerations^[196].

When patients with acute intestinal symptoms after allogeneic stem cell transplantation underwent esophago-gastroduodenoscopy (EGD) with duodenal biopsies, as well as CE within 24 h of the onset of their symptoms, acute intestinal graft-vs-host disease (GVHD) was diagnosed by EGD with biopsies in 7 out of 13 patients, 3 of whom would have been missed by EGD alone but were detected by CE. In all 7 patients with histologically confirmed acute intestinal GVHD, CE revealed the typical lesions of GVHD^[198]. The authors concluded that CE showed comparable sensitivity with EGD plus biopsies, and CE also demonstrated a high negative predictive value for diagnosing acute intestinal GVHD.

After the formation of an ileal pouch anal anastomosis (IPAA) in patients having a colectomy for ulcerative colitis, development of pouchitis is common. It is unknown how frequently lesions occur elsewhere in the small intestine. At small bowel follow-through of persons with pouchitis, 13% showed a focal ectasia of the middle ileum and a stenosis of the pouch, whereas CE performed in patients with chronic pouchitis after IPAA showed diffuse lesions from the duodenum to the ileum in all evaluable patients^[199]. These lesions included aphthous ulcers, erosions, redness, atrophy, cobblestoning, and deep ulcers or fissures.

A retrospective analysis of the charts of 562 patients who underwent CE at the Mount Sinai Medical Centre (NYC) for a variety of indications showed small bowel tumors in 9%^[200]. In a report of the largest series of patients with small bowel tumors detected by CE, half were identified in the jejunum and approximately a quarter were in the ileum or in the mid to distal small bowel^[201]. The most common malignant small bowel tumors were adenocarcinoma, carcinoids, melanomas, lymphomas and sarcomas. The most common benign tumors were GIST, hemangiomas, hamartomas, adenomas, and granulation tissue polyps. In a three-center report of Australian experiences with CE, the usefulness of CE was also confirmed, and the authors suggested that “in many patients, detection of a tumor alters management and improves outcomes”^[202].

CE found a median of four small bowel polyps greater than 1 cm in size in persons with Peutz-Jeghers syndrome, while barium follow-through detected a median of only one polyp^[203]. In persons with known familial adenomatous polyposis (FAP), regular examination of the small intestine for small intestinal tumors is part of the recommended management. CE detected ileal or jejunal polyps in 30% of patients, and all polyps were less than 5 mm in size. Upper gastrointestinal endoscopy detected duodenal polyps in 11 of 23 patients, and only four of these patients were identified as having duodenal polyps on CE. Thus, CE underestimated the number of polyps

and did not visualize the ampulla of Vater. This suggests that CE is useful for detection of jejunal and ileal polyps in patients with FAP, but standard forward- and side-viewing endoscopic procedures are advised for detection of duodenal polyps^[204].

While duodenal biopsy represents the gold standard for the diagnosis of celiac disease, CE has shown that over a third of celiac patients have mucosal changes extending beyond the duodenum, and in approximately 7% the entire small bowel is involved^[184]. As compared with duodenal biopsy for detecting celiac disease, the sensitivity of CE was 88%, specificity 91%, positive predictive value 97%, and negative predictive value 71%.

Although CE provides excellent visualization of the small intestinal epithelium, if a small bowel lesion is identified, it cannot be biopsied. DBE is clinically useful to identify and biopsy such lesions^[205-210].

Because of the varying values of the sensitivity and specificity of the various diagnostic methods available to diagnose small bowel disease (such as capsule endoscopy, CT- or MRI- enteroscopy, ileocolonoscopy, small bowel follow-through), it is suggested that except for CE, “...a combination of two of the other available imaging methods is the best diagnostic option for small-bowel Crohn’s disease...”^[211,212]. While CE gives a diagnostic yield in about two-thirds of patients with obscure gastrointestinal bleeding, DBE (when used within 1 mo after the last episode of overt bleeding) reveals positive findings in 84% and provides a means to control bleeding in 64%^[213].

CYSTIC FIBROSIS

Meconium ileus occurs in approximately 20% of newborns with cystic fibrosis (CF). The distal intestinal obstruction syndrome (DIOS) occurs in about 25% of CF adults. Mucus accumulation in the CF intestine is partly due to the dehydrated, acidic environment, as well as to the altered glycosylation of mucins. Mucin glycoprotein levels are increased, due to reduced mucus clearance rather than an enhanced synthesis. This is suggested by the lack of increase in the levels of expression of the major intestinal mucin genes (*Muc2*, *Muc3*). Interestingly, *Muc1* is not a major component of the accumulated mucus of CF mice^[214]. Mucin binds bacteria, both by nonspecific trapping as well as by binding to specific glycans, which help to carry bacteria aborally for efficient clearance from the small intestine.

In human CF, there are mutations in the *CFTR* gene that result in little or no cystic fibrosis transmembrane conductance regulator (CFTR) activity. Some 30%-50% of CF patients have small intestinal bacterial overgrowth (SIBO), thought to be due to slow small intestinal transit^[215]. This SIBO may be due to impaired migrating motor complexes, which lead to less removal of mucus and bacteria from the small intestine. Laxatives and N-acetylcysteine (NAC) reduce bacterial overgrowth in the CF intestine of mice, and this eradication is associated with normalized intestinal transit and a reduction in the innate

immune response^[215].

Exogenous pancreatic replacement enzyme therapy improves, but does not normalize, steatorrhea in CF patients. In CF mice, the crypt-villus axis height is decreased, there are fewer apoptotic cells in the intestinal crypts; there is also goblet cell hyperplasia and inflammatory cell infiltration^[216]. In humans with CF, there is more than just defective lipolytic enzyme activity leading to the malabsorption of lipids. Indeed, there is evidence for abnormal enterocyte intracellular lipid processing in intestinal biopsies from CF subjects, such as decreased lipid esterification and lipid secretion, decreased output of triglyceride-rich lipoproteins, as well as diminished synthesis of apoB48 and apoA-1^[217].

Factors that may contribute to the incomplete normalization of fat malabsorption in CF patients who are compliant with their intake of adequate amounts of pancreatic replacement enzymes include the use of outdated or inactivated enzymes, incorrect timing of the intake of the supplements with regards to meals, excessive duodenal acid inactivation of the enzymes, or reduced duodenal and pancreatic bicarbonate secretion leading to high intraduodenal acid concentrations, impaired formation of mixed micelles, altered composition of the BBM [decreased linoleic acid (18:2n-6) and docosahexaenoic acid (26:6n-3), as well as increased arachidonic acid (20:4n-6) and elevated (20:3n-9) to (20:4n-6) ES] with changes in its permeability and absorptive function, impaired esterification of lipids in the enterocytes, reduced apolipoprotein synthesis, chylomicron formation or secretion across the BLM. CFTR knockdown in Caco-2 cells stimulates both the synthesis and transport of fat but not cholesterol^[218].

Approaches are needed to prevent the CF-associated increased viscosity of the intestinal luminal contents, and hopefully reduce the frequency of meconium ileus in CF newborns, or DIOS in CF adults. The activation of proteinase-activated receptors on the BLM of enterocytes by EGFR activation, MAP kinase signaling, Ca^{2+} , PKA (and possibly PKC), causes chloride secretion. PKC enhances the activation of PKA, or increases BLM NKCCI, thereby enhancing the phosphorylation of CFTR, and thus Cl^- and water secretion. Basolateral PAR2-induced Cl^- secretion induces the activation of PKC β and PKC δ via a phospholipase C mechanism, which results in the stimulation of cRaf and ERK 1/2 signaling^[219]. Reduction of NHE3-mediated Na^+ and water absorption helps to increase the fluidity of the intestinal contents that would otherwise be very thick and dehydrated if NHE3 activity remained normal in the presence of reduced CFTR activity^[220]. Lubiprostone activates CIC-2 chloride channels, causing Cl^- and water secretion^[221].

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How can portal vein cavernous transformation cause chronic incomplete biliary obstruction?

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Abstract

Biliary disease in the setting of non-cirrhotic portal vein thrombosis (and similarly in portal vein cavernous transformation) can become a serious problem during the evolution of disease. This is mostly due to portal biliary ductopathy. There are several mechanisms that play a role in the development of portal biliary ductopathy, such as induction of fibrosis in the biliary tract (due to direct action of dilated peribiliary collaterals and/or recurrent cholangitis), loss of biliary motility, chronic cholestasis (due to fibrosis or choledocholithiasis) and increased formation of cholelithiasis (due to various factors). The management of cholelithiasis in cases with portal vein cavernous transformation merits special attention. Because of a heterogeneous clinical presentation and concomitant pathophysiological changes that take place in biliary anatomy, diagnosis and therapy can become very complicated. Due to increased incidence and complications of cholelithiasis, standard treatment modalities like sphincterotomy or balloon sweeping of bile ducts can cause serious problems. Cholangitis, biliary strictures and hemobilia are the most common complications that occur during management of these patients. In this review, we specifically discuss important issues about bile stones related to bile duct

obstruction in non-cirrhotic portal vein thrombosis and present evidence in the current literature.

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Key words: Portal vein cavernous transformation; Cholelithiasis; Hemobilia; Portal ductopathy; Portal biliopathy

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INTRODUCTION

The thrombosis occurring secondary to various causes results in acute and generally asymptomatic thrombosis in an extra-hepatic portion of portal vein transformation (PVT). Eventually, portal vein cavernomatous transformation (PVCT) forms as a compensatory reaction 3 wk to 3 mo after the initial event at the hilum of the liver. However, this salvage also results in inevitable anatomical or functional changes at surrounding structures including the biliary tree, gallbladder and pancreas^[1-4]. These changes are secondary to perfusion changes, neovascularization, fibrosis reaction or compression of ducts by newly formed but highly perfused vessels.

Recent terminology uses the term “portal biliopathy” to denote overall anatomical, morphological and functional changes in the biliary tree^[5]. The manufactured term “biliopathy” originates from a combination of the Latin word “bilis” (meaning bile) and the Greek word “pathos” (meaning disease) suggesting a disease of liq-



Figure 1 Liver ultrasound image showing stone (white arrow) and portal vein cavernomatous transformation (black arrow) in gallbladder.

uid bile or its constituents. However, current knowledge lacks compelling evidence of formation of diseased bile in patients with PVCT. Therefore in this review, the term “portal ductopathy” (PD) will be used instead of portal biliopathy as suggested by our group previously^[6].

The changes that take place in the biliary ducts during the course of PVCT can be summarized as strictures, dilatations and varicose veins located at the ductular walls and gallbladder. In the long term, these changes result in biliary stasis, jaundice, cholangitis and cholelithiasis adding more to morbidity and even resulting in mortality (Figures 1 and 2). The superimposition of cholelithiasis observed in the setting of PD is very important since these patients are prone to secondary biliary cirrhosis, a complication observed in 4% of patients^[7]. The clinical problem of cholelithiasis observed in chronic, non-cirrhotic PVCT patients is an under-mentioned topic and forms the basis of this compact review. Other clinical problems such as cirrhotic patients, biliary strictures or surgical management related to PD are beyond the scope of this paper.

PATHOGENESIS OF CHOLELITHIASIS IN PORTAL VEIN CAVERNOMATOUS TRANSFORMATION

Theoretically, cholelithiasis in the setting of PD and PVCT may be secondary to chronic cholestasis, changes in the constituents of bile or other factors such as reduced portal flow and associated liver atrophy.

Cholestasis and gallbladder functions

Cholestasis and related clinical findings such as jaundice, pruritus and cholangitis are the most common presenting symptoms in the setting of symptomatic PD and occurs in 5%-30% of all patients^[8,9]. Chronic biliary stasis (due to compression in the biliary outflow and loss of bile duct contractility due to either fibrosis or ischemia^[8]) may eventually result in stones formed *de novo* located in biliary ducts and/or gallbladder. The evidence for the proof of this hypothesis is obtained from clinical studies which generally observed pigment stones, located

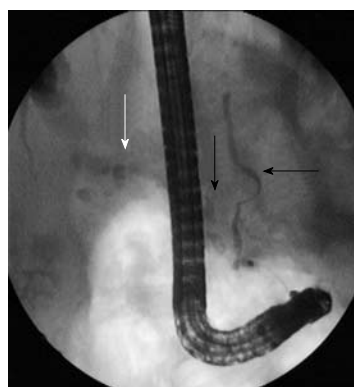


Figure 2 “Portal double ductopathy” sign with stones. Endoscopic retrograde cholangiopancreatography image of a patient with chronic portal vein cavernomatous transformation showing irregular pancreatic duct and biliary ducts (black arrows). Main bile duct shows local stricture and dilations with cholelithiasis (white arrow).

just proximal to the site of ductular stenosis without associated stones in the gallbladder^[10,11]. The reduced contractile function of the gallbladder may also contribute to development of stones due to the presence of varicose veins in the gallbladder wall, but in one study the contractile function of gallbladder has been found to be unchanged^[12]. Thus, cholestasis is believed to develop at later stages of PD secondary to fibrosis and compression of newly formed vessels, which are a major factors for development of stones. Once formed, stones further contribute to cholestasis and stricture formation by initiating recurrent cholangitis attacks.

Change in the constituents of bile

There is a small amount of evidence in the literature to indicate a change in the composition of bile resulting in a tendency for development of cholelithiasis. Previous studies investigating the contribution of reduced portal flow for development of cholelithiasis found that there is an increased tendency towards formation of stones after selective portal vein ligation in dogs^[13]. Also, temporary interruption of portal flow leads to decreased bile acid synthesis and biliary flow^[14]. There is no human study in the literature to indicate definitive changes in biliary composition take place during PVCT. Hypothetically speaking, presence of abnormal portal inflow might contribute to formation of lithogenic bile either by causing hypersplenism and increased pigment load in bile or abnormal enterohepatic circulation of bile acids due to portal hypertension.

FREQUENCY AND CLINICAL FINDINGS OF CHOLELITHIASIS IN PORTAL VEIN CAVERNOMATOUS TRANSFORMATION

The findings obtained from key studies concerning cholelithiasis observed in PVT-PVCT (in adult and pediatric populations) are summarized in Table 1. The frequency of cholelithiasis is highly variable (0%-84%). Probably, this variability is secondary to the problem of potential

Table 1 Cholelithiasis and important findings from selected studies

Author	Patient population	Frequency and location of cholelithiasis (%)	Notes
Bayraktar <i>et al</i> ^[1]	47 PVT patients evaluated by ERCP	8 (19) located in CBD	Unselected PVCT patients irrespective of symptoms, had a control group of 22 patients with other causes of portal hypertension
Condat <i>et al</i> ^[11]	25 consecutive patients in 2 yr evaluated by MRCP	4 (16)	Cholelithiasis associated with abrupt elevation of transaminase level, obstructive jaundice and cholangitis (2 patients)
Khare <i>et al</i> ^[27]	13 patients with obstructive jaundice evaluated by ultrasound	4 (31) located in GB, 4 (31) located in CBD	Retrospective selection of patients with obstructive jaundice
Sezgin <i>et al</i> ^[19]	10 consecutive patients in 6 yr presenting with jaundice and/or cholangitis evaluated by ERCP	0	Only patients with biliary symptoms are evaluated; Total number of patients or prevalence of cholelithiasis is not mentioned
Oo <i>et al</i> ^[16]	13 patients with symptoms related to PD in 13 yr	11 (84) located in GB, 9 (69) located both in GB and CBD	Symptomatic patients selected
Dumortier <i>et al</i> ^[28]	6 consecutive patients presented as case series	4 (66) located in GB, 2 also had stones in CBD	Upon follow up 3 patients suffered from cholecystitis and cholangitis requiring cholecystectomy
Chaudhary <i>et al</i> ^[20]	9 symptomatic PD patients managed surgically	2 (22) located in CBD	Selected patients with indications for surgery; Bile aspirates during surgery revealed <i>Escherichia coli</i> in patients with stones
Agarwal <i>et al</i> ^[29]	39 symptomatic PD patients managed surgically	12 (30) located in GB, 7 (18) located in CBD	Selected patients with indications for surgery
Dhiman <i>et al</i> ^[17]	53 symptomatic and asymptomatic patients	11 (20) (7 located in GB while 4 in CBD)	Stones were found more frequently in patients with symptoms than asymptomatic patients
Ozkavukcu <i>et al</i> ^[30]	16 patients diagnosed with PVT evaluated by MRCP	0 (there are 3 patients with history of cholecystectomy but the reasons are not given)	Patients with history of, chronic liver diseases, liver abscess and hydatid cyst were not eliminated with potential interference with findings of biliary system
Chiu <i>et al</i> ^[31]	29 children with PVT diagnosis evaluated for Rex shunt	5 (17) located in GB, 4 (14) had biliary sludge	Only children evaluated for possible surgery included
Yamada <i>et al</i> ^[32]	21 children with PVT	3 (14) located in GB	21 patients selected out of 35, selection criteria not mentioned

PVT: Portal vein transformation; ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography; PD: Portal ductopathy; CBD: Common bile duct; GB: Gallbladder; PVCT: Portal vein cavernomatous transformation.

bias in patient selection criteria. Most of the studies only included patients with symptoms in a retrospective design or included patients with chronic liver disease-cirrhosis or with mass-occupying lesions. In most of these studies, investigation methods [endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance cholangiopancreatography or ultrasound] were not uniform so it was not possible to make a definitive conclusion about incidence and prevalence of stones. The choice of investigation method has been found to have a clear impact on the results of anatomical location of cholelithiasis found in the biliary system. Therefore, current literature lacks prospective and well-designed studies to reveal the exact prevalence and incidence of cholelithiasis in a group of well selected PVCT patients irrespective of presence of symptoms.

The clinical findings related to cholelithiasis in PVCT patients are not different from patients who do not have PVCT, but it is evident that it results in a high frequency of PD symptoms. Cholelithiasis is found more commonly in patients with biliary findings such as jaundice, biliary colic, cholangitis and elevated liver enzymes compared with asymptomatic patients^[7,15].

TREATMENT

The management of stones in patients with PVCT merits special attention since concomitant changes in the biliary system may jeopardize standard therapeutic proce-

dures. The collaterals of Petren and Saint around major bile ducts enlarge in response to PVCT and they form the basis of collateral vessel compression to major bile ducts in PD^[6,7]. Also, secondary to ischemic injury and development of fibrous tissue in the major biliary ducts, biliary interventions should be performed with caution. Since these varicose collaterals and fibrous strictures may result in detrimental complications like hemobilia or recurrent cholangitis, clinicians should follow special management strategies being aware of these potential risks. Otherwise, therapeutic strategies for cholelithiasis in PD are not different from other patient groups.

In previous studies focusing on treatment of PD, the major concern and attention was given to the problem of biliary strictures. The treatment of strictures and associated cholestasis is important since resolving these factors also reduces the risk of formation or recurrence of cholelithiasis. Biliary stricture and associated jaundice can be managed by medical treatment with ursodeoxycholic acid^[11,16] or by interventions like biliary stenting^[16-18], balloon dilatation^[19] or portosystemic shunting^[20]. If present, cholelithiasis should first be managed by evaluation of varicose veins around the common bile duct, either by magnetic resonance angiography or endoscopic ultrasonography^[17]. After elimination of varicose veins, standard sphincterotomy and balloon trawl for extraction of common bile duct stones and/or sludge should be employed^[10]. The presence of any collateral varicose vein is very important since inadvertent interventions

like basket removal of stones or biliary stenting might result in bothersome hemobilia^[21-25] requiring massive transfusions, injection sclerotherapy^[26], terlipressin^[25] or urgent portosystemic shunting^[21]. In our personal experience, we propose that papillotomy should be performed at earlier stages of disease as a prophylactic measure to make future ERCP procedures safer and easier. If not performed earlier, varices and fibrosis in the distal main bile duct can preclude or complicate safe papillotomy.

In conclusion, cholelithiasis and choledocholithiasis in the setting of PVCT and PD is a specific problem that needs special attention. Stones can become very problematic and interventions may result in severe hemobilia. Stone extraction by balloon or basket trawl can be gruelling due to strictures and fibrosis in biliary system. If present, biliary strictures and severe vascular compression should be treated by medical or interventional treatments in order to facilitate stone extraction and reduce recurrence. Further well designed prospective studies are required to clearly understand the exact natural history of cholelithiasis in PVCT patients.

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Acute pancreatitis in aging animals: Loss of pancreatitis-associated protein protection?

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Abstract

AIM: To investigate the effect of age on severity of acute pancreatitis (AP) using biochemical markers, histology and expression of the protective pancreatitis-associated proteins (PAPs).

METHODS: AP was induced *via* intraductal injection of 4% sodium taurocholate in young and old rats. Sera and pancreata were assayed at 24 h for the parameters listed above; we also employed a novel molecular technique to assess bacterial infiltration using polymerase chain reaction to measure bacterial genomic ribosomal RNA.

RESULTS: At 24 h after induction of AP, the pancreata of older animals had less edema (mean $\pm$ SE histologic score of young *vs* old: 3.11 ± 0.16 *vs* 2.50 ± 0.11 , $P < 0.05$), decreased local inflammatory response (histologic score of stromal infiltrate: 3.11 ± 0.27 *vs* 2.00 ± 0.17 ,

$P < 0.05$) and increased bacterial infiltration ($174\% \pm 52\%$ increase from sham *vs* $377\% \pm 4\%$, $P < 0.05$). A decreased expression of PAP1 and PAP2 was demonstrated by Western blotting analysis and immunohistochemical staining. There were no differences in serum amylase and lipase activity, or tissue myeloperoxidase or monocyte chemoattractant protein-1 levels. However, in the most-aged group, serum C-reactive protein levels were higher (young *vs* old: 0.249 ± 0.04 mg/dL *vs* 2.45 ± 0.68 mg/dL, $P < 0.05$).

CONCLUSION: In older animals, there is depressed PAP expression related to a blunted inflammatory response in AP which is associated with worsened bacterial infiltration and higher C-reactive protein level; this may explain the more aggressive clinical course.

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Key words: Acute pancreatitis; Aging; Rats; Pancreatitis-associated protein; Molecular biology

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INTRODUCTION

Acute pancreatitis (AP) is a disease with significant impact in the United States. In 2003 there were about 226 000 admissions for this diagnosis, at a cost of over \$2.2 billion^[1]. The incidence of pancreatitis is higher^[2], and its severity is

worse^[3] in the older patient. While Frey *et al*^[3] showed that the increased comorbidities associated with age account, in part, for the worsened severity, he and others also showed that age is an independent prognostic indicator of survival, specifically in patients over the age of 70 years^[3-5].

The mechanism that puts the aged pancreas, and aged patient, at risk for severity in pancreatitis is unknown. Possibilities include: the immune response of the organism to the injury is impaired; the host organ response to the injury (e.g., lung or renal) is worsened; or protective mechanisms from the pancreas are depressed. The latter two concepts are supported by the observation that the aged patient with pancreatitis has a higher incidence of systemic complications, while there is a decreased local complication rate^[4].

We have recently defined that pancreatitis-associated proteins (PAPs) are proteins innate to the pancreas which are induced during AP. PAPs are members of the pancreatic regenerating (Reg) family of proteins, which are calcium-dependant lectins; all are secretory proteins^[6-8]. PAPs are homologues of pancreatic reg I, and in rats, mice and man, localize to the same chromosome^[7,9-12]. However, while reg I is constitutively expressed in normal pancreas, PAP mRNA expression is very low. PAPs are highly induced during AP^[7,13].

PAPs are endogenous protectors against pancreatic injury. We have shown that targeted inhibition of the three rat PAP isoforms, PAP1, 2 and 3, exacerbated AP^[14,15]. They likely protect by immunomodulation^[16-18]. In particular, PAP2 protects by inducing macrophages, and PAP1 has other anti-inflammatory and anti-apoptotic effects^[19].

Loss of protection by PAP with aging may put patients at risk for pancreatitis. The normal expression of the PAP homologue pancreatic regeneration gene I (*reg I*), decreases in aging^[20], resulting in impairment of its function in the aged animal. We postulate that PAP levels are also depressed in aging, resulting in less protection in AP and more severe disease. However, since baseline PAP levels are minimal in the normal pancreas, we needed to measure the extent of its induction after stimulation.

In this study, we used sodium taurocholate (NaT) to induce AP and determined: (1) the extent of pancreatitis in young and old rats; (2) the extent of PAP expression in young and old animals with pancreatitis; and (3) how PAP expression relates to severity of pancreatitis in the older animals. We employed a novel molecular method to measure bacterial infiltration.

MATERIALS AND METHODS

Animals

Sprague Dawley male rats weighing 260-1000 mg (Charles River, Wilmington, MA) were divided follows: animals in the young group had an average age of 91.9 ± 3.7 d (range 82-111 d, $n = 10$), and those in the older group averaged 339 ± 17.9 d (range 291-546 d, $n = 21$). Each group had 10 unoperated and 10 sham-operated animals as controls.

Animals were fed standard laboratory chow, given

water *ad libitum*, and were randomly assigned to control or experimental groups. All animal studies were approved by the Division of Animal and Laboratory Resources, SUNY Downstate Medical Center.

Pancreatitis

AP was induced with 4% NaT, by retrograde injection into the pancreatic duct as previously described^[14]. Briefly, under nembutal anesthesia (50 mg/kg intraperitoneally, Abbott Laboratories, North Chicago, IL), a midline incision was performed. The common bile duct was identified and cannulated in a retrograde direction with PE-10 tubing (Fisher Scientific, Pittsburgh, PA) through the ampulla of Vater *via* small needle puncture of the duodenum. The bile duct was ligated to prevent the flow of bile, and NaT was infused into the pancreatic duct at a rate of 1 mL/kg over 30 min. The abdomen was then closed with monofilament suture, and the animals allowed to recover.

Animals assigned to the sham group were anesthetized under the same protocol as the sodium taurocholate group. They were then subjected to a midline incision, pancreaticoduodenal manipulation, and then similarly closed and recovered.

Post-operative analysis

The animals were sacrificed 24 h after surgery. Swab cultures of the pancreata were taken, after which whole blood was taken *via* an 18-Gauge syringe in the inferior vena cava. The pancreas was then subdivided into 30 mg portions for pathology (10% formalin), RNA isolation (liquid nitrogen and rapid isolation), and protein isolation.

Qualitative cultures were performed in the clinical microbiology laboratory at SUNY Downstate, for which samples were plated on agar plates for 48-72 h.

To calculate edema, a pancreatic sample was immediately weighed on a coverslip and placed in a 37 °C dry oven. These samples were then re-weighed at 72 h on the same scale. The wet-to-dry ratio was then calculated.

Other pancreas samples were homogenized in 25 mmol/L Tris/0.5% Triton (Sigma, St. Louis, MO) in a glass homogenizer and centrifuged at 12 000 *g* for 10 min at 4 °C. After aspiration of the lipid layer, the supernatant was poured into a 1.5 mL tube and the pellet discarded. Proteins were assessed by the Bradford Assay (Bio-Rad Laboratories, Inc., Richmond, CA) and all samples were diluted to 2 mg/mL in phosphate buffered saline (PBS) (Lonza, Walkersville, MD), treated with protease inhibitor cocktail (Sigma) and stored at -20 °C until analyzed.

Ribonucleic acid isolation

Pancreas samples were flash frozen in liquid nitrogen and immediately processed using 600 µL Trizol reagent (Fisher Scientific, Piscataway, NJ). The samples were homogenized and mixed with 75% ethanol. The tubes were centrifuged at 10 000 *g*, and the clear lysate was applied to an RNeasy column (Qiagen, Valencia, CA). They were

centrifuged at 10 000 *g* for 15 s and then rinsed with wash buffer, and subjected to DNase digestion.

Blood

Whole blood was collected in red-top tubes without anticoagulant (Fisher, Piscataway, NJ) and allowed to clot for 30 min at room temperature. Tubes were centrifuged at 1800 rpm for 20 min. Sera were collected and stored at -80 °C until analyzed.

Serum amylase activity was measured using 4,6-ethyridene (G7)-p-nitrophenyl (G1)- α -D-maltoheptaoside as the substrate. Serum lipase was measured by the Clinical Laboratories, SUNY Downstate Medical Center. Similarly, C-reactive protein (CRP) levels were determined in the clinical laboratory using a conventional immunoassay system and Beckman nephelometer (Beckman Coulter, Inc., Galway, Ireland).

Myeloperoxidase, monocyte chemotactic protein-1 analysis

Pancreatic protein was assessed for rat monocyte chemotactic protein-1 (MCP-1) using the Rat MCP-1 ELISA Kit for tissue lysate (RayBiotech, Inc., Norcross, GA) and myeloperoxidase (MPO) by the rat MPO ELISA kit (Cell Sciences, Canton, MA) according to the manufacturer's protocol.

Polymerase chain reaction

One-step real-time quantitative reverse-transcriptase-polymerase chain reaction (PCR) for PAP 1, 2, 3, and *reg I* was performed as previously described^[13,14] using the LightCycler 480 (Roche, Indianapolis, IN) with β -actin as an endogenous control to standardize the amount of sample RNA added to a reaction. Primers and probes were designed as previously for PAP 1, 2, and 3^[13,14]; the primers for *reg I* were 5'-TACAGCTGCCAATGTCTGGATT-3' (forward), 5'-CAGTGTCCAGGATTTGTAGAGA-3' (reverse), and 5'-ATCCCAAAAATAATCGCCGCTGGC-3' (probe) (Applied Biosystems, Bedford MA). One hundred nanograms of total RNA was used to set up 20 μ L real-time quantitative PCRs that consisted of 1 $\times$ Master Mix, 500 nmol/L forward and reverse primers, and 250 nmol/L TaqMan probe (Roche). Polymerase chain reaction amplification was performed with the temperature profile of: 3 min at 45 °C, 7 min at 95 °C, and 45 cycles of 5 s at 95 °C and 30 s at 60 °C. Assays were performed in triplicate. Data were analyzed with the relative standard curve method. Standard curves of the genes of interest and β -actin were prepared with six ten-fold dilutions of total RNA from the sample that was expected to have the highest amount of mRNA for the gene of interest. For each reaction tube, the amount of target or endogenous reference was determined from the standard curves. The mean amount of each sample was calculated from the triplicate data and was normalized by division by the mean quantity of β -actin RNA for the same sample. The mean and standard error of each

treated group were calculated from the normalized value for each rat in the group.

Bacterial 16s ribosomal DNA from pancreatic tissue was amplified by PCR as described^[21], using probes specific for gram-positive cocci (GPC) and gram-negative rods (GNR). The primers are specific for bacterial 16sRNA, a gene highly conserved in bacterial strains. Briefly, 0.2 μ g of total DNA extracted from each pancreas tissue isolated above was added to pre-decontaminated PCR cocktail for PCR amplification with the primers. The product is a 370 bp fragment.

Western blotting analysis

After SDS-polyacrylamide gel electrophoresis (15%), 15 μ g and 30 μ g protein/well was electrophoretically transferred to nitrocellulose (Nytan; Schleicher and Schuell, Keene, NH) and monoclonal antibody to *reg I*^[22] was used at 1:10 000 dilution, polyclonal antibodies to PAP 1, and PAP 2^[22] were used at 1:1000 dilution. Secondary antibody to IgG at 1:100 000 dilution was used for detection of primary antibody.

Densitometry was performed using the Bio-Rad Gel Documentation System with Quantity One software. Units are presented as Intensity (INT) $\times$ area (mm²).

Histopathological analysis

The head of the pancreas from each rat was fixed in 10% buffered formaldehyde solution (Fisher Scientific, Pittsburgh, PA). Four to six micron section slides were generated for each pancreas, collected and stained with hematoxylin and eosin (H and E). Using previously described criteria, the histological severity of pancreatitis was examined in a blinded fashion by our pathologist. Using a scale ranging from zero (representing the least), to four (representing the greatest) severity, the degree of leukocytic infiltration (acinar and stromal) and tissue necrosis (acinar and stromal), edema and hemorrhage were determined for each specimen.

Immunohistochemical analysis was done as described in^[23] using PAP1 and 2 antibodies at 1:1500 dilution.

RESULTS

NaT experiments

There were three mortalities after surgery to induce pancreatitis; 2 in the young and one in the old group. Where appropriate, we show data from shams and unoperated controls for clarity and comparisons. Where noted, we performed subgroup analysis of the older animals, dividing them into: subgroup (a) aged, whose average age was 256.3 $\pm$ 2.1 d (*n* = 10); subgroup (b) very aged, 372.5 $\pm$ 0.4 d (*n* = 9); and subgroup (c) most aged, 544 $\pm$ 2.8 d (*n* = 3). The latter group had the single mortality of the older group.

Twenty four hours after induction of NaT pancreatitis, older animals had slightly less edema, as measured by ratio of wet to dry weight, when compared to younger ones (1.35 $\pm$ 0.04 *vs* 1.52 $\pm$ 0.08, *P* = 0.07). By compari-

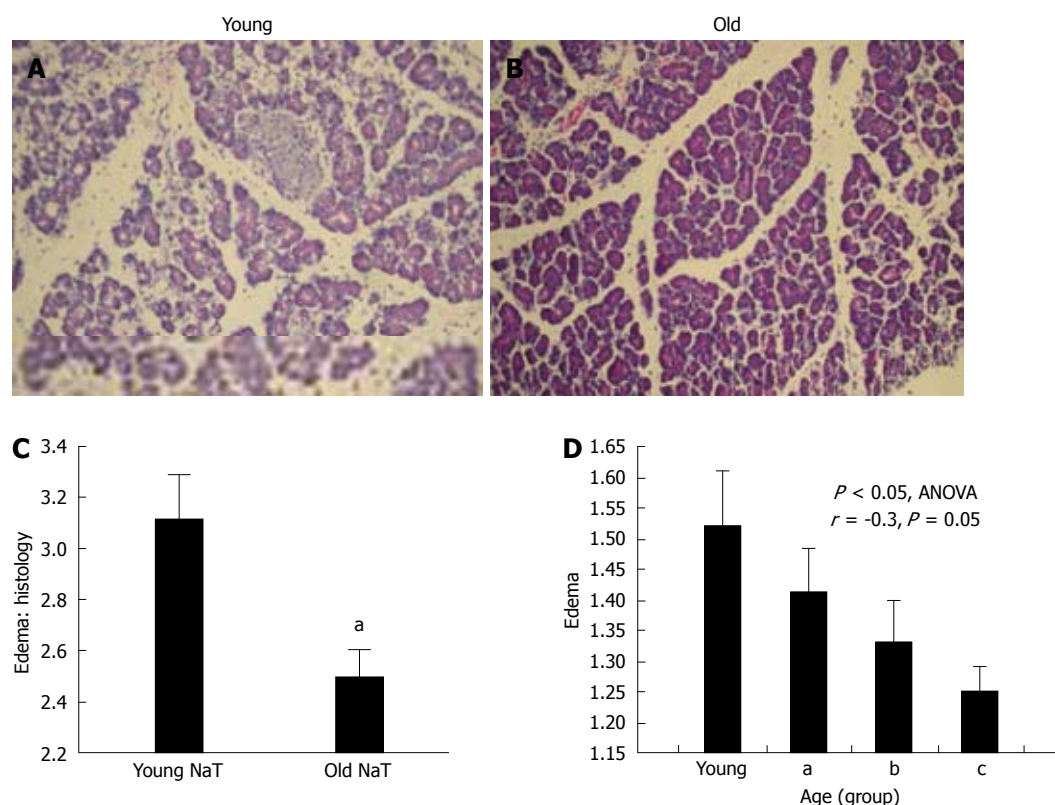


Figure 1 Effect of age on pancreatic edema 24 h after induction of sodium taurocholate pancreatitis. A: A young rat with severe, grade 4 edema [hematoxylin and eosin (H and E), 40×]; B: An old rat with moderate, grade 3 edema (H and E, 40×); C: Mean ± SE histologic scoring of edema for both groups; D: Edema data calculated by wet:dry ratio of pancreatic tissue. While young vs old ratios are discussed in the text, when animals were sorted into young, aged (group a), very aged (group b) and most aged (group c) groups, a progressive decrease in edema was seen with age. A significant negative correlation was also noted (^a*P* < 0.05, Student's *t* test). NaT: Sodium taurocholate; ANOVA: Analysis of variance.

son, sham-operated animals had 1.12 ± 0.03 wet to dry ratios; unoperated controls had 1.01 ± 0.08 (all statistically significantly lower than the NaT group).

Histopathologic examination also showed that the extent of edema was significantly depressed in the older group (Figure 1A-C, *P* < 0.05). When subgroups of age (subgroups A-C), were subjected to analysis of variance (ANOVA), there was a statistically significant decrease (Figure 1D, *P* = 0.02), and significant negative correlation (*r* = -0.3, *P* = 0.05) with aging.

Histopathologic examination also showed that the extent of parenchymal and stromal inflammatory cell infiltrates was depressed in aging (Figures 2A and B, 3B and C). The extent of necrosis (acinar, peripheral acinar or fat necrosis), cellular vacuolization and hemorrhage was not different in the older animals (Figure 3A).

Markers of pancreatitis

When compared to sham-operated controls, animals with NaT pancreatitis showed significant increases in amylase (628 ± 63 μg/L *vs* 7919 ± 2022 μg/L, respectively, *P* < 0.05), and lipase (37.7 ± 16 μg/L *vs* 864 ± 260 μg/L, respectively, *P* < 0.05). Also, pancreatic levels of MPO were very high (202 ± 49 ng/mL *vs* 2014 ± 628 ng/mL, respectively, *P* < 0.05), as were levels of MCP-1 (2172 ± 463 pg/mL *vs* 4508 ± 251 pg/mL, respectively, *P* < 0.05). However, when values from old and young NaT animals

Table 1 Effect of age on biochemical markers of sodium taurocholate pancreatitis

	Young rats	Old rats
Amylase (μg/L)	12 536 ± 6141	5941 ± 1162
Lipase (μg/L)	1288 ± 755	682 ± 194
MPO (ng/mL)	1962 ± 538	2066 ± 1219
MCP-1 (pg/mL)	4312 ± 350	4730 ± 376
CRP (mg/dL)	0.25 ± 0.04	0.61 ± 0.19

All serum levels were higher than sham and unoperated controls. No differences were seen between young and old animals. However, subgroup analysis of CRP levels revealed that the most-aged subgroup (c) had significantly higher levels (Figure 3). MPO: Myeloperoxidase; MCP-1: Monocyte chemoattractant protein-1; CRP: C-reactive protein.

were compared, no differences were noted (Table 1).

Serum CRP levels in control, unoperated animals were 0.17 ± 0.07 mg/dL. A significant increase in CRP was seen after NaT (0.25 ± 0.04 mg/dL, respectively, *P* = 0.05 compared to unoperated controls); but when young and old NaT animals were directly compared, no difference was seen between younger and older groups (Table 1). However, subgroup analysis of age groups showed that serum CRP was statistically higher in the most-aged (subgroup c) (Figure 4) (2.45 ± 0.69 mg/dL, *P* < 0.001 ANOVA compared to all groups, *P* < 0.001 compared to young, Student's *t* test). This phenomenon was noted only for

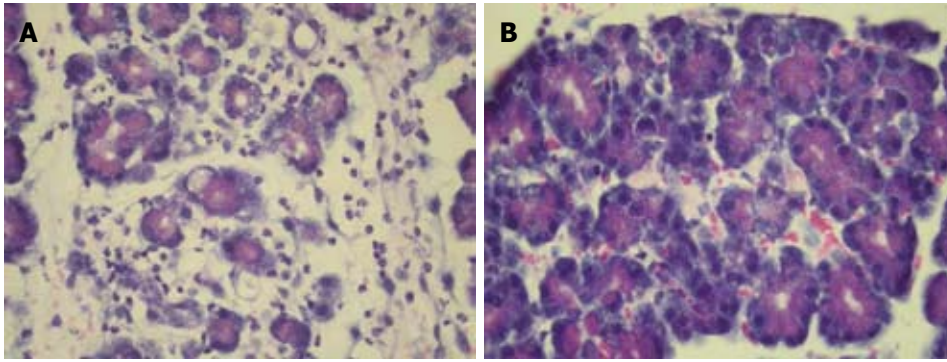


Figure 2 Histology (hematoxylin and eosin, 100 $\times$) of young (panel A) and old (panel B) inflammatory infiltrates 24 h after induction of sodium taurocholate pancreatitis. A: Severe, grade 4 parenchymal inflammation in the young animal; B: Mild, grade 2 inflammation in an older one.

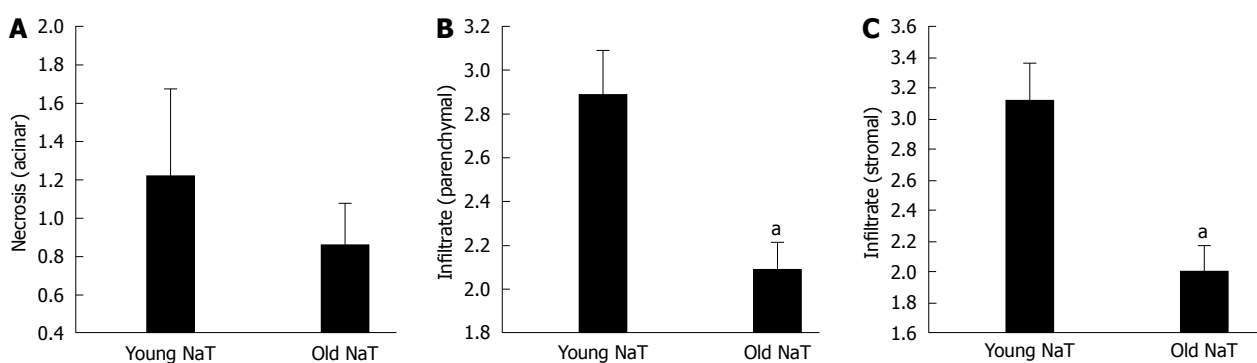


Figure 3 Histologic scores. There was no difference in necrosis scores between young and old (C), but significant decreases were observed in inflammatory infiltrates of the parenchymal (D) and stromal (E) tissue ($^aP < 0.05$, Student's *t* test). NaT: Sodium taurocholate.

CRP, not for any of the other markers measured above (amylase, lipase, MPO, MCP-1).

Real time PCR of mRNA isolated from rats with pancreatitis showed that *reg I*, PAP1, PAP2 and PAP3 all increased when compared to controls (not shown). The increase was similar to that which we have observed previously^[13,14] with *reg I* increasing twofold, and PAP1, 2, and 3 increasing 50-100 fold (not shown). Western analysis showed that protein levels increased but only minimally for *reg I* and by a factor of 3-8 fold for PAP1 and 2.

When young and old NaT animal mRNAs were compared, no differences were noted for pancreatic *reg I*, PAP1 and PAP2 mRNA (Table 2); and only PAP3 levels were significantly decreased. Conversely, when protein levels were compared, older animals had lower levels of PAP1 ($P < 0.05$); PAP2 proteins were depressed as well ($P = 0.07$) (Figure 5). PAP3 proteins were not measured since antibodies were not readily available.

Since we have previously shown *in vitro* that PAP2 directly activates leukocytes^[17], we postulated that its expression would correlate with MPO or MCP-1 activity. While there were no correlations between mRNAs or proteins and MCP-1 levels, a significant positive correlation of PAP2 mRNA levels with MPO protein was observed ($r = 0.81$, $P = 0.004$).

Immunohistochemistry

PAP1 and 2 immunohistochemistry showed no staining in normal pancreas, and positive staining pattern in the acinar cells (Figure 6A-C) after induction of NaT pancreatitis. Similar to our observation in the Western analysis, both PAP1 and 2 immunohistochemistry showed more intensity in the younger animals when compared to older (Figure 7); preparation of the slides was done side by side.

In young animals, PAP1 staining in AP was typically intracellular, uniform, and oriented in a base-to-apical intensity (Figures 6B, 7A). In contrast, PAP2 staining in AP was heterogeneous (Figures 6C, 7C): it too was intracellular, but was occasionally visible as dense, clumped proteins towards the base of the cells.

In older animals the staining patterns reversed: PAP1 staining became more heterogeneous (Figure 7B), and the pattern for PAP2 became more homogenous (Figure 7C).

Interestingly, in many sections, PAP2 staining was found in areas which corresponded to parenchymal and stromal inflammatory infiltrates (Figure 8).

Bacteriology

Qualitative cultures of the pancreata from almost all the animals showed colonization by GPC and GNR, with no difference between old and young animals. For example,

Table 2 Effect of age on pancreatic Reg1 and pancreatitis-associated protein mRNA levels

	Young rats	Old rats
Reg1	0.727 ± 0.19	1.27 ± 0.22
PAP1	119 ± 46	116 ± 18
PAP2	690 ± 267	475 ± 142
PAP3	57.8 ± 19	26.4 ± 4.3 ^a

Real time polymerase chain reaction analysis of RNA isolated from pancreata from young and old rats 24 h after induction of pancreatitis by sodium taurocholate. All were done against a β -actin control. No difference in RNAs were found except for pancreatitis-associated protein (PAP)3 (^a $P < 0.05$, Mann Whitney *U* test).

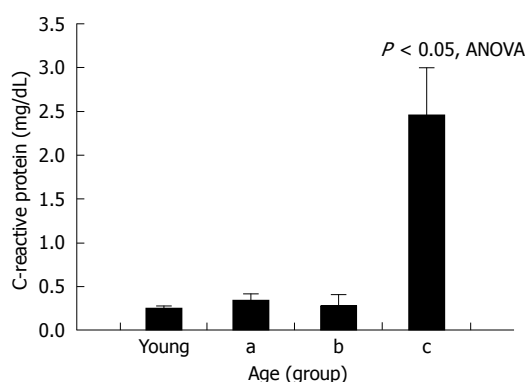


Figure 4 Effect of age on serum C-reactive protein level 24 h after induction of sodium taurocholate pancreatitis. Data for young vs old animals were not significantly different and are shown in the text and Table 1. However, when animals were sorted into young, and subgroups of aged (subgroup a), very aged (subgroup b) and most aged (subgroup c), a significant increase in C-reactive protein was seen in the most aged group (subgroup c). ANOVA: Analysis of variance.

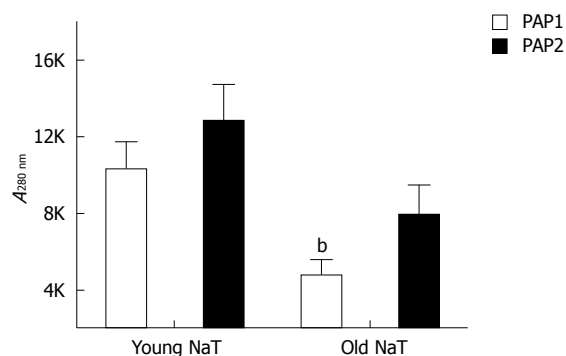


Figure 5 Western blotting analysis of pancreatitis-associated protein 1 and pancreatitis-associated protein 2 protein levels from pancreatic tissue 24 h after induction of sodium taurocholate pancreatitis. Both decreased with age, with statistical significance in the pancreatitis-associated protein (PAP)1 group (^b $P = 0.003$), and near significance in the PAP2 group ($P = 0.06$). Units are based on densitometry and expressed as intensity $\times$ area (mm^2). NaT: Sodium taurocholate.

5 of 9 young animals with NaT pancreatitis grew GPC, while 8 of 9 grew GNR; only one animal had no culture positive. In the older group, 8 of 19 grew GPC, while 18 of 19 grew GNR; only one had no culture positive.

In order to accurately quantitate bacterial infiltration of the pancreas, we employed a novel molecular technique to discern the amount of bacterial genomic ribosomal RNA (rRNA) in the pancreata. As shown in Figure 9,

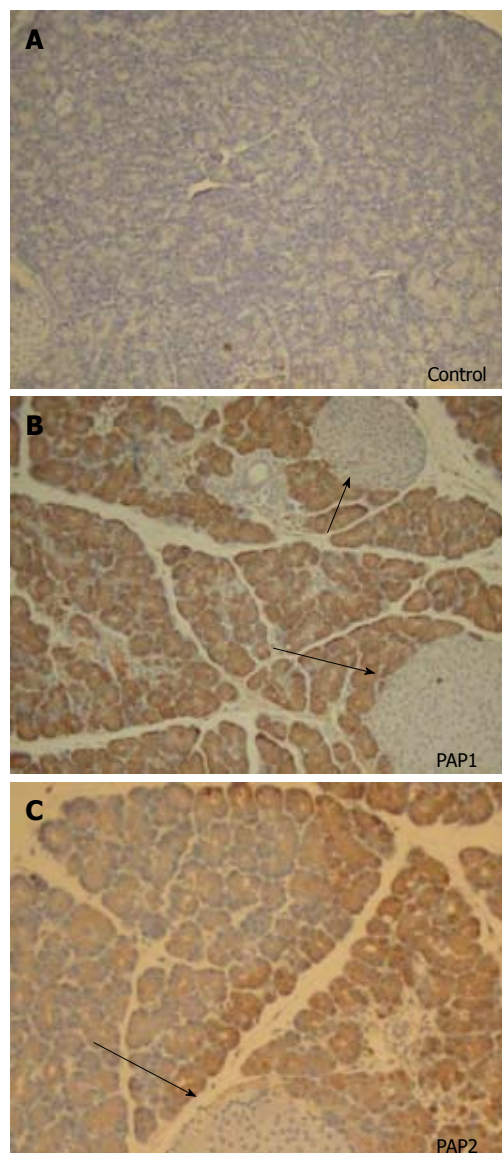


Figure 6 Immunohistochemistry for pancreatitis-associated proteins (40 $\times$) in a young control animal without pancreatitis (A) compared to pancreatitis-associated protein 1 (B) and pancreatitis-associated protein 2 (C) in young animals 24 h after sodium taurocholate pancreatitis. No staining is seen in controls, uniform brown colored staining is seen for pancreatitis-associated protein (PAP)1 (B). Preparation of the slides was done side by side. A different, heterogeneous pattern of staining is seen for PAP2 (C). Note that islets of Langerhans (arrows) do not stain at all for PAPs in normal animals or animals with acute pancreatitis.

compared to sham-operated animals, there was an increase in bacterial genomic rRNA in both the young and old NaT animals, and a more intense amount of bacterial rRNA in the older animals when compared to young.

DISCUSSION

In the present study, we found that 24 h after induction of AP, the pancreata of older animals have less edema, decreased inflammatory response and increased bacterial infiltration. This is associated with decreased expression of PAPs as measured by Western analysis and immunohistochemistry. While there were no differences between young

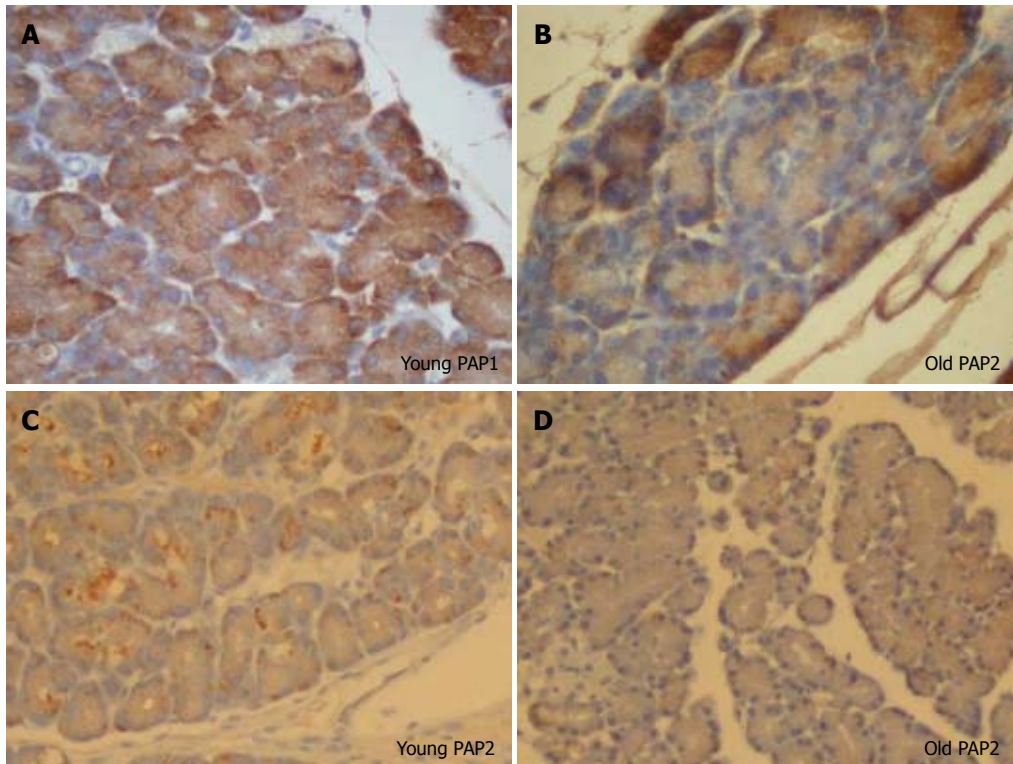


Figure 7 Immunohistochemical staining for pancreatitis-associated protein 1 and 2, high power (100×). Young animals (A, C) are compared to older (B, D). A decreased intensity of staining is observed in aging, and while pancreatitis-associated protein (PAP)1 staining in younger animals is uniform, in older animals it is heterogeneous. In contrast, while PAP2 staining patterns are heterogeneous in younger animals, they are more uniform in older ones. Note the clumping of PAP2 within the cells of the younger animals, which is absent in the older group.

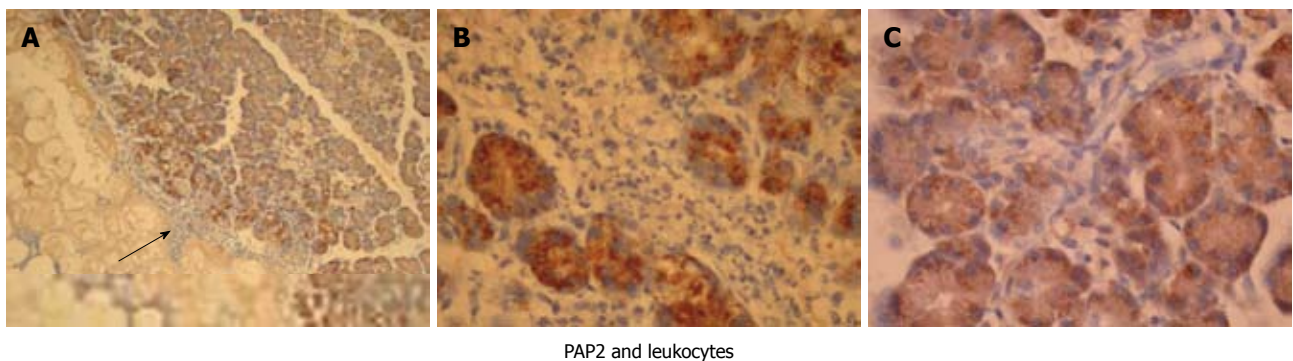


Figure 8 Association of pancreatitis-associated protein2 staining with inflammatory infiltrates. A: Immunohistochemical staining in animals 24 h after sodium taurocholate. The arrow points to intense stromal infiltration near the periphery of intense pancreatitis-associated protein (PAP2) staining (40×); B: Association of PAP2 positive cells with stromal leukocyte infiltration depicted by arrow in A (100×); C: Association of PAP2 positive cells with parenchymal leukocyte infiltration (100×). This association was not seen for PAP1 (not shown).

and old animals in serum amylase and lipase activity, in the most-aged subgroup (c), there was an increase in CRPs.

These data suggest that the local response to AP is less severe in the older animals, and the systemic response may be worse. PAPs are endogenous protectors against pancreatic injury^[14-18], and it appears that the decrease in PAPs may not be related to the decreased inflammatory infiltrate. As will be described below, the effect may be a result of an altered balance of PAP 1 and 2. The decrease of PAPs may also result in increased bacterial infiltration into the pancreas, and ultimately a worsened outcome.

While the pancreas progressively atrophies with age,

its reserve capacity results in minimal clinical changes. Studies comparing baseline and stimulated levels of pancreatic enzymes have shown no significant differences^[24,25]. However, the regenerative capacity of the pancreas is diminished^[26,27], as evidenced by decreased recovery from pancreatitis. At the molecular level, this has been attributed to decreased phosphorylation of Akt^[27].

Very little is known about the aged pancreas in AP. As we have observed in animals, in humans there seems to be a blunted local response and an exaggerated systemic one. Fan *et al*^[4] noted decreased local complications but increased systemic complications, leading to death.

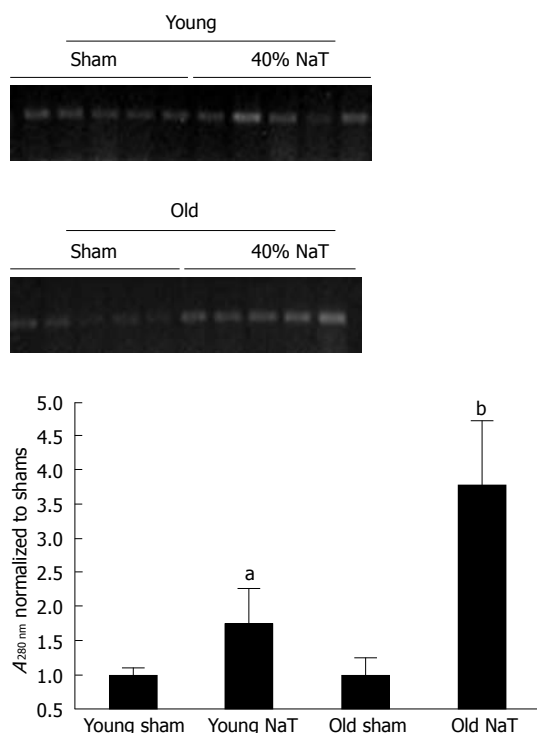


Figure 9 Molecular quantitation of bacterial levels in pancreatic tissue in unoperated controls, sham-operated animals and 24 h after sodium taurocholate pancreatitis. DNA from pancreas was subjected to semiquantitative polymerase chain reaction (PCR) using probes for the genomic sequences of bacterial ribosomal RNA. Top Panel shows representative polyacrylamide gels showing the 370 base pair PCR product; Bottom graph shows quantitated values from multiple experiments. No differences were seen between controls and sham-operated animals (not shown). There were significant differences in bacterial genomic levels between sham and sodium taurocholate (NaT) animals (^a $P < 0.05$), and between young and old (^b $P = 0.008$) NaT animals, when percent increases were compared (Mann-Whitney U test).

Kimura *et al.*^[28] described elderly patients with high mortality from AP, but autopsy evidence revealed only mild interstitial pancreatitis, where the inflammation was centered in the ductal and interstitial tissues regions.

We postulated that as animals age, there would be loss of protective mechanisms from the pancreas which would lead to worsened fate after pancreatitis. We focused on the PAP family since we and others have shown that they are endogenous proteins which are protective in AP^[14-16,29]. We know that expression of other genes of the PAP family decrease with aging^[20], and we formulated experiments to determine whether PAPs decreased too.

We showed that in older rats subjected to NaT, there is blunted PAP1 and 2 protein response in both Western analysis and immunohistologic staining patterns. How does this correlate with the histologic and biochemical data above?

PAP1's protective activity may be local or systemic. Locally, it has both mitogenic^[30] and anti-apoptotic^[31] effects, which affect regeneration after pancreatitis. PAP1 is a known anti-inflammatory agent^[16], and a potent modulator of lung injury in pancreatitis^[29,32]. Its loss with aging

may worsen the systemic response after acute pancreatitis.

However, our results differ from others who observed increased local inflammatory infiltrates in PAP1 knockout mice^[19]. Our model differs not only in that we employed a different rodent species, but in the fact that other proteins, such as PAP2, 3 and other inflammatory modulators, are likely also depressed.

PAP2's protective activity, on the other hand, is likely local. It can directly activate macrophages^[17], and macrophage activation has been shown to be protective in pancreatitis^[33-35]. PAP2 is a more potent immunomodulator than PAP1^[36]. In our present study we even noted that PAP2-positive pancreatic cells co-localize with leukocytes (Figure 8); this was not observed with PAP1. We also noted a positive correlation of PAP2 with pancreatic MPO activity. Activation of macrophages can induce MPO activity^[37,38], and it is possible that a decrease of PAP2 will be associated with decreased macrophages or macrophage activity, which is detrimental for the pancreas and host. The decrease in PAP2 may really be the reason for the decreased leukocyte infiltration seen in older animals.

Finally, PAP proteins directly interact with bacteria. They can bind gram-negative^[7] and gram-positive bacteria^[39], and we have discovered a bacteriocidal action of PAP1 and 2 (unpublished results). Our data suggest there is more bacterial infiltration in the older pancreata, as evidenced by increased quantity of bacterial rRNA. The blunted PAP responses may result in more acute pancreatic infection.

Increased bacterial colonization could lead to an increased systemic response to the injury, which is worsened by decreased PAP1 level. It is possible that PAPs are endogenous bacteriostatic or bacteriocidal agents that protect against infection during AP. Whether the loss of PAP with aging leads to increased clinical pancreatic infection remains to be seen. To study this, longer term experiments along with qualitative analysis of bacterial species will be necessary.

In conclusion, this is the first study to show a physiologic difference in AP between young and old animals, and the first to show that endogenous pancreatic molecules are related to the diathesis of the disease. In older animals, there is decreased edema and leukocyte infiltration, but increased bacterial infiltration and even serum CRP level. All are associated with decreased endogenous PAPs in the pancreas. Further studies are planned on the direct effect of all three PAPs in modulating the severity of pancreatitis, by focusing on their local immunomodulatory effect and effect on bacterial infiltrates, as well as the systemic response of the host.

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COMMENTS

Background

Acute pancreatitis (AP) is a disease with significant impact and expense worldwide. In the United States, the cost is significant, and only recently has the impact of this disease on the elderly begun to emerge.

Research frontiers

The mechanism that puts the aged pancreas, and aged patient, at risk for severity in pancreatitis is currently being explored. Impaired immune system, host organ response to the injury or protective mechanisms from the pancreas are possibilities. A molecular mechanism is the likely source.

Innovations and breakthroughs

The authors recently showed that pancreatitis-associated proteins (PAPs) are protective in pancreatitis. The authors postulated that their expression would be depressed in elderly animals with pancreatitis, resulting in worsened disease.

Applications

By understanding how PAPs affect the outcome of pancreatitis, the authors suggest that physicians can then employ strategies to enhance their expression in elderly patients with the disease and prevent complications. The authors also employed a new way of monitoring the infiltration of bacteria into the pancreas using polymerase chain reaction (PCR), which is much more sensitive than culture techniques.

Terminology

PAPs are members of the Reg family of pancreatic regenerative proteins. They have been shown to be important in pancreatic disease, including AP and gastrointestinal malignancies. Expression was monitored by standard techniques such as Western blot and immunohistochemical staining, as well as PCR. Myeloperoxidase and monocyte chemoattractant protein-1 analysis was used to monitor inflammation. Bacterial infiltration was monitored by PCR, using their 16S ribosomal RNA as the unique target.

Peer review

The overall goal of the paper is relevant. The data presented are solid and credible. The results are interesting and clinically important.

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Osteopontin increases hepatocellular carcinoma cell growth in a CD44 dependant manner

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Abstract

AIM: To investigate the role of osteopontin (OPN) and its splice variants in the proliferation of hepatocellular carcinoma (HCC).

METHODS: The expression of OPN variants in HCC cell lines as well as HCC tissue samples and non-tumour tissue was studied using polymerase chain reaction. OPN variant cDNAs were cloned into a mammalian expression vector allowing both transient expression and the production of stable OPN expressing cell lines. OPN expression was studied in these cells using Western blotting, immunofluorescence and enzyme linked immunosorbent assay. A CD44 blocking antibody and siRNA targeting of CD44 were used to examine the role of this receptor in the OPN stimulated cell growth observed in culture. Huh-7 cells stably expressing either OPN-A, -B or -C were injected subcutaneously into the flanks of nude mice to observe *in vivo* tumour

growth. Expression of OPN mRNA and protein in these tumours was examined using reverse transcription-polymerase chain reaction and immunohistochemistry.

RESULTS: OPN is expressed in HCC in 3 forms, the full length OPN-A and 2 splice variants OPN-B and -C. OPN variant expression was noted in HCC tissue as well as cognate surrounding cirrhotic liver tissue. Expression of these OPN variants in the HCC derived cell line Huh-7 resulted in secretion of OPN into the culture medium. Transfer of OPN conditioned media to naïve Huh-7 and HepG2 cells resulted in significant cell growth suggesting that all OPN variants can modulate cell proliferation in a paracrine manner. Furthermore the OPN mediated increase in cellular proliferation was dependent on CD44 as only CD44 positive cell lines responded to OPN conditioned media while siRNA knockdown of CD44 blocked the proliferative effect. OPN expression also increased the proliferation of Huh-7 cells in a subcutaneous nude mouse tumour model, with Huh-7 cells expressing OPN-A showing the greatest proliferative effect.

CONCLUSION: This study demonstrates that OPN plays a significant role in the proliferation of HCC through interaction with the cell surface receptor CD44. Modulation of this interaction could represent a novel strategy for the control of HCC.

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Key words: Osteopontin; Hepatocellular carcinoma; CD44 antigen; Nude mice; Xenograft

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly aggressive carcinoma of the liver, and is the fifth most common cancer worldwide, and the third leading cause of cancer related death^[1,2]. Risk factors for HCC include; infection with either hepatitis B virus (HBV) or hepatitis C virus (HCV), alcoholic cirrhosis and exposure to environmental toxins such as aflatoxin. HCC is a global disease most prevalent in Southeast Asia and sub-Saharan Africa^[3], however, in countries such as the United States and Japan, HCC incidence is on the increase, primarily as a result of infection with HCV^[4,5]. Despite intense investigation, therapeutic intervention for HCC is extremely limited, with a poor prognosis due to high rates of recurrence and intrahepatic metastasis following surgical resection. Furthermore, there is a significant gap in our knowledge of the molecular mechanisms responsible for HCC development and progression.

Osteopontin (OPN) is a secreted multifunctional matrix-glycoprotein that is emerging as a significant protein in the biology of HCC. It is involved in normal tissue remodelling processes and is secreted to high levels in numerous tumours including HCC^[6]. OPN is overexpressed in HBV-related metastatic HCC^[7], while OPN antibodies can suppress pulmonary metastasis of HCC cells in a nude mouse model, suggesting that it plays a significant role in the metastatic potential of HCC^[8]. However, its role in development and proliferation of HCC is relatively unexplored, although a recent study involving the silencing OPN mRNA expression in HCC cell lines, suggested it may also have proliferative effects^[9]. OPN binds to the family of $\alpha\beta$ integrins, and the cell-surface adhesion molecule CD44, to initiate cellular signals that enable tumour progression^[10-12]. However, the role, if any, of CD44 and OPN in modulating HCC cell growth, is unknown.

Osteopontin is expressed as a heterogenous protein, dependent on glycosylation patterns and the type of cell from which it is expressed. For example, OPN derived from osteosarcoma-derived cells is smaller than that from non-transformed bone cells, with expression of the smaller form correlating with anchorage independence, suggesting that different forms of OPN have different phenotypic effects^[13]. In addition, the existence of two OPN splice variants have been described, with deletions in exon 4 (termed OPN-C) and 5 (termed OPN-B)^[14]. These variants were originally described in glioma cells and more recently OPN-C has been implicated in the invasiveness of breast cancer cell lines^[15] while in HCC

derived cell lines OPN-C promotes the extracellular cleavage by matrix metalloproteinase (MMP)-9, releasing a distinct 5 kDa OPN fragment that is essential for HCC cellular invasion^[16]. However, the relative expression of the OPN variants in HCC has not been formally demonstrated, nor have their effects on HCC cell growth been studied. In this study we demonstrate that all splice variant forms of OPN are expressed in HCC at the mRNA level and that all have the ability to stimulate the growth of HCC derived cell lines *in vitro* and in an *in vivo* ectopic xenograft mouse model. Furthermore this growth promoting effect was mediated by interaction of OPN with CD44 and adds significantly to our understanding of the role of OPN in HCC.

MATERIALS AND METHODS

Cells and tissue samples

The human hepatoma-derived cell lines used in this study were Huh-7, Hep G2 and Hep3B, while Hepa 1-6 cells are of mouse hepatoma origin. All cells were maintained in Dulbecco's Modified Eagle Medium, containing 4.5 g/L D-Glucose, 25 mmol 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid and 2 mmol/L L-glutamine (Invitrogen, CA, United States). Media was supplemented with 10% fetal calf serum, 12 μ g/mL penicillin and 16 μ g/mL gentamycin. To monitor cell growth, cultured cells were seeded at a density of 7×10^4 cells per well in a 12-well plate and cell numbers monitored daily using trypan blue exclusion. All experiments were performed at least in triplicate. Human HCC tissue and cognate surrounding tissue were collected from patients undergoing HCC resection at the Royal Adelaide Hospital (collection was approved by the Hospital's ethics committee).

Construction of OPN expression plasmids and transfection

Full-length OPN cDNA and splice variants were amplified from Huh-7 cells by reverse transcription polymerase chain reaction (RT-PCR). Total RNA and cDNA synthesis were performed as described elsewhere^[17]. The coding sequence for OPN was amplified using the primers 5'-GTTGAAGCTTCTCACTACCATGAGA-ATTGCAGTG-3' and 5-TAGTTCTAGACCTTTTA-ATTGACCTCAGAAGATG-3' and cloned into the mammalian expression vector pRC-CMV using *Xba* I and *Hind* III to generate pRC-CMV-OPN-A. During sequence verification, two smaller cDNAs were isolated that corresponded to OPN-B and OPN-C. These vectors were used in transient expression assays and to generate stable OPN expressing clones in Huh-7 cells using the transfection reagent Fugene (Roche, Australia) and G418 selection.

RNA extraction, cDNA synthesis and PCR analysis

Total cellular RNA was isolated from cells using Trizol (Invitrogen) and first strand cDNA was synthesized

and standard PCR performed as mentioned previously^[17]. Primers used for OPN cDNA detection were 5'-ATGAGA ATTGCAGTGATTTGCTTTTGCCT-3' and 5'-CATGGTCATCATCATCTTCATCATC-3'; primers utilised for the detection of CD44 were 5'-GACACATATTGCTTCAATGCT TCAGC-3' and 5'-GATGCCAAGATGATCAGCCATTCTGGAAT-3'; and glyceraldehyde-3-phosphate dehydrogenase primers were 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. Amplicons were electrophoresed through a 1% agarose gel before visualisation on a ultraviolet transilluminator.

Detection of OPN and CD44

Huh-7 cells, either stably selected or transiently expressing OPN, were lysed using RIPA buffer and total protein separated *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose as previously described^[18]. Membranes were blocked with 5% skim milk in 0.1% phosphate buffered saline Tween-20 (PBS-T) and incubated overnight at 4 °C with either 400 ng/mL of goat anti-human OPN antibody (K-20; SCBT, SantaCruz, CA) or mouse anti-human CD44 antibody (Labvision, Fremont, CA, United States) at 200 ng/mL followed by either 33 ng/mL of anti-goat or anti-mouse horseradish peroxidase (HRP) antibody (Rockland, Gilbertsville, PA, United States). Washes between antibody binding were with 0.1% PBS-T. Protein bound to antibody was visualised *via* chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ, United States).

Cellular localisation of transiently expressed OPN was performed *via* indirect immunofluorescence as previously described^[19] with the exception that cells were incubated in 1 µg/mL of anti-OPN antibody followed by 10 mg/mL anti-goat Alexa 488-conjugated antibody (Molecular probes, Eugene, OR). CD44 expression was visualised using a mouse anti-human CD44 antibody at 4 µg/mL on cells that had been fixed in 5% formalin but not permeabilised for detection of surface CD44 only. Cells were visualised using a BioRad Radiance 2100 confocal microscope.

OPN concentration in cell culture supernatants was determined using an "in house" sandwich enzyme linked immunosorbent assay (ELISA) as described previously^[17]; where plates were coated with a monoclonal anti-OPN antibody (3 µg/mL R and D Systems, Minneapolis, MN, United States) and detection performed with a polyclonal anti-OPN antibody (200 ng/mL R and D Systems). CD44 blocking antibody (sc-7946; Santa Cruz, CA, United States) for 30 min at room temperature. The blocking antibody is a polyclonal antiserum raised against amino acids 21-320 of CD44^[20].

siRNA knockdown of CD44

StealthTM siRNA double stranded RNA oligonucleotides (Invitrogen) designed to "knock down" or minimise expression of the OPN receptor CD44 were transfected into Huh-7 and HeLa cells using the reverse transcription method of LipofectamineTM 2000 (Invitrogen) as

per the manufacturer's instructions. Cells were assayed for CD44 expression (and growth rate when required) 24-96 h post transfection *via* immunoblot assay. Three different siRNA oligonucleotides, designed to target the conserved exons present in all CD44 isoforms, were tested for their ability to minimise CD44 expression in a transient system using both RT-PCR and immunoblotting as described above. Of the three, two demonstrated consistent knockdown of CD44 at the RNA level by RT-PCR and immunoblot analysis and were pooled for further experiments.

In vivo tumour growth

Five week-old female Balb/c nude mice were implanted subcutaneously with 5×10^6 cells from Huh-7 OPN-A expressing cell lines and the Huh-7 parent cell line as a control. Cells were diluted in 100 µL of PBS and injected into both dorsal flanks ($n = 6$). Tumour growth was monitored by calculation of tumour volume [$V \text{ (mL)} = (\text{width}^2 \times \text{length}^2)/2$] and measured daily for approximately 25 d. The mice were sacrificed and tumours removed for histological analysis. These experiments were approved by the University of Adelaide and Institute of Medical and Veterinary Science animal ethics committees.

Immunohistochemical staining of tissue sections

Paraffin embedded sections of liver and tumour tissue were processed as previously described^[17]. Five micron sections were re-hydrated and incubated in either anti-OPN (LabVision, Fremont, CA, United States), or normal rabbit IgG (Cell signalling, Boston, MA, United States) (diluted to 1 µg/mL in PBS + 3% (v/v) normal horse serum) for 30 min, before being incubated in ADVANCETM HRP-Link (Dako, Glostrup, Denmark), and then in ADVANCETM HRP Enzyme (Dako) for 30 min each. Slides were then incubated in diluted DAB + chromagen (Dako; 20 µL DAB + chromagen in 1 mL DAB + substrate buffer) for 5 min and then following a 5 min wash in H₂O, slides were counterstained with haematoxylin.

Statistical analysis

Student *t* tests were performed to analyse the distributions of 2 independent data sets and all statistical analysis were performed using SPSS 10.0.

RESULTS

Multiple OPN forms are expressed in HCC

The existence of multiple forms of OPN, termed OPN-A, OPN-B and OPN-C, has been previously described^[14], however, their expression and physiological roles in tumour biology, including HCC has not been well studied. We therefore investigated expression of these OPN variants in the HCC cell lines, Huh-7, HepG2, Hep3b and the mouse HCC line Hepa1-6, using PCR primers that spanned exons 2-6 (Figure 1A). As predicted, all HCC cell lines expressed the full-length OPN-A mRNA (Figure 1B). In addition, two smaller transcripts were identified

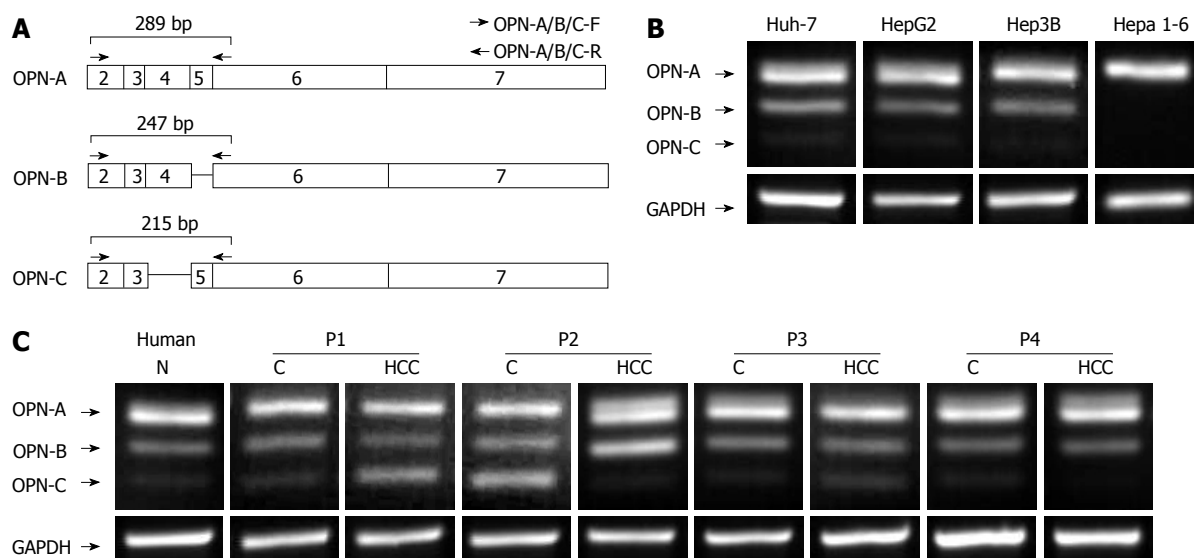


Figure 1 Osteopontin splice variants are expressed at different levels in the liver of hepatocellular carcinoma patients and in various cell lines. A: Schematic representation of the three osteopontin (OPN) splice variants; B: Polymerase chain reaction (PCR) performed on cDNA from different cell types shows varying distribution patterns of OPN mRNA; C: PCR performed on cDNA taken from patient hepatocellular carcinoma (HCC), and its surrounding tissue indicates altered OPN splice variant expression in HCC. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

in all cell lines (with the exception of Hepa 1-6 cells) with OPN-B consistently expressed to a higher level than OPN-C (OPN-C cDNA is readily visible on longer exposure). These smaller transcripts were identified by cloning and sequencing and shown to be specific for OPN-b and OPN-C that lack exons 5 and 4 respectively (Figure 1A). OPN-A is clearly the dominantly expressed OPN transcript followed by OPN-B and OPN-C respectively. Interestingly mouse tissue only expressed the full length OPN-A and this is consistent with a previous report indicating that OPN-A is exclusively expressed in mouse tissues^[15].

We next investigated OPN mRNA expression in HCC tissue samples and cognate non-tumour tissue using the primers described above. Attempts to generate a quantitative real-time assay for detection of all OPN variant simultaneously was unreliable. Nevertheless, it is clear that normal human liver tissue expressed the 3 OPN transcripts to variable degrees, while OPN variant expression was variable between HCC samples. For example, OPN-C expression was increased in HCC P1 compared to cognate liver tissue, while in P2, expression of OPN transcripts in non-tumour tissue was significantly different from that of the corresponding tumour (Figure 1C). This suggests that OPN transcript expression is variable and that individual OPN variants may have specific physiological roles. The detection of all OPN splice variants in normal liver tissue (OPN-C visible on long exposure) is in contrast to previous reports in which it was suggested that OPN-C expression is specific to tumour cells^[14,15].

Stable expression of OPN splice variants in Huh-7 cells

In order to study the physiological roles of the distinct OPN splice variants, cDNAs representing OPN-A,

OPN-B and OPN-C, were cloned into a mammalian expression vector to allow both transient expression and the production of stable OPN expressing cell lines. Following transfection of cDNA constructs representing the OPN variants, detection of OPN expression by confocal microscopy was unsuccessful, suggesting efficient secretion of OPN into the media. However, following treatment of transiently transfected cells with brefeldin A, which inhibits cellular secretion pathways and causes retention of OPN within the cell, we could detect intracellular expression of OPN variants (Figure 2A). OPN is predominantly thought to act at the extracellular level, however, OPN can be detected in tumourigenic hepatocytes by immunohistochemistry suggesting it may, at least in part, have a role at the intracellular level^[6]. Following brefeldin-A treatment, OPN variants exhibited different cytoplasmic distribution in Huh-7 cells, suggesting that each variant may have different effects on tumourigenic hepatocytes, however, this requires further investigation.

To investigate the role of the OPN variants on cell growth, stable clones were produced in a Huh-7 cell line that has low OPN mRNA expression and undetectable protein expression by ELISA (results not shown) and immunoblot. Multiple clones were isolated after G418 selection with no adverse effect on cell morphology or growth. Intracellular OPN was not detected in any clones (results not shown) consistent with efficient expression of OPN, however, OPN mRNA, specific for the OPN variant of interest, was detected by RT-PCR (Figure 2B), while OPN protein was detected by immunoblot (Figure 2C). Furthermore, OPN ELISA performed on media from stable cell lines revealed secretion of OPN in parent Huh 7 and vector transfected cells to be below the limit of detection (< 7.81 ng/mL).

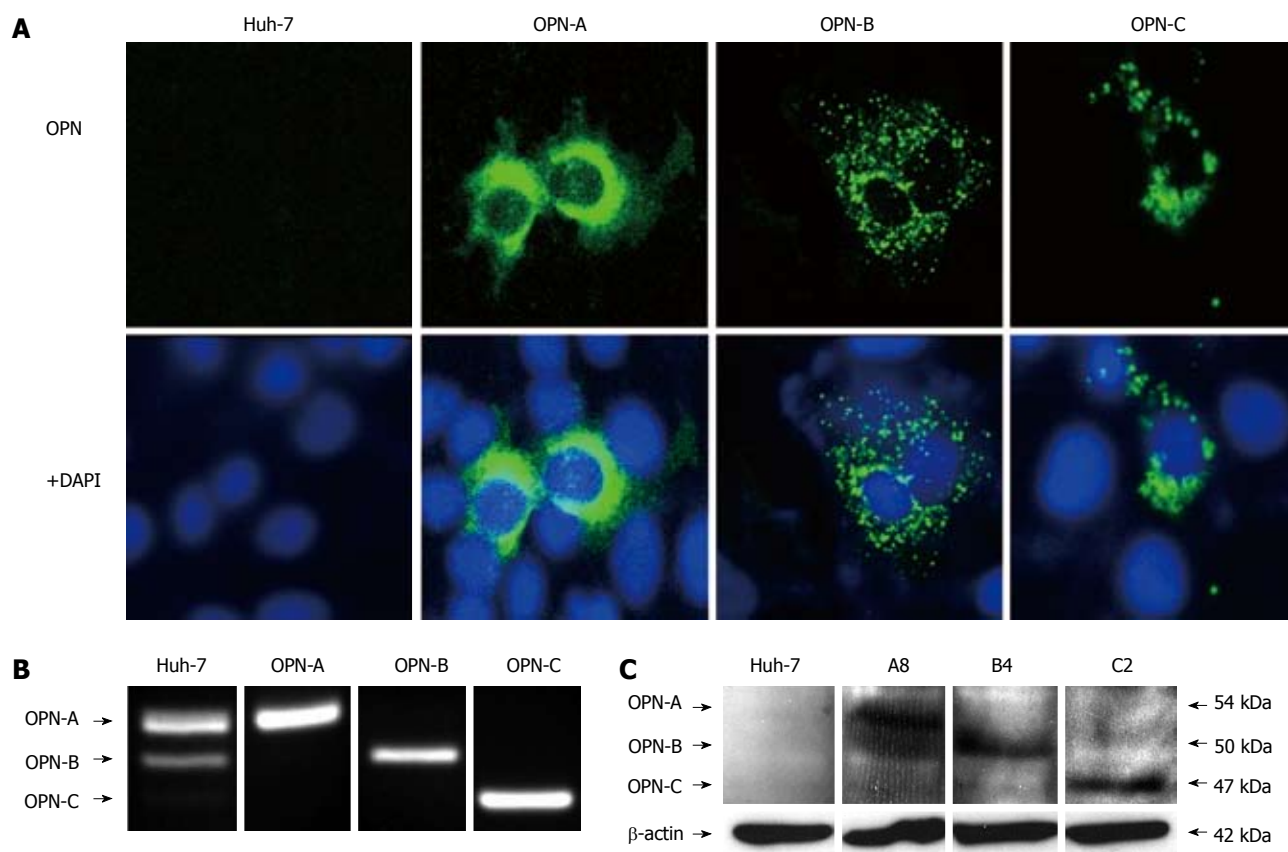


Figure 2 Stable expression of osteopontin splice variants demonstrates unique localisation patterns. Huh-7 cell stably overexpressing osteopontin (OPN)-A, -B, -C, or the control, were stained for OPN expression and visualised fluorescently to demonstrate localisation of the three splice variants (A); Stable expression was confirmed using polymerase chain reaction (B), and Western blotting (C). DAPI: 4',6-diamidino-2-phenylindole.

In contrast OPN-A was detected at 160.4 ng/mL and OPN-B at 127.1 ng/mL. We could not detect OPN-C by ELISA for reasons that are not entirely clear, however the OPN detection ELISA Ab was raised against OPN purified from human milk in which OPN-C is not present^[21]. However, OPN-C is clearly expressed in our system as detected by RT-PCR, immunofluorescence and immunoblotting (Figure 2).

OPN variants facilitate proliferation of HCC derived cell lines

Little work has been done regarding the role of OPN and associated splice variants on cell growth. However recent work from Sun *et al.*^[9] suggests that OPN can enhance cell growth of liver-derived cell lines, although the role of OPN splice variants was not investigated. Therefore, using our stably transduced OPN Huh-7 cells, we determined the impact of the individual variants on Huh-7 cell growth. Huh-7 cells expressing either OPN-A, -B or -C grew significantly faster after 4 d of culture compared to cells transfected with the vector only and the parent cell line (Figure 3A). This increase in growth rate was seen in multiple selected OPN transformants, removing the possibility of clonal selection bias. OPN is readily secreted from these Huh-7 cells and to determine if OPN was exerting its effect by either an autocrine or paracrine mechanism, we incubated Huh-7

cells with conditioned media from OPN-A, -B or -C stable transformants. As can be seen in Figure 3B, conditioned media from all stable transformants increased Huh-7 cell growth, suggesting OPN was exerting its effect in a paracrine manner. This increase in Huh-7 cell growth was directly attributable to the action of OPN because the addition of an anti-OPN antibody, but not a control immunoglobulin, to the cultures, suppressed the increase in cell growth when OPN-A conditioned media was added to Huh-7 cells (Figure 3C). Similar results were obtained for OPN-B and -C conditioned media (results not shown). These results suggest that all variants of OPN can enhance Huh-7 cell proliferation *in vitro*.

OPN exerts its growth proliferative effect through CD44

The C-terminal region of OPN binds the CD44 variant v6/7 to induce cellular signals responsible for tumour progression^[22]. To investigate if OPN was exerting its growth effect in a CD44-dependent manner, we stimulated a number of cell lines possessing different levels of CD44 with OPN conditioned media. As can be seen in Figure 4A, Huh-7, HepG2 and HeLa cells all express cell surface CD44 as determined by immunofluorescence microscopy of unpermeabilised cells, while Hep3b cells do not express CD44. Consistent with this observation, Huh-7, HepG2 and HeLa cells, when stimulated with OPN-A conditioned media, all showed a significant in-

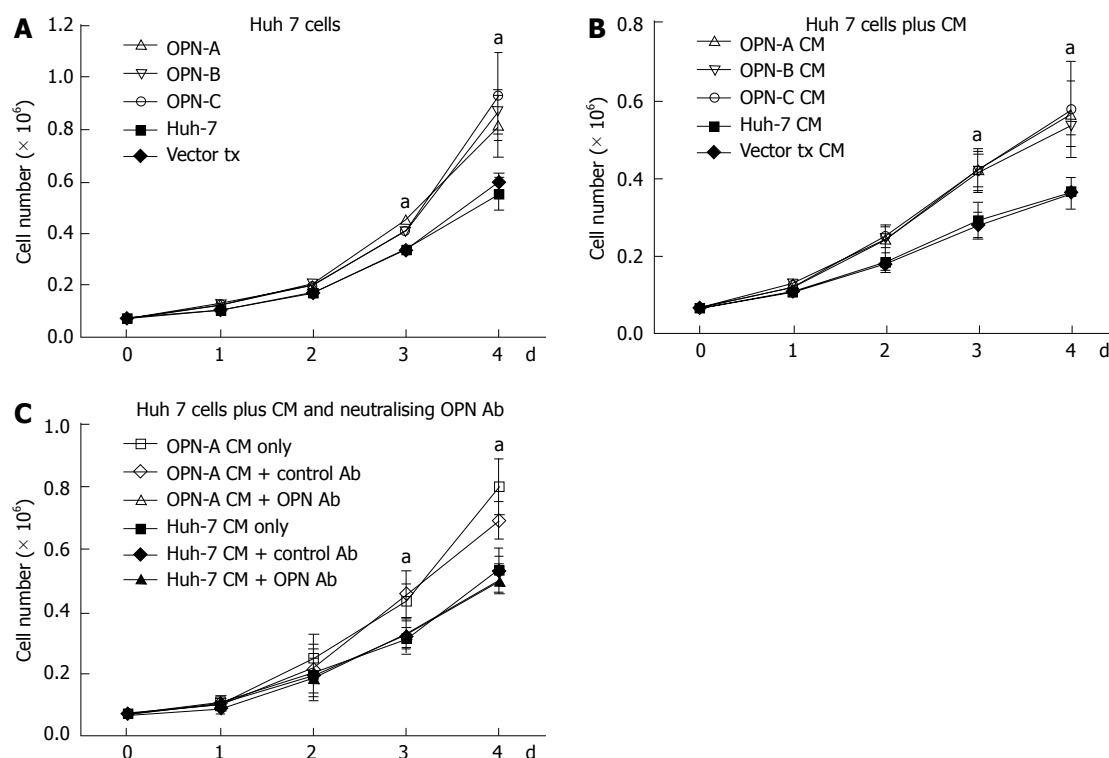


Figure 3 Osteopontin splice variants increase Huh-7 cell growth. A: Osteopontin (OPN)-A, -B, -C and control-expressing Huh-7 stable cell lines in conjunction with Huh-7 cells, were seeded at a density of 7×10^4 cells per well in a 12-well plate and cell numbers monitored daily using trypan blue exclusion; B: Conditioned media (CM) was removed from OPN-A, -B, -C and control-expressing Huh-7 stable cell lines in conjunction with Huh-7 cells and placed onto Huh-7 cells. Cells were monitored as mentioned above; C: Huh-7 cells were seeded at a density of 7×10^4 cells per well in a 12-well plate and conditioned media from OPN-A, -B, -C and control expressing-Huh-7 stable cell lines placed onto the cells in conjunction with either anti-OPN (OPN Ab) or an control immunoglobulin. $^aP < 0.05$.

crease in cell growth (Figures 3B, 4B and C). However no cell growth was noted in Hep3Bs consistent with a lack of CD44 expression (Figure 4D). These observations were confirmed following the addition of CD44 blocking antibody to the conditioned media that completely suppressed the OPN growth effect (Figure 5A and B). Addition of control immunoglobulin to the cultures had no effect on OPN induced cell growth. To further confirm the role of CD44, we suppressed CD44 expression using an siRNA approach. Transfection of an siRNA targeting CD44 resulted in a significant decrease in CD44 mRNA and CD44 protein expression as determined by PCR and immunoblot in Huh-7 and HeLa cells (Figure 6A and B). This knockdown, in turn, correlated with a decrease in OPN-mediated dependent cell growth of both cell lines (Figure 6C and D). Collectively, these experiments indicate that the increase in OPN induced cell growth results from an interaction between OPN and CD44.

OPN-A induced tumour growth in an ectopic xenograft mouse model

Our *in vitro* data prompted us to examine whether the different OPN splice variants regulate tumour growth in an ectopic xenograft nude mouse model. Accordingly, Huh-7 cells stably expressing either OPN-A, -B or -C were injected subcutaneously into the flanks of nude mice. Mice injected with OPN-A expressing Huh-7 cells were sacrificed approximately 18 d post injection

as the tumour size was causing mice discomfort, while all other mice were sacrificed at approximately 22 d post injection. The typical gross appearance of the tumours is shown in Figure 7A, while the mean tumour volume throughout the experiment is shown in Figure 7B. Compared to parent control Huh-7 cells, mice injected with OPN expressing cell lines showed an increase in tumour growth. This increase in cell growth was most noticeable in mice injected with Huh-7 cells expressing full-length OPN-A (Figure 7B). These mice developed tumours at day 10, after which the growth rate was faster than that of the control tumours. To confirm OPN expression in isolated tumours, RNA was isolated and mRNA expression of the different OPN splice variants was confirmed by RT-PCR (Figure 7C). Detection of OPN *in situ* was performed by immunohistochemistry in which OPN-A expressing tumours expressed significantly more OPN-A than control Huh-7 cells (Figure 7D). OPN-B and -C were similarly detected by immunohistochemistry (results not shown). No differences were noted histologically between the tumours, with the exception that OPN-A tumours contained a greater number of cells undergoing various stages of the mitotic pathway. These data clearly suggest a role for OPN in promoting the growth of Huh-7 cells in an ectopic xenograft mouse model.

DISCUSSION

OPN is a secreted phosphoglycoprotein that binds to

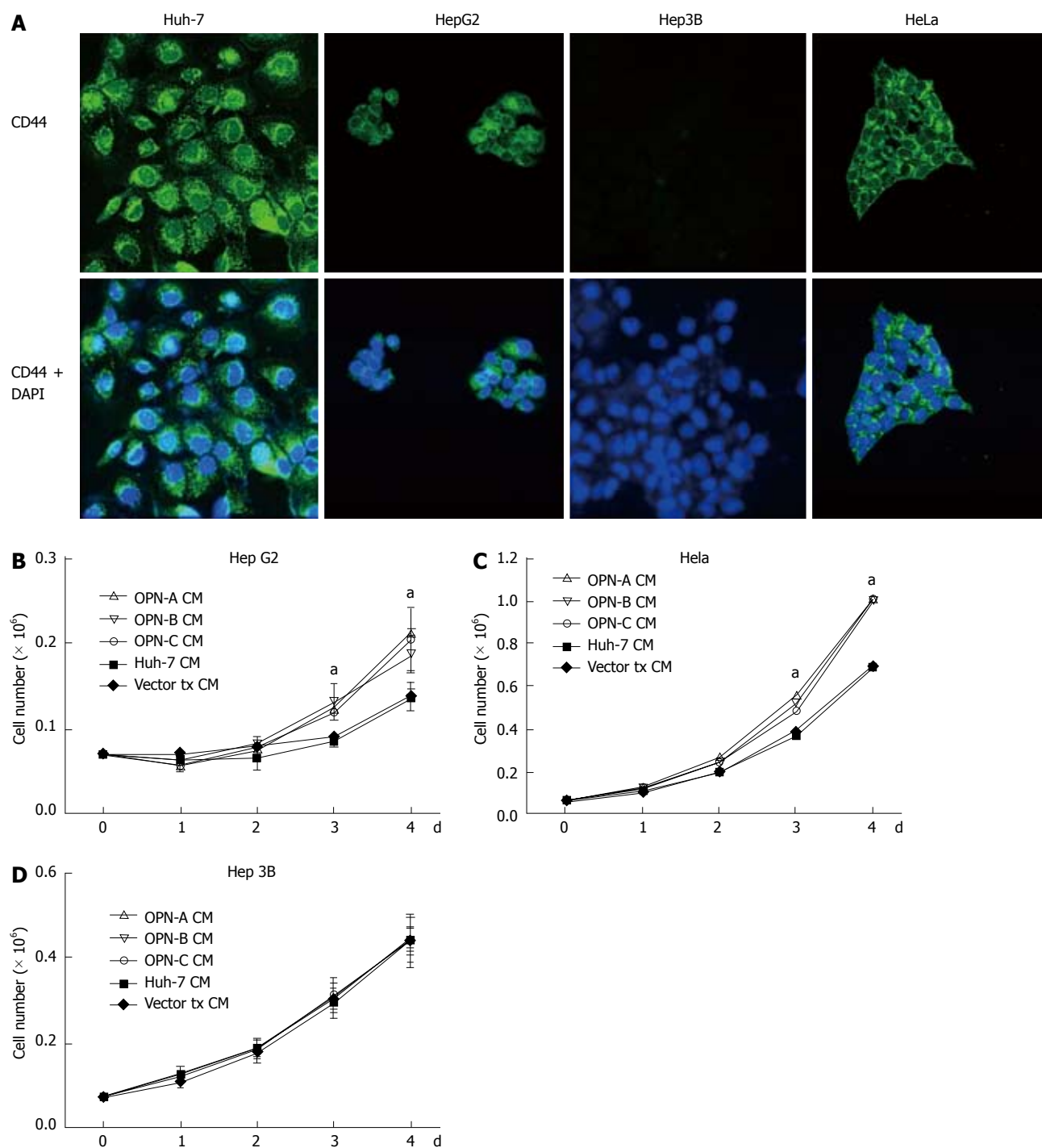


Figure 4 CD44 expression is necessary for increased cellular growth due to osteopontin expression. Huh-7, HepG2, Hep3B and HeLa cells were immunostained for 4',6-diamidino-2-phenylindole (DAPI) and CD44 expression (A); Conditioned media (CM) was removed from osteopontin (OPN)-A, -B, -C and control-expressing Huh-7 stable cell lines in conjunction with Huh-7 cells and placed onto Hep G2 cells (B); HeLa cells (C) or Hep 3B cells (D); cell numbers were monitored daily using a trypan blue exclusion. * $P < 0.05$.

the family of cellular receptors integrin $\alpha v \beta$ and CD44 to exert its biological effects, and of late has gained attention as its expression is associated with invasion and metastasis of a number of malignant tumours, including HCC^[8,23-26]. In many of these tumours, increased plasma levels of OPN have shown promise as potential prognostic biomarkers as OPN expression correlates with metastatic potential, early recurrence and decreased

patient survival. While our understanding of the role of OPN in HCC biology is expanding, there is still a paucity of information regarding the role of OPN (and its splice variants) in proliferation of HCC and the molecular mechanisms responsible.

OPN undergoes post-translational modifications *via* phosphorylation, glycosylation and cleavage by thrombin and MMPs to produce differentially active forms^[10].

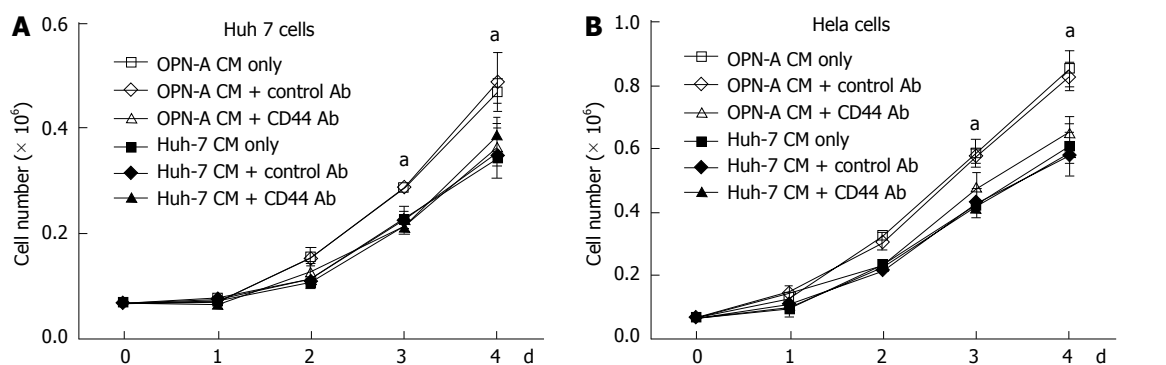


Figure 5 CD44 engagement is necessary for osteopontin-a induced cell growth in Huh-7 and HeLa cells. (A) Huh-7 or (B) HeLa cells were seeded at a density of 7×10^4 cells per well in a 12-well plate and conditioned media from osteopontin (OPN)-A Huh-7 stable cell lines or Huh-7 cells alone placed onto the cells in conjunction with either anti-CD44 or a control immunoglobulin. Cell numbers were monitored daily using trypan blue exclusion. CM: Conditioned media. $^aP < 0.05$.

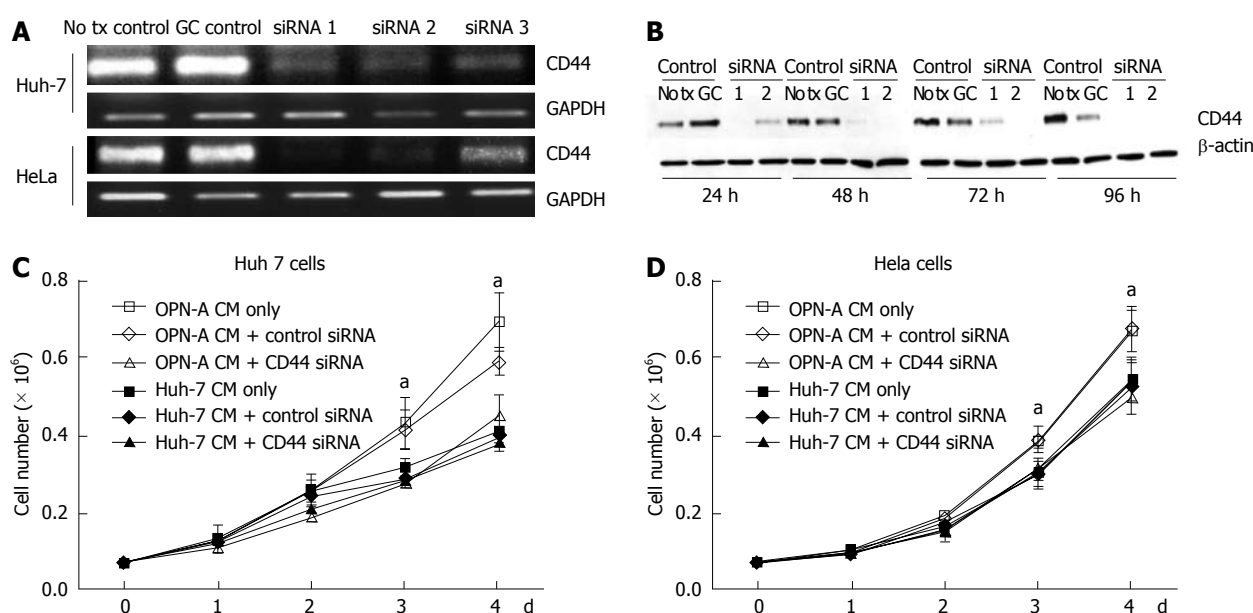


Figure 6 CD44 knockdown inhibits osteopontin-a-induced cell growth in Huh-7 and HeLa cells. A panel of 3 Stealth siRNA's were investigated for their ability to limit CD44 mRNA expression using polymerase chain reaction (A), further validation was achieved by examining protein reduction of CD44 using Western blotting (B). (C) Huh-7 or (D) HeLa cells were seeded at a density of 7×10^4 cells per well in a 12-well plate and a CD44 siRNA pool containing Stealth siRNA1 and 2 was transfected into the cultures 24 h prior to placing conditioned media from osteopontin (OPN)-A Huh-7 stable cell lines or Huh-7 cells alone onto the cells. Cell numbers were monitored daily using trypan blue exclusion. CM: Conditioned media; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. $^aP < 0.05$.

For example, the MMP-9 cleaves OPN into specific fragments of which a 5 kDa fragment induces HCC cellular invasion *via* the CD44 receptor^[16]. Furthermore, stromelysin (MMP-3) and matrilysin (MMP-7) have been reported to cleave OPN at residues 166 and 210, resulting in tumour cell adhesion, while thrombin cleavage of OPN mediates increased adhesion and migration of tumour cells, *via* binding of the OPN RGD motif to cell surface integrins^[27,28]. Clearly the role of OPN and its derivatives in tumour biology is complex, which is further complicated by the expression of OPN splice variants. In humans, the existence of two OPN splice variants with deletion of exon 4 (OPN-C) or exon 5 (OPN-B) were first described in glioma cells^[29] and more recently in breast cancer cell lines^[15], however, in HCC, the roles of the OPN splice variants is relatively unde-

fined, although a recent report suggests that expression of OPN-A and -B in HCC-derived cell lines confers a robust migratory phenotype through activation of the p42/44 Map kinase pathway^[30].

There has been little investigation of the expression of OPN splice variants and the mechanisms by which OPN regulates cellular proliferation. Our initial investigations focused on OPN splice variant expression in various HCC cell lines, and in HCC and its cognate surrounding normal liver. At the mRNA level, we observed expression of all three OPN splice variants in human HCC cell lines, although OPN-A was clearly the dominant species followed by OPN-B and to a lesser extent-c. This observation is consistent with the work of Takafuji *et al*^[16] who showed that greater levels OPN-C were expressed in HCC cell lines with high metastatic potential

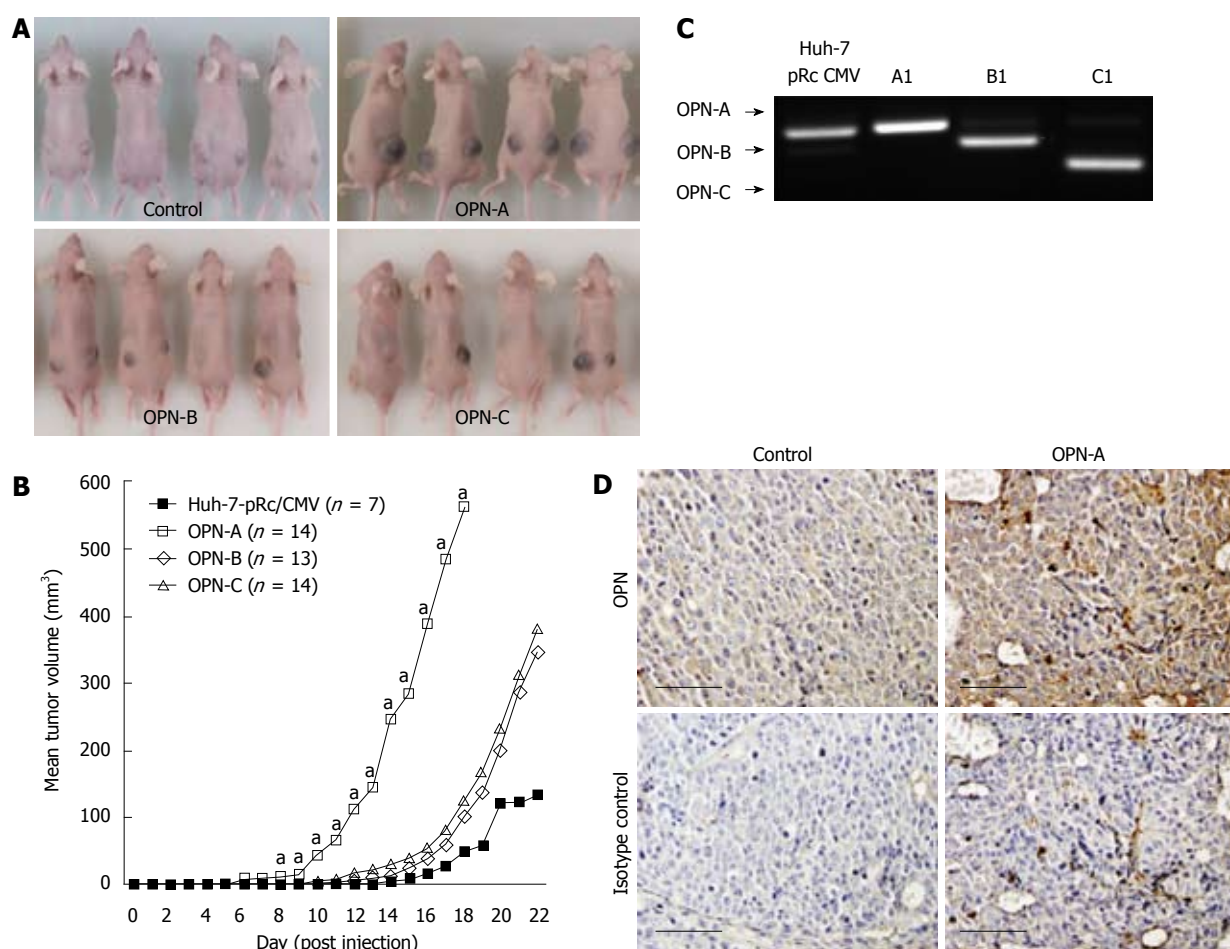


Figure 7 Over expression of the three osteopontin splice variants promotes Huh-7 tumour growth in Balb/c nude mice. A: Growth of tumours in mice injected in the hind flanks with osteopontin (OPN)-A, -B, -C or control-expressing stable cell lines, with mean tumour volumes at the time of euthanasia expressed graphically in (B) $^aP < 0.05$ OPN-A/Huh-7 vector only; C: Polymerase chain reaction analysis of a representative tumour from each of the 4 groups showing OPN mRNA expression in all tumours; D: OPN-a expressing tumours display increased levels of OPN protein. Control and OPN-A tumour sections were immunostained for OPN in conjunction with a rabbit isotype control. Bar represents 100 μ m.

(the cell lines studied here are not of high metastatic potential). However, the expression of OPN-B and other splice variants was not investigated in primary HCC. Thus this study represents a comprehensive description of complete OPN variant expression *in vivo* and *in vitro*. Transcripts for all OPN variants were expressed in both primary HCC and cognate surrounding tissue, however, levels of expression varied between paired samples and between different patient HCCs. While our RT-PCR is only semi-quantitative (development of a quantitative real time assay for all OPN variants was not reliable) these results in-part confirm the work of He *et al.*^[15] and show that expression of OPN splice variants can be detected in surrounding non-cancerous tissue. It has been reported that the OPN splice variants are specific to tumour cells and are rarely, if ever, expressed in normal tissue^[15]. However, we noted expression of all OPN variants in non-diseased liver tissue, suggesting that OPN splice variant expression is not restricted to tumour cells, and different forms of OPN may play different roles depending on the cell type that expresses OPN and the context in which it is expressed.

A significant finding of this study was the role of OPN and splice variants in *in vitro* proliferation and *in vivo* tumour growth of HCC. It is becoming increasingly clear that in addition to the role of OPN in the metastatic process it also has a role in cellular proliferation of a number of tumours. However, its role in HCC is not well studied, although, recently, an siRNA OPN knock-down approach suggested that OPN is important for HCC proliferation. Nevertheless, the relative roles of OPN variants or the cellular receptor responsible were not investigated^[9]. In contrast to the above OPN RNA interference study, we exogenously expressed OPN variants in the HCC Huh-7 cell line, and investigated cellular proliferation *in vitro* and in an ectopic xenograft nude mouse model. Stable ectopic expression of OPN-A, -B and -C resulted in secretion of all OPN forms into the culture media, and significant proliferation of Huh-7 cells, either directly or through the addition of conditioned media to naive Huh-7 cells. This suggests that at least *in vitro* OPN exerts its proliferative effect in either an autocrine or paracrine manner, which is consistent with the secreted nature of this protein, and was con-

firmed through the use of a neutralizing OPN antibody that abolished the proliferative effect of conditioned media. This proliferation effect was also seen in an *in vivo* HCC proliferation model in which Huh-7 cell lines, expressing either OPN-A, -B or -C, all proliferated faster than the control Huh-7 line. Interestingly compared to the *in vitro* proliferation assays, OPN-A showed the most significant proliferation advantage over OPN-B and -C. The reason for this discrepancy is unclear as all cell lines expressed similar amounts of OPN but may be related to stability or processing of OPN in an *in vitro* environment. To our knowledge, this is the first report that OPN and its splice variants play a significant role in cellular proliferation and growth of HCC, in addition to its role in invasiveness. Our collective studies strongly suggest that OPN plays a role in cellular proliferation however we cannot exclude the possibility that OPN expression also plays a role in increased cell survival given the recent observations from Zhao *et al.*^[31].

The CD44 family are cell surface adhesion molecules that mediate cell-matrix interactions, and in addition to the standard form, multiple isoforms arise from differential splicing of ten variant exons, and are designated CD44v6-15^[32]. CD44 variants, especially CD44v6, have been identified as protein markers for metastatic behaviour in a number of cancers, including HCC^[33-36]. A C-terminal region of OPN can specifically interact with CD44v6 and or v7 to mediate cellular chemotaxis^[37] and a thrombin-cleaved C-terminal OPN fragment induces macrophage migration *via* CD44^[38]. While OPN CD44v6-7 binding has been associated with metastasis and invasion, little work has been done in regard to the CD44 OPN interaction and tumour growth, although one study revealed that knockdown of CD44 expression reduces tumour growth in colon carcinoma cells^[39]. In this study, we show that the increased growth rate of Huh-7 cells in response to OPN conditioned media was as a result of CD44 expression because blocking CD44 with a neutralising antibody completely abolished this growth effect. Furthermore, HepG2 and HeLa cells, which both express CD44v6/7, also showed this growth effect while the Hep3B cell line, which is negative for CD44v6/7, was unresponsive to OPN in the conditioned media. To conclusively demonstrate a role for CD44, a siRNA approach was employed that successfully reduced CD44 expression in both Huh-7 and HeLa cells. Consistent with the above, this reduction in CD44 in both cell lines resulted in a decrease in cellular proliferation in response to OPN conditioned media. To our knowledge this is the first report that the interaction between OPN and CD44 results in signals that drive cellular proliferation in HCC. This is not surprising considering the recent work describing a role for OPN in regulating MKK3/6 and p38 dependent nuclear factor kappa B activation in cervical cancer leading to furin expression, a proprotein convertase that plays crucial roles in regulation of tumour progression metastasis and angiogenesis^[40]. Cervical cancer cells over expressing OPN

showed enhanced cell growth, while shRNA-mediated silencing of OPN suppressed cell growth in a CD44-mediated MKK3/6 dependent p38 activation mechanism^[40]. While similar mechanisms may be at play in HCC, the signalling pathways driven by OPN and CD44 activation in HCC require further investigation. Thus, understanding the molecular mechanisms that underpin increased HCC cell proliferation through the OPN/CD44 interaction and activation of downstream signalling pathways could provide potential targets for therapeutic strategies for the treatment of HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a highly aggressive carcinoma of the liver, and is the fifth most common cancer worldwide. It is the third leading cause of cancer related death. Osteopontin (OPN) is a secreted multifunctional matrix-glycoprotein involved in normal tissue remodelling processes but is secreted at high levels in numerous tumours, including HCC. OPN is expressed as 3 isoforms and their role in HCC biology is not entirely known.

Research frontiers

Human OPN is known to be expressed in three different isoforms with potentially differing functions. Despite evidence of elevated OPN in several cancers and correlation of levels to malignant invasiveness, the expression of OPN isoforms in the context of HCC has not previously been investigated. Similarly the affect of OPN and its isoforms on HCC growth is unexplored.

Innovations and breakthroughs

This study demonstrates that all isoforms of OPN are expressed in HCC tissues and cell lines, at the mRNA level, and that each isoform can stimulate the growth of HCC derived cell lines both *in vitro* and *in vivo*, in an ectopic xenograft mouse model. Furthermore, this growth promoting effect has been shown to occur through a mechanism requiring functional CD44. These cellular proliferation signalling pathways, driven by OPN and CD44 activation, require further investigation.

Applications

Understanding the molecular mechanisms that underpin increased HCC cell proliferation could provide potential targets for therapeutic strategies for the treatment of HCC.

Peer review

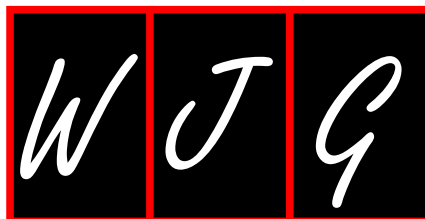
Authors elegantly and convincingly demonstrated that OPN plays a relevant role in the expansion of HCC cells through interaction with the cell surface receptor CD44; this information is potentially interesting for novel anti HCC therapeutic approaches.

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Retrograde-viewing device improves adenoma detection rate in colonoscopies for surveillance and diagnostic workup

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Abstract

AIM: To determine which patients might benefit most from retrograde viewing during colonoscopy through subset analysis of randomized, controlled trial data.

METHODS: The Third Eye® Retroscope® Randomized Clinical Evaluation (TERRACE) was a randomized, controlled, multicenter trial designed to evaluate the efficacy of a retrograde-viewing auxiliary imaging device that is used during colonoscopy to provide a second video image which allows viewing of areas on the proximal aspect of haustral folds and flexures that are difficult to see with the colonoscope's forward view. We performed a post-hoc analysis of the TERRACE data to determine whether certain subsets of the patient population would gain more benefit than others from use of the device. Subjects were patients scheduled for colonoscopy for screening, surveillance or diagnostic workup, and each underwent same-day tandem examinations with standard colonoscopy (SC) and Third Eye colonoscopy (TEC), randomized to SC followed by TEC or *vice versa*.

RESULTS: Indication for colonoscopy was screening in 176/345 subjects (51.0%), surveillance after previous polypectomy in 87 (25.2%) and diagnostic workup in 82 (23.8%). In 4 subjects no indication was specified.

Previously reported overall results had shown a net additional adenoma detection rate (ADR) with TEC of 23.2% compared to SC. Relative risk (RR) of missing adenomas with SC *vs* TEC as the initial procedure was 1.92 ($P = 0.029$). Post-hoc subset analysis shows additional ADRs for TEC compared to SC were 4.4% for screening, 35.7% for surveillance, 55.4% for diagnostic and 40.7% for surveillance and diagnostic combined. The RR of missing adenomas with SC *vs* TEC was 1.11 ($P = 0.815$) for screening, 3.15 ($P = 0.014$) for surveillance, 8.64 ($P = 0.039$) for diagnostic and 3.34 ($P = 0.003$) for surveillance and diagnostic combined. Although a multivariate Poisson regression suggested gender as a possibly significant factor, subset analysis showed that the difference between genders was not statistically significant. Age, bowel prep quality and withdrawal time did not significantly affect the RR of missing adenomas with SC *vs* TEC. Mean sizes of adenomas detected with TEC and SC were similar at 0.59 cm and 0.56 cm, respectively ($P = \text{NS}$).

CONCLUSION: TEC allows detection of significantly more adenomas compared to SC in patients undergoing surveillance or diagnostic workup, but not in screening patients (ClinicalTrials.gov Identifier: NCT01044732).

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Key words: Colonoscopy; Colorectal cancer; Adenomas; Miss rates; Retrograde-viewing

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INTRODUCTION

Colonoscopy is generally regarded to be the “gold standard” for the detection of colorectal neoplasia, and is the only method that allows detection and removal during a single procedure^[1,2]. If the colon were a straight pipe, the standard colonoscope would be the perfect instrument for examining its lining, but lesions can be missed during colonoscopy, especially when located on the proximal aspect of haustral folds or on the inner curve of flexures^[3-8].

The Third Eye Retroscope (TER) (Avantis Medical Systems, Sunnyvale, California) provides an additional, retrograde view that helps to visualize areas behind folds and flexures^[9-11]. We recently showed in the Third

Eye Retroscope Randomized Clinical Evaluation (TER-RACE) study that Third Eye colonoscopy (TEC) detected 23.2% additional adenomas compared to standard colonoscopy (SC)^[12].

As concerns about medical costs increase, there is growing interest in finding ways to target interventions and new technologies to specific patient sub-populations that will experience the greatest benefit^[13,14]. In this follow-up of our initial report of TERRACE results, we performed a post-hoc subset analysis of the data to determine whether specific indications for colonoscopy (screening, surveillance or diagnostic workup) or other patient characteristics were associated with higher additional adenoma detection rates (ADR) with TEC. Such information may be useful for targeting those segments of the colonoscopy population that would benefit most from use of this new technology. We also wished to evaluate the clinical significance of the additional adenomas detected through TEC and determine how finding those additional adenomas would affect recommendations for follow-up intervals.

MATERIALS AND METHODS

The TERRACE study was a prospective, randomized, controlled trial that used a “tandem” design involving two same-day, back-to-back procedures to provide a head-to-head comparison between TEC and SC.

Between March 2009 and February 2010, 15 experienced endoscopists at 4 European and 5 United States sites enrolled 448 patients who were scheduled to undergo colonoscopy for routine screening, surveillance after previous polypectomy, or diagnostic workup for symptoms possibly related to the lower gastrointestinal tract. Subjects were excluded for history of inflammatory bowel disease, colonic resection, polyposis syndrome or radiation therapy to abdomen or pelvis, or for active diverticulitis, suspicion of colonic stricture or concurrent enrollment in another study.

Upon arrival in the endoscopy suite, subjects were randomized to one of two groups by a research assistant utilizing a Web-based randomization module stratified both by center and by individual endoscopist. Each subject then underwent two complete examinations by the same endoscopist, who was informed of the order of exams immediately before beginning intubation.

Group A underwent SC first, followed by TEC, using the same colonoscope for both procedures. Group B underwent TEC first, then SC.

For TEC, following intubation of the cecum with a standard colonoscope, the TER was inserted through the biopsy channel of the colonoscope. When the TER emerges, its distal tip automatically turns 180 degrees so its miniature video camera and light are oriented backwards. As the colonoscope and TER are withdrawn together, the device provides a continuous retrograde view that complements the forward view of the colonoscope. The two video images are displayed side-by-side on a

monitor.

Tandem miss rate studies have demonstrated a “second-pass effect” - i.e., looking a second time generally yields additional lesions^[3-5]. In Group B the detection rate for SC as the second procedure served as a proxy for second-pass effect. Subtracting that from the additional detection rate with TEC in Group A yielded the net additional detection rate attributed to TEC.

Polyps were removed when detected and sent for histological evaluation. Advanced adenomas were defined as adenomas that measured at least 1 cm in diameter or those with villous or tubulovillous components, high-grade dysplasia or adenocarcinoma. An assistant recorded withdrawal time (from cecum to anal verge, subtracting pauses for polypectomy or extensive irrigation/suctioning) and total procedure time (from initial insertion of the colonoscope to withdrawal through the anal verge, not subtracting pauses). Bowel preparation was graded with the Ottawa Bowel Preparation Quality Scale Score^[15]. Telephone interviews were performed 24-72 h after procedures to assess each subject for adverse events.

Subjects were withdrawn from the study if they withdrew consent, had inadequate bowel preparation or developed complications, if the endoscopist was unable to intubate the cecum or identified a condition in which back-to-back examinations might create risk for the subject, or if there were device malfunctions, technical errors or deviations from the protocol.

The primary outcome measure was per-polyp detection rates for adenomas and for all polyps with SC and TEC. Secondary outcome measures were polyp size, polyp histology, withdrawal time and total procedure time.

Patients presenting for routine screening were regarded as having a low-to-average risk of developing colorectal cancer (CRC), while the non-screening patients - those presenting for surveillance colonoscopy as a follow-up after previous removal of adenomas or for diagnostic workup of symptoms - were considered to have an above-average risk for CRC.

The protocol was approved by the Institutional Review Board of each institution, and all patients signed an informed consent. The study was registered with ClinicalTrials.gov (Identifier Number NCT01044732) and was reported in compliance with the Consolidated Standards of Reporting Trials (CONSORT) 2010 Statement.

Statistical analysis

Statistical analyses were performed on the per-protocol population using the SAS statistical package, version 9.1 (SAS Institute, Cary, NC). Because non-adenomatous polyps are generally regarded as having limited clinical significance, subset analysis was applied only to adenomas. Sample size determination was discussed in our earlier report of TERRACE results^[12].

Because it is not possible to directly determine *P* values for the net additional ADR, we evaluated the statistical significance of the detection rates indirectly by calculating the relative risk (RR) of missing an adenoma

during first exams with SC *vs* first exams with TEC. RR for missing adenomas during the first exam was estimated by Poisson regression including only study group (TEC first *vs* SC first) as an explanatory variable. *P* values < 0.05 were considered statistically significant.

To further assess the effect of the order of procedures on adenoma miss rates during the first exam, an exploratory analysis was performed to examine the influence of the independent variables including study group, age, gender, bowel prep quality (Ottawa Score), withdrawal time and indication for procedure (screening, surveillance, or diagnostic workup). Interactions between study groups (i.e., order of exams) and each of the explanatory variables were also examined. A multivariate Poisson regression with backward selection was performed to determine whether the above variables predicted differences in the number of adenomas missed during first exams with SC *vs* TEC. Independent variables with *P* values < 0.10 were included in the model for the multivariate analysis. Independent covariates included in the final model from the multivariate Poisson regression were further evaluated through subset analysis, where *P* values < 0.05 were considered statistically significant.

For the subsets of subjects by indication for procedure, Fisher's exact test was performed to compare results for second exams in terms of additional subjects found to have at least one adenoma and additional subjects found to meet criteria for shortened surveillance intervals as a result of findings during the second exam.

RESULTS

Patient demographics and a study flow diagram describing withdrawal of subjects to yield a per-protocol population of 349 subjects are shown in Figure 1.

Indication for colonoscopy was screening in 176/345 subjects (51.0%), surveillance after previous polypectomy in 87 (25.2%) and diagnostic workup in 82 (23.8%). In 4 subjects no indication was specified.

Previously reported overall TERRACE results had shown a net additional ADR with TEC of 23.2% compared to SC (Table 1). The RR of missing adenomas with SC *vs* TEC was 1.92 (*P* = 0.029) (Table 2).

Using a multivariate Poisson regression, we determined the risk of missing adenomas during the first exam. Study group (TEC first *vs* SC first) and gender were selected as possibly significant factors, and the interaction between indication for procedures and study group was found to be predictive. Age, bowel prep quality and withdrawal time did not significantly affect the risk.

Subset analysis segmenting the population by indication showed that additional ADRs for TEC compared to SC were 4.4% in the screening group, 35.7% in the surveillance group, 55.4% in the diagnostic group and 40.7% in the non-screening patients (surveillance and diagnostic groups combined) (Table 1). The RR of missing adenomas with SC *vs* TEC was 1.11 (*P* = 0.815) for screening

Table 1 Additional adenoma detection rates for second exams by indication for procedure

Indication	Group A (SC, then TEC)			Group B (TEC, then SC)			Net additional detection with TEC (%)
	SC 1st	TEC 2nd	Additional in 2nd exam (%)	TEC 1st	SC 2nd	Additional in 2nd exam (%)	
All indications	107	49	45.8	115	26	22.6	23.2
Screening	54	19	35.2	52	16	30.8	4.4
Surveillance	37	20	54.1	49	9	18.4	35.7
Diagnostic	16	10	62.5	14	1	7.1	55.4
Surveillance + diagnostic	53	30	56.6	63	10	15.9	40.7

TEC: Third Eye colonoscopy; SC: Standard colonoscopy.

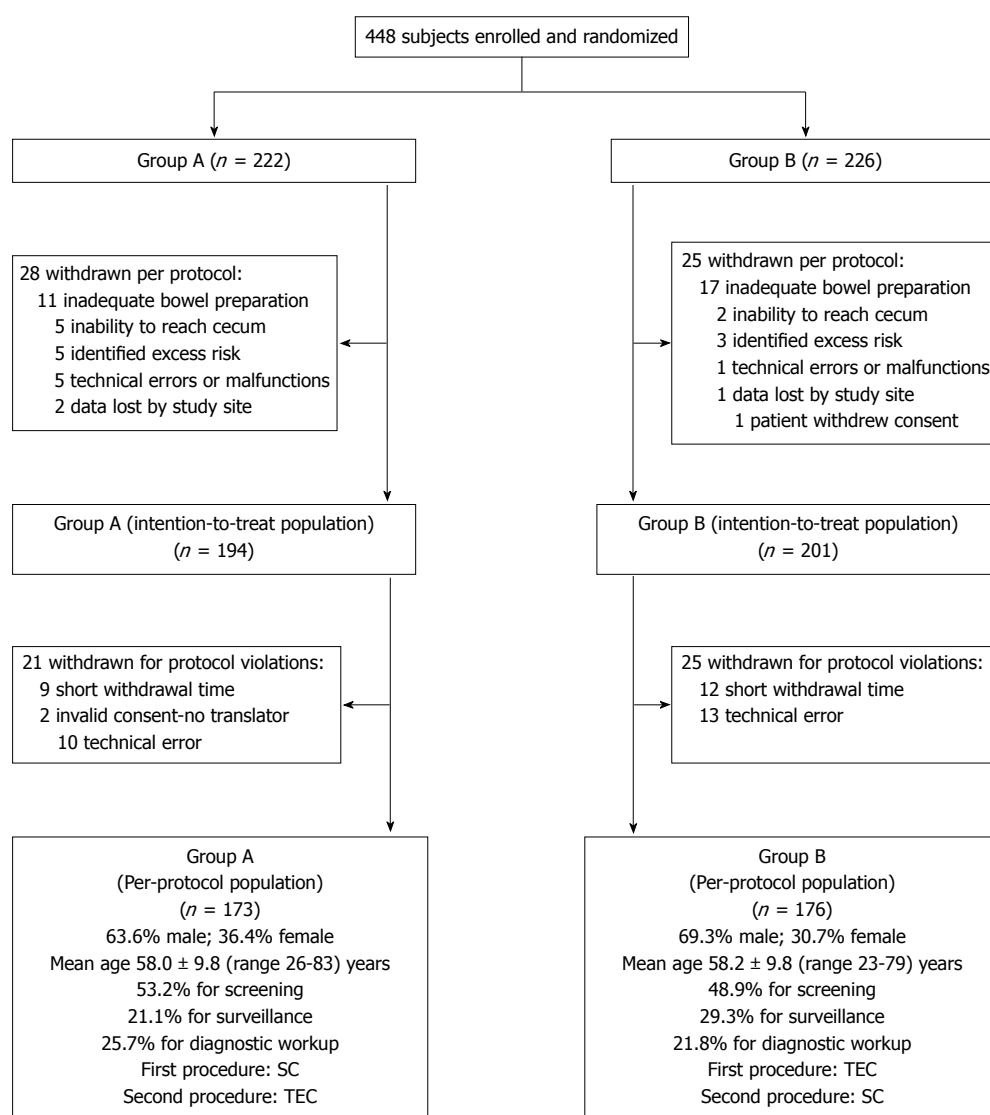


Figure 1 Study flow diagram and patient demographics. SC: Standard colonoscopy; TEC: Third Eye colonoscopy.

subjects, 3.15 ($P = 0.014$) for surveillance subjects, 8.64 ($P = 0.039$) for diagnostic subjects and 3.34 ($P = 0.003$) for the surveillance and diagnostic subjects combined (Table 2).

In the diagnostic group, due to detection of lesions with TEC that had been missed with SC (after correction for second-pass effect), 75.0% additional subjects were found to have at least 1 adenoma. In the surveillance group, 42.9% additional subjects were found to have at

least 1 advanced adenoma or at least 3 small adenomas criteria for 3-year follow-up according to joint guidelines published by the United States Multi-Society Task Force on CRC, American College of Radiology and American Cancer Society^[16]. However, the numbers of subjects with these findings were small, and the differences did not reach statistical significance.

Segmenting the population by gender, additional

Table 2 Adenoma miss rates¹ by indication for procedure

Indication	Group A standard colonoscopy first	Group B Third Eye colonoscopy first	Relative risk ² (95% CI)	P
All Indications ³	49/173 = 0.283	26/176 = 0.148	1.92 (1.25-2.94)	0.029
Screening	19/91 = 0.209	16/85 = 0.188	1.11 (0.62-2.04)	0.815
Surveillance	20/36 = 0.556	9/51 = 0.176	3.15 (1.63-6.10)	0.014
Diagnostic	10/44 = 0.227	1/38 = 0.026	8.64 (1.16-64.41)	0.039
Surveillance + diagnostic	30/80 = 0.375	10/89 = 0.112	3.34 (1.74-6.39)	0.003

¹Expressed in adenomas per patient; ²Relative risk of missing an adenoma during first exams with standard colonoscopy *vs* first exams with Third Eye colonoscopy, 95% CI calculated using 2 × 2 frequency table; ³In 4 subjects (2 in group A and 2 in group B), no indication was specified.

Table 3 Per-polyp miss rates for standard colonoscopy and Third Eye colonoscopy by size of adenoma and indication for procedure

	Adenomas missed with standard colonoscopy during 1st exams in group A (%)			Adenomas missed with Third Eye colonoscopy during 1st exams in group B (%)		
	< 10 mm	≥ 10 mm	All sizes	< 10 mm	≥ 10 mm	All sizes
All indications	33.8	11.8	31.4	20.8	0	18.4
Screening	27.7	12.5	26.0	28.1	0	23.5
Surveillance	37.7	0	35.1	16.7	0	15.5
Diagnostic	42.9	20.0	38.5	7.1	0	6.7
Surveillance + diagnostic	39.2	11.1	36.1	14.7	0	13.7

Table 4 Mean procedural times (min)

Indication	All exams with SC		All exams with TEC	
	Withdrawal time	Total procedure time	Withdrawal time	Total procedure time
All indications	7.6	17.0	9.5	20.9
Screening	7.5	16.0	9.6	19.4
Surveillance	7.9	16.4	9.5	21.8
Diagnostic	7.5	19.8	9.6	23.2
Surveillance + diagnostic	7.7	18.1	9.5	22.5

Total procedure time: Total time from initial insertion of the colonoscope to withdrawal through the anal verge; Withdrawal time: Time from cecum to anal verge, excluding pauses for polypectomy or extensive suctioning; TEC: Third Eye colonoscopy; SC: Standard colonoscopy.

ADRs for TEC compared to SC were 25.9% for males and 8.5% for females ($P = \text{NS}$). Segmenting by age, additional ADRs for TEC compared to SC were 22.0% for subjects less than 65 years of age and 25.8% for those at least 65 years ($P = \text{NS}$).

Per-polyp miss rates in the surveillance and diagnostic groups were lower for TEC than for SC, and no large adenoma (at least 10 mm) was missed with TEC in any subgroup (Table 3).

Mean sizes of adenomas detected with TEC and SC were similar at 0.59 cm and 0.56 cm, respectively ($P = \text{NS}$). Mean procedure times are shown in Table 4.

No adverse events were reported.

DISCUSSION

We report a subset analysis of results from the TER-RACE study, which was the first “head-to-head” comparison of TEC with SC and was designed to provide correc-

tion for the “second-pass effect” that invariably occurs in tandem studies^[12]. Reversing the order of procedures in the control group (TEC first, followed by SC) provided a proxy for the second-pass effect, which was then subtracted from the additional detection rate in the study group (SC first, followed by TEC) to yield the net additional ADR for TEC compared to SC.

This subset analysis provides additional insight regarding which segments of the population are likely to benefit most from TEC and the clinical significance of the adenomas that were detected with TEC after having been missed during SC.

As shown in Table 1, overall results demonstrated a 23.2% net additional ADR for TEC compared to SC. This was consistent with the outcome of a previous study by DeMarco *et al*^[11], in which investigators using a different methodology found a 25% additional ADR with the TER after each endoscopist had gained experience through performing 15 procedures.

Subset analysis segmenting the population by indication for procedure showed that in subjects undergoing colonoscopy for surveillance or diagnostic workup, TEC demonstrated additional ADRs of 35.7% and 55.4%, respectively, compared to SC. Pooling the results for those two groups of patients, who can be considered to have above-average risk for CRC, TEC showed an additional ADR of 40.7% compared to SC. In subjects undergoing screening colonoscopy, who can be regarded as having a low-to-average risk for CRC, TEC showed an additional ADR of 4.4% after correction for second-pass effect (Table 1).

In order to determine the statistical significance of these differences, we calculated the RR of missing an adenoma during first exams with SC *vs* first exams with TEC. The advantage with TEC proved to be statistically

significant for the surveillance and diagnostic groups, both separately and combined. However, the difference for the screening population was not significant (Table 2).

It is not surprising to find proportionally more lesions behind folds in surveillance patients because, by definition, they have had at least one previous colonoscopy during which polyps that were seen with the colonoscope were removed, while lesions that were hidden behind folds may have been left behind. In contrast, screening patients have not had adenomas removed, so whatever polyps they have are likely to be more evenly distributed. However, the reason for the difference in results between diagnostic patients and screening patients is not clear.

A multivariate analysis indicated that more adenomas were found during second exams with TEC than with SC, and more were found during second exams in males than in females. However, subset analysis showed that while the net additional ADR for TEC compared to SC trended lower for females than for males, the total number of adenomas detected in females during all exams was relatively small, and the difference between genders was not statistically significant.

The multivariate Poisson regression showed no significant influence of age, bowel preparation quality or withdrawal time on adenoma miss rates. These findings were confirmed by subset analysis in which patient age and bowel prep quality had no significant impact.

Mean withdrawal times for TEC were essentially identical for the groups segmented by indication for procedure, suggesting that the amount of time spent examining the mucosa could not explain the differences among those groups. However, total procedure times for TEC were somewhat longer for the surveillance and diagnostic populations, most likely because many more polypectomies were performed in those subjects compared to the screening group.

In this study, overall adenoma miss rates for SC were 31.4% for all-size and 11.8% for large adenomas (at least 10 mm). These miss rates are higher than suggested by previous studies involving two same-day colonoscopies. One such study found miss rates of 24% for all-size and 6% for large adenomas^[3]. Another found miss rates of 21% for all-size and 11% for advanced adenomas (but did not specify how many of those advanced adenomas were at least 10 mm)^[5]. These discrepancies are not surprising, since many authors have acknowledged the limitation inherent in comparing two examinations with identical technology: lesions that are hidden from the colonoscope during one exam are likely to be hidden during the second exam as well, causing them to be missed on the second exam and thus underestimating the true miss rate^[3-5,17].

Our 11.8% miss rate for large adenomas with SC is similar to the finding of a 12% miss rate for large adenomas in one study comparing colonoscopy with CT colonography (CTC)^[8]. Another comparison with CTC showed a 17% miss rate with SC for all polyps at least 10 mm, but did not indicate a miss rate specifically for large

adenomas^[17]. Due to the limited sensitivity of CTC for smaller lesions, neither study could reliably estimate the all-size adenoma miss rate for SC.

In Tables 1 and 2, we show that, except in the screening group, TEC provided a significant improvement in ADR on a per-polyp basis (i.e., number of adenomas per patient). As we currently think that each adenoma has the potential to develop into malignancy, it is desirable to detect and remove as many adenomas as possible except in the relatively uncommon circumstance in which the patient has a high risk for complications from a polypectomy of small lesions.

One might argue that it is important to determine ADR on a per-patient basis (i.e., the number of patients in whom at least one adenoma is found), because the detection of even one adenoma identifies the patient as a “polyp-former” who might be followed more carefully than a “non-polyp-former.” However, for example, if a patient had one diminutive tubular adenoma (5 mm or smaller) and two large advanced adenomas, this measure would attribute the same quality to an exam whether the endoscopist detected all 3 lesions or found only the small adenoma and missed the 2 more significant lesions.

Furthermore, the finding of a single small adenoma does not necessarily dictate close follow-up according to joint guidelines published by the United States Multi-Society Task Force on CRC, American College of Radiology and American Cancer Society^[16], which recommend follow-up at 3 years only for those patients who are at a high risk, defined as those with at least 1 advanced adenoma or at least 3 small adenomas. For patients with only 1-2 small tubular adenomas, who are at a low risk, the guidelines recommend repeat colonoscopy in 5-10 years.

Therefore, if we wished to consider a quality metric other than the per-polyp ADR, it might be more useful to calculate the number of subjects who are determined to be at high risk, i.e., those with at least 1 advanced adenoma or at least 3 small adenomas. In the surveillance group, due to detection of lesions with TEC that had been missed with SC, 42.9% additional subjects would be advised to return for 3-year follow-up per guidelines. However, the numbers were small and were not statistically significant.

Our results suggest that TEC offers an improvement in adenoma detection that can currently not be matched by other techniques or technologies. There is growing interest in improving quality in colonoscopy, and substantial evidence indicates that optimizing bowel preparation^[18-20], spending adequate time examining the mucosa^[21-24] and practicing high-quality withdrawal technique^[21,25,26] are associated with improved detection rates. However, none of these measures fully addresses the difficulty of detecting neoplasia located behind folds and flexures. According to one study comparing SC with CTC, two-thirds of adenomas missed with the colonoscope were located behind folds^[8], where they would likely be missed again during repeated exams.

CTC can reveal lesions on both sides of folds, but lacks sensitivity for small and flat lesions, and requires

referral for colonoscopy when abnormalities are identified^[27,28]. Cap-assisted colonoscopy does not require a second procedure, but studies have shown mixed results^[29-32] and even the most encouraging study demonstrated an advantage only for the detection of diminutive adenomas^[32]. Retroflexion of a colonoscope in the proximal colon has been found to be feasible by highly-experienced endoscopists, but results of studies so far have been mixed^[33-35]. Moreover, the safety, practicality and time requirement for routine retroflexion proximal to the rectum still need to be established.

Reducing the prevalence of CRC in a cost-effective manner requires use of the proper examination for each patient, so it is important to identify which segments of the population are most likely to benefit from various technologies. The findings in this subset analysis may have implications for evidence-based patient selection for TEC. In our study, TEC demonstrated a clinically and statistically significant benefit for the subsets of patients who presented for surveillance in follow-up of previous polypectomy and for diagnostic workup of symptoms. However, our results did not provide evidence for efficacy in patients who presented for routine screening.

Cost-effectiveness also requires appropriate timing of examinations. There is evidence that many endoscopists perform surveillance colonoscopy at shorter intervals than those recommended by guidelines^[16,36,37]. Their decisions regarding surveillance intervals may be influenced by the growing recognition that limitations in technology result in substantial miss rates during SC and by associated medico-legal concerns^[36,37]. Improving the quality of colonoscopy through use of a retrograde-viewing device could instill confidence in physicians and patients that the likelihood of missing significant neoplasia is very low and thus foster greater compliance with guidelines for surveillance intervals.

Limitations

The TERRACE study was not powered for analysis of subsets, and some numerical trends revealed by this post-hoc analysis, such as difference in additional detection rates between genders, did not reach statistical significance.

Only 33.5% of the subjects were female. Some of this discrepancy was due to one of the sites being a Veteran's Administration Medical Center, where 55 of the 57 subjects were male. Another, probably more significant, factor was that the TERRACE study was performed with the first-generation Retroscope, which was too large to fit through the working channel of "pediatric" size colonoscopes. With the growing trend toward using these smaller colonoscopes when examining female patients, it is likely that some investigators may have chosen not to enroll some of their female patients in the study on the basis that use of an "adult" colonoscope might have made the examination more difficult and/or uncomfortable (The second-generation device that is currently in use differs in that it has a higher-resolution camera and a smaller diameter that allows its use with all colonoscopes).

In conclusion, the results of this subset analysis demonstrated that adenoma miss rates for SC are even higher than those previously estimated through performance of back-to-back colonoscopy exams.

Use of a retrograde-viewing device significantly improves the quality of colonoscopy by increasing the detection of adenomas of all sizes. Use of the retrograde-viewing device along with a colonoscope appears to have the greatest value for patients undergoing colonoscopy for surveillance or for diagnostic workup of symptoms, and the study results do not provide evidence for efficacy of the device in routine screening colonoscopy. These findings could provide endoscopists with an evidence-driven basis for selection of patients most likely to benefit from this new technology.

COMMENTS

Background

This article expands on a previously-published report of the Third Eye Retroscope Randomized Clinical Evaluation (TERRACE), which was a large randomized, controlled trial. TERRACE evaluated the effectiveness of a device that provides an additional, retrograde (backward) view to allow examination of areas located behind folds in the colon wall that hide them from the forward-viewing camera during standard colonoscopy (SC). The previously-published overall TERRACE results showed that Third Eye colonoscopy (TEC) detected 23.2% additional pre-cancerous adenomas not found with SC.

Research frontiers

This new report focuses on post-hoc analyses of the TERRACE data to determine whether some subsets of patients might benefit more than others from TEC, to evaluate the clinical significance of the additional adenomas detected through TEC and to determine how finding those additional adenomas would affect recommendations for follow-up intervals.

Innovations and breakthroughs

These new analyses of the TERRACE data showed that, of all the variables that might influence the effectiveness of TEC, only the indication - i.e., the reason for performing the colonoscopy procedure - was significant. Patients who were having "surveillance" colonoscopy because they were found to have adenomas during previous exams, or "diagnostic" colonoscopy to look for a cause for symptoms, are considered to have above-average risk for colorectal cancer (CRC), and pooled results for those groups showed a 40.7% net additional adenoma detection rate (ADR) for TEC compared to SC. In contrast, patients who were having routine "screening" colonoscopy - who are considered to have low-to-average risk - had a 4.4% net additional ADR with TEC. In all of the groups combined, SC missed 11.8% of large adenomas, while none were missed during TEC.

Applications

These results confirm that use of a retrograde-viewing device allows detection of substantial numbers of clinically-significant adenomas that would be missed with the colonoscope alone. The finding that use of the device appears to offer more benefit for patients undergoing surveillance or diagnostic colonoscopy than for screening patients may provide physicians with a basis for selecting patients in whom they will use the device.

Terminology

Adenomas are polyps that have the potential to transform into CRC, and most cases of CRC arise from adenomas. The term "post-hoc" refers to additional analyses that were not anticipated in the original study protocol.

Peer review

The results of this post-hoc analysis will be helpful in supporting use of the Third Eye Retroscope (TER) in high-risk patients, which will allow for a more cost-effective approach as endoscopists continue to improve quality in colonoscopy. The authors are congratulated on demonstrating how TEC can detect additional adenomas during colonoscopy. Furthermore, the post-hoc analysis answers the question of who should have a TER used during colonoscopy. Based on the data from this manuscript, it would be those undergoing surveillance and diagnostic exams.

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Importance of early diagnosis of pancreaticobiliary maljunction without biliary dilatation

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Abstract

AIM: To clarify the strategy for early diagnosis of pancreaticobiliary maljunction (PBM) without biliary dilatation and to pathologically examine gallbladder before cancer develops.

METHODS: The anatomy of the union of the pancreatic and bile ducts was assessed by using endoscopic retrograde cholangiopancreatography (ERCP). Patients with a long common channel in which communication between the pancreatic and bile ducts was maintained even during sphincter contraction were diagnosed as having PBM. Of these, patients in which the maximal diameter of the bile duct was less than 10 mm were

diagnosed with PBM without biliary dilatation. The process of diagnosing 54 patients with PBM without biliary dilatation was retrospectively investigated. Histopathological analysis of resected gallbladder specimens from 8 patients with PBM without biliary dilatation or cancer was conducted.

RESULTS: Thirty-six PBM patients without biliary dilatation were diagnosed with gallbladder cancer after showing clinical symptoms such as abdominal or back pain ($n = 16$) or jaundice ($n = 12$). Radical surgery for gallbladder cancer was only possible in 11 patients (31%) and only 4 patients (11%) survived for 5 years. Eight patients were suspected as having PBM without biliary dilatation from the finding of gallbladder wall thickening on ultrasound and the diagnosis was confirmed by ERCP and/or magnetic resonance cholangiopancreatography (MRCP). The median age of these 8 patients was younger by a decade than PBM patients with gallbladder cancer. All 8 patients underwent prophylactic cholecystectomy and bile duct cancer has not occurred. Wall thickness and mucosal height of the 8 resected gallbladders were significantly greater than controls, and hyperplastic changes, hypertrophic muscular layer, subserosal fibrosis, and adenomyomatosis were detected in 7 (88%), 5 (63%), 7 (88%) and 5 (63%) patients, respectively. Ki-67 labeling index was high and K-ras mutation was detected in 3 of 6 patients.

CONCLUSION: To detect PBM without biliary dilatation before onset of gallbladder cancer, we should perform MRCP for individuals showing increased gallbladder wall thickness on ultrasound.

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Key words: Pancreaticobiliary maljunction; Pancreatobiliary reflux; Ultrasound; Gallbladder cancer; Endoscopic ultrasonography

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INTRODUCTION

Pancreaticobiliary maljunction (PBM) is a congenital anomaly defined as a junction of the pancreatic and bile ducts located outside the duodenal wall, and usually forming a markedly long common channel. As the action of the sphincter of oddi does not have a functional impact on the junction of the pancreatic and bile ducts in PBM cases, PBM causes a continuous reciprocal reflux of pancreatic juice and bile^[1,2]. This results in various pathological conditions of the biliary tract and pancreas. Given that the hydropressure in the pancreatic duct is usually greater than that in the bile duct, pancreatic juice frequently flows back into the biliary duct (pancreatobiliary reflux), and this becomes a high risk factor for biliary cancer^[3-5]. In a Japanese analysis^[6], biliary cancer was associated with 131 (10.6%) of 1239 cases with PBM with biliary dilatation (congenital choledochal cyst); 44 (33.6%) of the 131 cases had cancer of the extrahepatic bile duct and 85 cases (64.9%) had gallbladder cancer. On the other hand, PBM without biliary dilatation was associated with biliary cancer in 147 (37.9%) of 388 cases, and 137 (93.2%) of these cancers were gallbladder cancer.

Most PBM cases detected in childhood are associated with bile duct dilatation, but one third of PBM detected in adults do not show dilatation of the bile duct. Many patients with PBM with biliary dilatation have clinical symptoms due to cholangitis or pancreatitis in childhood, whereas in PBM without biliary dilatation, few patients have symptoms in childhood and they are usually not diagnosed until adulthood^[7]. Furthermore, many patients with PBM without biliary dilatation are diagnosed in association with advanced-stage gallbladder cancer, which carries a poor prognosis^[2,6,7]. It is necessary to clarify a strategy to diagnose PBM without biliary dilatation early, before cancer occurs. In the present study, we investigated the process for diagnosing patients with PBM without biliary dilatation and examined histopathological findings from the gallbladder of patients with PBM without biliary dilatation before cancer developed.

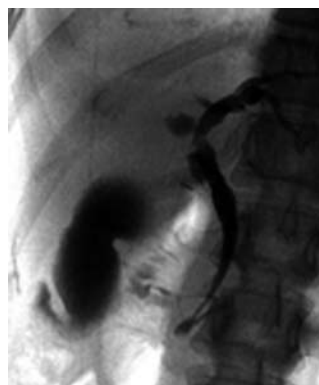


Figure 1 Endoscopic retrograde cholangiopancreatography of a patient with pancreaticobiliary maljunction without biliary dilatation showing a long common channel and deformity with fuzzy irregularity of the gallbladder.

MATERIALS AND METHODS

Study patients

We studied the anatomy of the union of the pancreatic and bile ducts using endoscopic retrograde cholangiopancreatography (ERCP). Patients with a long common channel in which communication between the pancreatic and bile ducts was maintained in both relaxation and contraction of the sphincter under serial observation during ERCP were diagnosed as having PBM^[1,2]. Of these, patients in whom the maximal diameter of the bile duct was less than 10 mm were diagnosed with PBM without biliary dilatation (Figure 1). When the common bile duct was involved with associated gallbladder cancer, diameter of the intact distal portion of the bile duct was measured.

Between January 1975 and December 2010, 104 patients were diagnosed with PBM with ERCP. Endoscopic ultrasonography (EUS) and magnetic resonance cholangiopancreatography (MRCP) were performed from 1990 and 2001, respectively. Of these, 54 patients (12 men and 42 women; median age at initial diagnosis of PBM 56.6 years, range 30-77 years) were diagnosed with PBM without biliary dilatation. The process leading to diagnosis of these patients was retrospectively investigated. This study was approved by the institutional review board.

Histopathological and immunohistochemical examinations

We conducted histopathological analysis of resected gallbladder specimens from 8 patients with PBM without biliary dilatation or cancer. Tissues were fixed in 10% buffered formalin and embedded in paraffin. Serial 3-μm sections were stained with hematoxylin and eosin for evaluation. Thickness of the gallbladder wall and mucosal height of each specimen were measured using a semiautomatic image analyzer. Hyperplastic changes were defined as an increased number of mucosal folds that were longer than normal, irregular, and frequently branched. Immunohistochemistry was performed us-

Table 1 Clinical features of 8 pancreaticobiliary maljunction patients without biliary dilatation or cancer

Case	Age (yr)	Sex	Process leading to ultrasound	Amylase level in bile (IU/L)	Follow-up
1	30	F	Screening for hepatitis A	528 000	Follow-up after 236 mo
2	55	F	Screening for chronic hepatitis C	69 850	Follow-up after 47 mo
3	65	F	Cholangitis with mild acute pancreatitis	17 272	Follow-up after 40 mo
4	41	F	Right hypochondralgia	117 240	Follow-up after 12 mo
5	70	F	Screening for diabetes mellitus	254 900	Follow-up after 3 mo
6	42	M	Diarrhea	185 000	Unknown after 75 mo
7	47	M	Abdominal pain	300 000	Unknown after 18 mo
8	36	M	Abdominal discomfort	672 100	Unknown after 1 mo

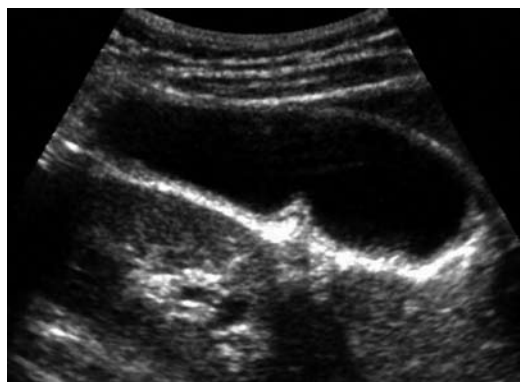


Figure 2 Abdominal ultrasound of a patient with pancreaticobiliary maljunction without biliary dilatation showing uniform smooth thickness of the gallbladder wall.

ing antisera for Ki-67 (clone MIB-1: Immunotech SA, Marseille, France) by the avidin-biotin horseradish peroxidase method (Vectastain Elite ABC kit; Vector, Burlingame, CA, United States). Ki-67-positive cells were defined as a cell with brown staining of the nucleus, and Ki-67 labeling index was determined by counting a minimum of 500 cells in the area representing the most homogenous region of positive cells.

Gallbladder epithelium was microdissected from 20- μ m formalin-fixed paraffin-embedded sections. Genomic DNA was extracted from the tissues by using Takara Dexpattm (Takara Shuzo, Otsu, Japan). A point mutation of K-ras codon 12 was analyzed by polymerase chain reaction enzyme-linked minisequence assay (Sumitomo Metal Industry, Tokyo, Japan). The precise methodology of this assay is described elsewhere^[8].

Ten cases of normal gallbladder that were resected at pancreatoduodenectomy for pancreatic diseases were selected as controls.

Statistical analysis

Statistical analysis was performed using chi-squared analysis or Mann-Whitney's *U* test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Process of diagnosing PBM without biliary dilatation

Clues associated with a diagnosis of PBM without biliary dilatation were the presence of gallbladder cancer (*n* =

36), other malignancies (*n* = 4), chronic pancreatitis (*n* = 1), intrahepatic stones (*n* = 1), gallstones (*n* = 1), gastritis (*n* = 1), previous cholecystectomy (*n* = 2), and gallbladder wall thickening (more than 3 mm) on ultrasound (US; *n* = 8) (Figure 2). Thirty-one patients with gallbladder cancer had clinical symptoms such as abdominal or back pain (*n* = 16), jaundice (*n* = 12), and abdominal discomfort (*n* = 3), and the other 5 gallbladder cancers were detected using screening tests such as US (*n* = 4) and fluorode-oxyglucose positron emission tomography (*n* = 1). Radical surgery was possible in only 11 (31%) of 36 cases of gallbladder cancer and only 4 patients (11%) survived for 5 years. Three PBM patients without biliary dilatation associated with benign diseases (chronic pancreatitis, gallstones and gastritis) were diagnosed before 1985, and they were lost to follow up without surgery.

PBM patients without biliary dilatation diagnosed from gallbladder wall thickening on US

PBM without biliary dilatation was suspected in 8 patients based on finding of gallbladder wall thickening on US. Abdominal US was performed for symptoms such as abdominal pain in 5 patients to screen for associated diseases. Following US, computed tomography (*n* = 8), EUS (*n* = 5), MRCP (*n* = 4), and ERCP (*n* = 8) were done. The median age of patients was 48.2 years (range 30-70 years; 3 men and 5 women), which was significantly younger than that of PBM patients with gallbladder cancer (median age, 58.5 years; *P* < 0.01). Based on the diagnosis of PBM without biliary dilatation, all patients underwent prophylactic cholecystectomy. Amylase levels in the bile were markedly elevated in all patients. Five patients have been regularly followed, and 3 patients were lost to follow up 1-75 mo after surgery. Bile duct cancer has not occurred in any patient (Table 1). On EUS, thickening of the inner hypoechoic layer and outer hyperechoic layer was detected in 2 patients (Figure 3), and thickening of the inner hypoechoic layer, a middle, more hypoechoic layer, and the outer hyperechoic layer was observed in 3 patients.

Histopathological findings of the gallbladder of PBM patients without biliary dilatation diagnosed from gallbladder wall thickening on US

On histological findings of the resected gallbladder, the gallbladder wall was more thickened in PBM (7.7 ± 7.0 mm, mean $\pm$ SD) than in control cases (2.0 ± 0.5 mm, *P*

Table 2 Histopathological findings of gallbladders from patients with pancreaticobiliary maljunction without biliary dilatation diagnosed based on gallbladder wall thickening on ultrasound

Case	Thickness of the wall (mm)	Mucosal height (mm)	Hyperplastic change	Hypertrophic muscular layer	Subserosal fibrosis	Adenomyomatosis	Gallstone	Ki-67 labeling index (%)	K-ras mutation
1	7	2.4	+	-	+	-	-	8	GAT (3+)
2	6	1.8	+	-	+	+	-	5	NA
3	3	0.6	-	-	-	-	+	6	-
4	5	2.2	+	+	+	+	-	4	-
5	5	1.7	+	+	+	+	-	5	NA
6	8	1.9	+	+	+	+	-	8	AGT (1+)
7	6	1.4	+	+	+	-	+	9	AGT (2+)
8	25	0.9	+	+	+	+	-	3	-
PBM, %, mean $\pm$ SD	7.7 $\pm$ 7.0 ^b	1.06 $\pm$ 0.7 ^b	88 (7/8) ^b	63 (5/8) ^b	88 (7/8) ^b	63 (5/8) ^b	25 (2/8)	6.0 $\pm$ 2.1 ^b	50 (3/6) ^a
Controls, %, (n = 10), mean $\pm$ SD	2.0 $\pm$ 0.5	0.58 $\pm$ 0.24	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/10)	10 (1/10)	1.2 $\pm$ 0.3	0 (0/10)

PBM: Pancreaticobiliary maljunction; NA: Not amplified. ^a*P* < 0.05, ^b*P* < 0.01 *vs* controls.



Figure 3 Endoscopic ultrasonography of a patient with pancreaticobiliary maljunction without biliary dilatation showing thickening of the inner hypoechoic layer and the outer hyperechoic layer.

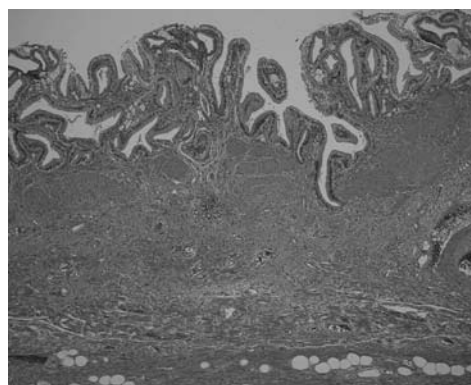


Figure 4 Histological findings of the gallbladder of a patient with pancreaticobiliary maljunction without biliary dilatation showing wall thickness composed of epithelial hyperplasia, hypertrophic muscular layer, and subserosal fibrosis.

< 0.01). Mucosal height was significantly higher in PBM (1.0 $\pm$ 0.7 mm) than in control cases (0.5 $\pm$ 0.2 mm, *P* < 0.01). Hyperplastic changes, hypertrophic muscular layer, subserosal fibrosis, and adenomyomatosis were detected in 7 (88%), 5 (63%), 7 (88%) and 5 (63%) of PBM patients, respectively, compared with no control cases (Figure 4). No dysplastic changes were observed. Ki-67 labeling index was higher in PBM (6.0% $\pm$ 2.1%) than control cases (1.2% $\pm$ 0.3%, *P* < 0.01). K-ras mutation was detected in 3 (50%) of 6 PBM patients (Table 2). Adenomatous changes were not observed in any patient.

DISCUSSION

Because PBM patients have a high incidence of biliary cancer due to pancreatobiliary reflux, once PBM is diagnosed, prophylactic biliary surgery is recommended before the onset of malignant change^[2]. In particular, PBM without biliary dilatation is frequently associated with gallbladder cancer. However, PBM cases without biliary dilatation rarely evoke symptoms, and most patients are not diagnosed until the onset of advanced-stage gallbladder cancer^[2,6,7].

In this series, 36 (67%) of 54 PBM patients without biliary dilatation were diagnosed based on associated gallbladder cancer. Most gallbladder cancers were in an advanced stage, and resection was possible in only 11 patients (31%). Since a paper^[9] published in 1991 reported that gallbladder wall thickening was sometimes observed in PBM cases without biliary dilatation, we have prospectively checked for PBM after finding gallbladder wall thickening on US. This additional investigation using MRCP and ERCP allowed us to identify 8 patients with PBM without biliary dilatation before the onset of gallbladder cancer. All 8 patients underwent prophylactic cholecystectomy, and bile duct cancer has not occurred in any patient. The median age of the 8 patients was about 10 years younger than PBM patients with associated gallbladder cancer. The age of PBM patients with gallbladder cancer at the time of diagnosis peaked in the 50s, and was younger by a decade than that of gallbladder cancer patients without PBM^[10]. Chronic injury to the epithelium of bile duct by pancreatic juice induces severe inflammation and malignant changes in PBM patients; therefore, age can be a major determinant of

risk. To eliminate the risk of gallbladder cancer, we must detect PBM without biliary dilatation early in adults.

Epithelial hyperplasia of the gallbladder induced by longstanding continuous stasis of the bile intermingled with refluxed pancreatic juice has been reported to be one of the characteristic pathological changes in PBM patients^[11-13]. The incidence of epithelial hyperplasia of the gallbladder of PBM patients without biliary dilatation was reported to be 72%^[11] to 91%^[12]. Tanno *et al*^[12] reported that the Ki-67 labeling index of epithelial hyperplasia of PBM patients was elevated to $6.1\% \pm 1.5\%$ and K-ras mutation was detected in 2 (13%) of 15 patients. Histopathological findings of our 8 cases were similar to the above findings. Considering that increased cell proliferation is linked to the development of cancer by means of tumor promotion and an increased rate of random mutations, gallbladder epithelium of PBM patients can be considered to represent a premalignant lesion.

Interestingly, hypertrophic muscular layer, subserosal fibrosis, and adenomyomatosis were detected in 63%, 88% and 63%, respectively. Sugai *et al*^[14] reported that subserosal fibrosis with chronic inflammation was detected in 33% of gallbladders of 27 PBM patients, and Tsuchida *et al*^[11] found K-ras mutations in 5 (50%) of 10 gallbladders of PBM without biliary dilatation. Long-standing continuous stimulation with bile containing pancreatic juice may induce these three changes as well as epithelial hyperplasia.

EUS shows the normal gallbladder wall to be a two-layered structure consisting of an inner hypoechoic layer composed of the mucosa and the muscular layer, and an outer hyperechoic layer composed of the subserosal layer and the serosa^[15]. On EUS, the gallbladder wall of patients with PBM appeared as two thickened layers showing epithelial hyperplasia and subserosal fibrosis or three thickened layers containing a middle, more hypoechoic, layer showing a hypertrophic muscular layer.

Although ERCP is the gold standard for diagnosis of PBM, MRCP has recently become a common non-invasive method for obtaining quality images of the pancreaticobiliary tract. MRCP can diagnose many PBM cases based on findings of an anomalous union between the common bile duct and the pancreatic duct, although some atypical PBM cases with relatively short common channel cannot be diagnosed only by MRCP, and should be confirmed by ERCP^[16]. To detect patients with PBM without biliary dilatation early requires that certain patients undergo MRCP before gallbladder cancer occurs. Gallbladder wall thickness on US during medical checkups may serve as an indication for MRCP and EUS before ERCP for suspected PBM without biliary dilatation. Once PBM is diagnosed in patients with increased gallbladder wall thickness, prophylactic biliary surgery is recommended before the onset of malignant change.

In conclusion, hyperplastic gallbladder mucosa of PBM patients represents a premalignant lesion. To detect PBM without biliary dilatation before onset of gallbladder cancer, we should perform MRCP for individuals showing gallbladder wall thickness on US.

COMMENTS

Background

Pancreaticobiliary maljunction (PBM) causes a continuous reciprocal reflux of pancreatic juice and bile. PBM cases without biliary dilatation rarely evoke symptoms, and most patients are not diagnosed until the onset of advanced-stage gallbladder cancer. It is necessary to clarify a strategy to diagnose PBM without biliary dilatation early, before cancer occurs.

Research frontiers

The authors investigated the process for diagnosing patients with PBM without biliary dilatation and examined histopathological findings from the gallbladder of patients with PBM without biliary dilatation before cancer developed.

Innovations and breakthroughs

Thirty-six (66%) out of 54 PBM patients without biliary dilatation were diagnosed with gallbladder cancer. Radical surgery for gallbladder cancer was only possible in 11 patients and only 4 patients survived for 5 years. Eight patients were suspected as having PBM without biliary dilatation from the finding of gallbladder wall thickening on ultrasound and the diagnosis was confirmed by endoscopic retrograde cholangiopancreatography and/or magnetic resonance cholangiopancreatography (MRCP). The median age of these 8 patients was younger by a decade than PBM patients with gallbladder cancer. All 8 patients underwent prophylactic cholecystectomy and bile duct cancer has not occurred. Wall thickness and mucosal height of the 8 resected gallbladders were significantly greater than controls, and hyperplastic changes were detected in 7 (88%) patients. Ki-67 labeling index was high and K-ras mutation was detected in 3 of 6 patients.

Applications

To detect PBM without biliary dilatation before onset of gallbladder cancer, people should perform MRCP for individuals showing increased gallbladder wall thickness on ultrasound.

Peer review

The study has a good-sized patient cohort and involves appropriate methods of analysis. The interpretation and presentation of the results are both appropriate. The findings of this study should contribute in the effective management of patients with PBM.

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Effect of sumatriptan on gastric emptying: A crossover study using the BreathID system

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in this randomized, 2-way crossover study. The subjects fasted overnight and were randomly assigned to receive a test meal (200 kcal/200 mL) 30 min after pre-medication with sumatriptan 50 mg (sumatriptan condition), or the test meal alone (control condition). Gastric emptying was monitored for 4 h after administration of the test meal by the ¹³C-acetic acid breath test performed continually using the BreathID system. Then, using Oridion Research Software (β version), the time taken for emptying of 50% of the labeled meal ($T_{1/2}$) similar to the scintigraphy lag time for 10% emptying of the labeled meal (T_{lag}), the gastric emptying coefficient (GEC), and the regression-estimated constants (β and κ) were calculated. The statistical significance of any differences in the parameters were analyzed using Wilcoxon's signed-rank test.

RESULTS: In the sumatriptan condition, significant differences compared with the control condition were found in $T_{1/2}$ [median 131.84 min (range, 103.13-168.70) vs 120.27 min (89.61-138.25); $P = 0.0016$], T_{lag} [median 80.085 min (59.23-125.89) vs 61.11 min (39.86-87.05); $P = 0.0125$], and β [median 2.3374 (1.6407-3.8209) vs 2.0847 (1.4755-2.9269); $P = 0.0284$]. There were no significant differences in the GEC or κ between the 2 conditions.

CONCLUSION: This study showed that oral sumatriptan significantly delayed gastric emptying of a liquid meal.

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Key words: Sumatriptan; Gastric emptying; Breath test; Liquid meal; BreathID system

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Abstract

AIM: To determine the effect of oral sumatriptan on gastric emptying using a continuous ¹³C breath test (BreathID system).

METHODS: Ten healthy male volunteers participated

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INTRODUCTION

Sumatriptan, a selective 5-HT₁ receptor agonist, was launched in the market in 1991, representing the single most remarkable advance to date in the treatment of migraine^[1]. It was suspected that the drug might delay gastric emptying of liquids in healthy subjects by prolonging the lag phase of gastric emptying^[2,3]. Several studies have reported that sumatriptan restores gastric accommodation in patients with functional dyspepsia^[4-9].

A recent study showed that the accelerated gastric emptying of nutrient liquids can be a surrogate marker of impaired distension-induced accommodation^[10]. After aggressive filling of the stomach with a nutrient liquid, the resultant increase in intragastric pressure determines the rate of gastric emptying; higher luminal pressure caused by defective accommodation accelerates gastric emptying^[10,11]. Conversely, it would be logical to consider that the gastric emptying of a nutrient liquid would be delayed when distension-induced accommodation is enhanced. Compared with second-generation 5HT_{1B/1D} receptor agonist antimigraine drugs, sumatriptan shows poor oral bioavailability and a relatively short elimination half-life^[6]. According to a previous report, the T_{max} of sumatriptan is 2-3 h, the T_{1/2} is 2 h, and the oral bioavailability is 14%^[6]. The aim of this preliminary study was to test our hypothesis that oral sumatriptan intake would cause a delay in the gastric emptying of nutrient liquids. If the hypothesis were proven to be true, it would lend indirect support to the suggestion that sumatriptan enhances distension-induced gastric accommodation^[11].

In this study, we investigated the physiologic effect of sumatriptan on the rate of gastric emptying using a continuous real-time ¹³C breath test (BreathID system: Exalenz Bioscience Ltd., Israel).

MATERIALS AND METHODS

Ethics

The study was conducted in accordance with the principles laid down in the Declaration of Helsinki. The study protocol using the BreathID system was approved by the Ethics Committee of Yokohama City University School of Medicine (No. A110929010).

Subjects

The subjects were 10 asymptomatic male volunteers, all of whom were students of Yokohama City University,

non-smokers, none were habitual drinkers, and none had a history of gastrointestinal disease or abdominal surgery. None of the subjects were on any routine medications at the time of the study.

¹³C-acetic acid breath test

Ten subjects participated in this randomized, 2-way crossover study. The 2 tests were conducted as follows: (1) The subjects were assigned to receive a test meal 30 min after intake of a 50 mg tablet of sumatriptan; and (2) The subjects only received the test meal. The two test protocols were administered in crossover fashion separated by a washout period of at least 7 d. In both experiments, the protocols were started after the patients had fasted overnight (for at least 8 h), and the breath test was performed for 4 h while the subjects were seated.

In experiment A, subjects were asked to take a 50 mg tablet of sumatriptan prior to the administration of a test meal. The test meal consisted of a 200 kcal/200 mL liquid meal (Racol with milk flavor, Otsuka Pharmaceutical, Tokyo, Japan) containing 100 mg of ¹³C-acetic acid (Cambridge Isotope Laboratories, Boston, MA, United States)^[12-23], which the patients were requested to consume within 5 min. In experiment B, subjects underwent the breath test after ingestion of the test meal alone. Breath samples were continuously collected *via* a nasal tube using the BreathID system (Exalenz Bioscience Ltd., Israel) from baseline, before administration of the test meal, until 4 h after completion of the test meal ingestion (time 0)^[14-23].

Analysis of the ¹³C-acetic acid breath test data

The data from the ¹³C acetic acid breath test were analyzed using the Oridion Research Software, version β (Oridion Medical Ltd., Israel)^[14-23]. The time *vs* ¹³CO₂ excretion rate curve was fitted to the conventional formula of $z(t) = m(1 - e^{-\kappa t})^\beta$, and the regression-estimated constants κ and β were determined^[24]. After the mathematical analysis, the time required for emptying of 50% of the labeled meal (T_{1/2}) similar to the scintigraphy lag time for 10% emptying of the labeled meal (T_{lag}), the gastric emptying coefficient (GEC), and the regression-estimated constants (β and κ) were also calculated. A larger (smaller) value of β indicates slower (faster) emptying in the early phase, and a larger (smaller) value of κ indicates faster (slower) emptying in the later phase.

Statistical analysis

Statistical evaluation was carried out using the Wilcoxon's signed-rank test. The level of significance was set at $P < 0.05$. The statistical analysis were performed with the StatView software (SAS Institute, Cary, NC, United States).

RESULTS

The study was completed in 10 male subjects (mean age, 30.7 years; median age, 31 years; range, 24-38). No adverse events occurred during the study. The subjects'

Table 1 Comparison of breath test parameters

	Sumatriptan	Control	P value
T _{1/2}	131.84 (103.13-168.70)	120.27 (89.61-138.25)	0.0166
T _{lag}	80.085 (59.23-125.89)	61.11 (39.86-87.05)	0.0125
GEC	3.545 (3.20-3.97)	3.55 (3.19-4.03)	0.6460
β	2.3374 (1.6407-3.8209)	2.0847 (1.4755-2.9269)	0.0284
κ	0.5833 (0.3689-0.7836)	0.6180 (0.4661-0.8872)	0.3329

T_{1/2}: Time to emptying of 50% of the labeled meal (min); T_{lag}: Very similar to the percentage dose recovery peak time (min); GEC: Gastric emptying coefficient; β and κ: Regression-estimated constants. A larger (smaller) value of β indicates slower (faster) emptying in the early phase, and a larger (smaller) value of κ indicates faster (slower) emptying in the later phase.

mean height was 171.5 cm, their median height was 171 cm (range, 165-180 cm), their mean weight was 72.6 kg, and their median weight was 69.5 kg (range, 62-92 kg).

Table 1 summarizes the sumatriptan-induced changes in the breath test parameters. In the sumatriptan condition, significant differences compared with the control condition were found in T_{1/2}, [median, 131.84 min (range, 103.13-168.70) *vs* 120.27 min (89.61-138.25); *P* = 0.0016], T_{lag}, [median, 80.085 min (59.23-125.89) *vs* 61.11 min (39.86-87.05); *P* = 0.0125], and value of β [median 2.3374: 1.6407-3.8209) *vs* 2.0847 (1.4755-2.9269); *P* = 0.0284]. On the other hand, there were no significant differences in GEC [median, 3.545 (3.20-3.97) *vs* 3.55 (3.19-4.03); *P* = 0.6460] or κ [median, 0.5833 (0.3689-0.7836) *vs* 0.6180 (0.4661-0.8872); *P* = 0.3329] between sumatriptan and control conditions, respectively.

DISCUSSION

In this study, we examined the changes in the rate of gastric emptying during the first 4 h after oral administration of a 50 mg tablet of sumatriptan half an hour prior to a test meal in healthy subjects. The rate of gastric emptying was measured by the ¹³C-acetic acid breath test.

The ¹³C-acetic acid breath test is a non-invasive and well-established test for measuring the rate of gastric emptying of liquid meals, and the results of this test have been shown to be significantly correlated with those of scintigraphy^[12-24]. The subject ingests ¹³C-labeled acetic acid, which passes through the stomach and is absorbed in the duodenum and upper small bowel. The ¹³C-labeled acetic acid is then metabolized in the liver and excreted from the lung as ¹³CO₂. This test thus enables gastric emptying to be measured in a non-invasive manner^[12-24]. The BreathID system allows continuous evaluation of gastric emptying. In patients, it allows real-time breath analysis with a shortened examination time and minimal patient discomfort. Continuous analysis also provides quick, immediate results^[14-23].

In general, administration of sumatriptan, a 5HT₁ receptor agonist, has been reported to delay gastric emptying of calorie-containing liquids, and to restore gastric accommodation. In other words, sumatriptan allows both increased gastric relaxation after ingestion of

a meal and an increased caloric intake at maximum satiety^[9,25,26]. However, several studies have reported these effects of sumatriptan after subcutaneous sumatriptan injection^[3,9,26-28]. As previously mentioned, sumatriptan shows poor oral bioavailability and a relatively short elimination half-life profile. To the best of our knowledge, no researchers have reported the aforementioned effects following administration of sumatriptan *via* the oral route. Therefore, we studied the effect of oral sumatriptan on the rate of gastric emptying.

Sumatriptan has been reported to be effective in relieving nausea associated with a migraine attack^[7], although specific antiemetic agents are often required to control the symptoms and to restore normal gastrointestinal motor patterns^[7,9].

In the present study, the amount of sumatriptan administered was not adjusted to the body weight of each individual, with the drug being administered *via* the oral route. T_{lag} of sumatriptan has been reported to be 2 or 3 h. In a recent study, the BreathID examination was started half an hour after the subjects took sumatriptan orally, and was continued for 4 h. Therefore, the T_{lag} of sumatriptan would have been reached while the BreathID examination was still ongoing.

In the present study, an increase in T_{lag} and T_{1/2}, and also in the value of β, was observed in the subjects administered sumatriptan prior to the test meal. Thus, sumatriptan delayed gastric emptying. In addition, the increase in the value of β indicated that sumatriptan in particular delayed the early phase of gastric emptying. We think that these findings were attributable to restoration of distention-induced gastric accommodation by sumatriptan. Several studies have shown that in patients with functional dyspepsia, who showed impaired accommodation of the proximal stomach, subcutaneously administered sumatriptan restored gastric accommodation, thereby significantly improving meal-induced satiety^[9,26,27]. In healthy volunteers, accommodation of the proximal stomach may be thought to increase after administration of sumatriptan. A previous study using real-time ultrasonography and computed tomography demonstrated that subcutaneous administration of sumatriptan, after distention of the stomach with liquids, produced a reduction in the proximal and distal transverse area and an increase in the sagittal axis of the proximal stomach^[28]. Another study using duplex sonography showed that subcutaneous administration of sumatriptan 10 min postprandially caused a significant widening of both the gastric antrum and the proximal stomach. Therefore, the authors concluded that the time to commencement of peristalsis-related emptying is delayed following administration of sumatriptan^[26].

The effect of 5-HT on gastric fundus tone has not been studied. However, Tack *et al.*^[29] recently showed that sumatriptan, which is a 5-HT₁ receptor agonist, is an agonist at 5-HT_{1P} receptors on nitrergic myenteric neurons in the stomach. We consider this action was induced through 5-HT_{1P} receptors as one of the side effects of

sumatriptan.

In the present study, we found that despite its low bioavailability, oral administration of even one tablet of sumatriptan consistently affected the rate of gastric emptying. Delayed gastric emptying may represent an increase in gastric accommodation.

In conclusion, this study suggests that sumatriptan, which can restore gastric accommodation, may be of benefit to many patients suffering from the distress of early satiety.

COMMENTS

Background

The incidence of functional gastrointestinal disorders is currently increasing worldwide though the cause of this disease is largely unknown. Rome III criteria included postprandial distress syndrome, which might come from impaired gastric accommodation and gastric emptying.

Research frontiers

It is accepted that both gastric emptying and gastric accommodation are important in functional gastrointestinal disorders. However, how gastric accommodation affects gastric emptying in a breath test has not been researched. In this study, the authors demonstrated that oral sumatriptan significantly delayed gastric emptying and suggest that delayed gastric emptying may represent an increase in gastric accommodation.

Innovations and breakthroughs

This is the first study to report that oral administration of sumatriptan affected the rate of gastric emptying. Furthermore, the studies would suggest that delayed gastric emptying may represent an increase in gastric accommodation.

Applications

By understanding both gastric emptying and gastric accommodation, this study may represent a future strategy for therapeutic intervention in the treatment of patients with functional dyspepsia.

Terminology

The BreathID system allows measurement of gastric emptying by a breath test. This allows continuous and real-time evaluation of gastric emptying. Continuous analysis also provides quick, immediate results.

Peer review

This paper demonstrated a new way to identify the gastric emptying in human subjects; this is the main merit of this study.

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Safety and effectiveness of propofol sedation during and after outpatient colonoscopy

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Abstract

AIM: To study the safety and effectiveness of propofol sedation for outpatient colonoscopy.

METHODS: Propofol was given by bolus injection with an age-adjusted standard protocol consisting of 60 mg for patients < 70 years old, 40 mg for patients age 70-89 years, and 20 mg for those ≥ 90 years, and additional injections of 20 mg propofol were given up to a maximum of 200 mg. The principal parameters were the occurrence of adverse events within 24 h after colonoscopy and overall satisfaction for this procedure. Secondary parameters included successful procedure, respiratory depression, and other complications.

RESULTS: Consecutive patients were entered prospectively and all 2101 entered successfully completed

outpatient colonoscopy. The mean dose of propofol used was 96.4 mg (range 40-200 mg). Younger patients required higher doses of propofol than older patients (20-40 years vs ≥ 61 years: 115.3 ± 32 mg vs 89.7 ± 21 mg, $P < 0.001$). Transient supplemental oxygen supply was needed by five patients (0.2%); no other complications occurred. The questionnaires were completed by 1820 (87%) of 2101 patients and most rated their overall satisfaction as excellent (80%) or good (17%). The majority (65%) of patients drove home or to their office after their colonoscopy. Most (99%) were willing to repeat the same procedure. No incidents occurred within 24 h after colonoscopy.

CONCLUSION: Propofol sedation using a dose < 200 mg proved both safe and practical for outpatient colonoscopy.

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Key words: Colonoscopy; Propofol; Colorectal cancer

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INTRODUCTION

Colorectal cancer is one of the main causes of death

from cancer in western countries and in Japan. There is no clear strategy for reducing the incidence of colorectal cancer, therefore, reduction in mortality relies on removal of premalignant lesions and detection of cancer at an early stage by colonoscopy^[1,2]. In the face of increasing demands for gastroenterological services, the success of colonoscopic screening in the outpatient setting depends on patient acceptability^[3]. CO₂ insufflation and the water method for unsedated colonoscopy have been shown to be more acceptable than unsedated conventional colonoscopy^[4,5]. We previously demonstrated that use of a small-caliber pediatric colonoscope resulted in completed colonoscopies in patients who had an unsuccessful procedure using a standard colonoscope^[6]. The use of variable stiffness colonoscopes significantly reduced procedure-related pain and the dose of propofol required for sedation during colonoscopy^[7].

Recently, the feasibility and safety of endoscopist-directed propofol administration was confirmed in a study of 646 080 cases from 28 centers^[8]. Endoscopist-directed propofol sedation refers to administration of propofol by a non-anesthesia specialist under the direct supervision of the endoscopist performing the endoscopic procedure. Propofol sedation for colonoscopy was shown to be superior to other sedation methods in that propofol was associated with a low incidence of cardiopulmonary complications and was superior to benzodiazepines with regard to rapidity of both induction of sedation and recovery^[9-11].

These results have been confirmed by meta-analysis^[12]. We have previously reported that propofol sedation was both safe and practical for diagnostic esophago-gastroduodenoscopy and endoscopic procedures in patients aged ≥ 90 years^[13-16]. In addition, we have allowed our patients to drive home after colonoscopy, based on the experience with diagnostic esophagogastroduodenoscopy in our endoscopy unit^[13,14]. Here, we report a prospective evaluation of the safety and effectiveness of propofol sedation with follow-up for 24 h after outpatient colonoscopy.

MATERIALS AND METHODS

Patients

The study was done at the Showa Inan General Hospital and included outpatients who underwent only colonoscopy. Emergency procedures were excluded. Patients were also excluded if they were < 20 years old, pregnant, assigned to American Society of Anesthesiologists (ASA) class III and IV, overweight (> 100 kg), or allergic to the drugs used or their components (soybeans or eggs). Routine standard monitoring at this unit included continuous assessment of peripheral oxygen saturation (SpO₂) and heart rate. Clinical assessment of the patients included measurement of respiratory effort by visual assessment and by palpation of the chest wall and abdominal excursion and/or palpation of exhaled breath. When oxygen desaturation (SpO₂ $< 90\%$) continued for

> 20 s, supplemental oxygen was given. The endoscopic team consisted of the nurse who administered the drugs and was responsible for the patient, the endoscopist, and a second nurse to assist the endoscopist and the patient-monitoring nurse.

Propofol was administered for endoscopic sedation by nurses supervised by endoscopists. Both the nurses and endoscopists had advanced cardiac life support certification, advanced airway training, didactic training on propofol, observation of cases, and supervised administration of propofol by anesthesiologists before beginning propofol administration supervised by the endoscopist. The training period typically lasted about 2 wk.

The study was conducted in accordance with the Helsinki Declaration and was approved by the ethics committee at the hospital. Verbal and written informed consent was obtained from all patients.

All colonoscopies were performed by six skilled endoscopists (Horiuchi A, Nakayama Y, Kajiyama M, Kato N, Kamijima T, Ichise Y) who each perform > 300 colonoscopies/year. All the procedures were conducted under propofol sedation (AstraZeneca, Osaka, Japan)^[13,14]. The standard bowel preparation was a polyethylene glycol solution (Ajinomoto Pharmaceutical Co, Tokyo, Japan).

Study design

Colonoscopy was performed in the lateral decubitus position. A butterfly needle for the bolus injection of propofol was placed on the patient's forearm shortly before the start of colonoscopy and was removed after completion of the procedure. Propofol was given by bolus injection with an age-adjusted standard protocol of 60 mg for patients aged < 70 years, 40 mg for patients aged 70-89 years, and 20 mg for those aged ≥ 90 years. Adequate sedation was considered achieved when the patients passed through the following sequence: eyes closing, one or two yawns, and cessation of body movements. The target level of sedation was moderate conscious sedation with the patients still being able to respond purposefully to verbal commands. When the target level was not obtained or the patients were under-sedated, additional injections of 20 mg propofol were given up to a maximum of 200 mg.

A decline in SpO₂ to $< 90\%$ that continued for > 20 s was regarded as respiratory depression associated with sedation. Vital signs were frequently assessed but not on a periodic basis. In addition to monitoring of vital signs, the patient's condition was assessed more globally by visual inspection. Monitoring and complications were recorded by a registered nurse. SpO₂ was routinely captured by visual inspection of the monitor and the value was recorded on the vital sign sheet.

After the procedure, patients were moved to a waiting room after they could stand by themselves and they were discharged after they were fully awake. Full recovery, including consciousness and psychomotor function was assessed using three criteria: (1) Level of consciousness (fully awake and responding to questions from the recov-

Table 1 Demographic and clinical data in 2101 patients who underwent colonoscopy using propofol sedation

	<i>n</i> (%)
Sex: Male	1149 (55)
Mean age (range) (yr)	66 (20-94)
Mean body weight (range) (kg)	56.7 (32-98)
Indication	
Screening	785 (37)
Hemopositive stool	538 (26)
Abdominal symptoms	406 (19)
Hematochezia	140 (7)
Surveillance	133 (6)
Anemia	70 (3)
Others	29 (1)
Propofol dose (mg):	
About 40	110 (5)
60	239 (11)
80	623 (30)
100-120	973 (46)
140-160	131 (6)
180-200	25 (1)
Successful procedure	2101 (100)
Mean procedure time (range) (min)	14 (8-46)
Oxygen administered	5 (0.2)
Mask ventilation required	0 (0)
Heart rate < 50 beats/min	0 (0)
Complications	0 (0)
Full recovery 60 min after the procedure	2101 (100)

Values are numbers (percentages) of patients except for mean age, mean body weight, and mean procedure time.

ery room nurse); (2) Ability to stand on one-foot; and (3) Ability to walk in a straight line without instability for 5 m. These three criteria were assessed every 15 min starting 30 min after the procedure; full recovery was defined as meeting all three criteria. The nurses reconfirmed the absence of re-emerging sedative effects and finally permitted patients to leave the endoscopic unit.

In addition, we provided questionnaires. Within 2 wk after the procedure, patients were contacted by telephone and asked about overall satisfaction for this procedure, whether they drove home or to their office after colonoscopy, the occurrence of any accidents within 24 h after colonoscopy and their willingness to repeat the same procedure next time (yes/no).

Study parameters

The principal parameters were the occurrence of adverse events within 24 h after colonoscopy and overall satisfaction for the procedure. Secondary parameters included successful procedure, respiratory depression, and other complications. Respiratory depression was defined as the need of oxygen supply due to an oxygen desaturation ($\text{SpO}_2 < 90\%$) that continued more than 20 s.

Instruments

The Olympus PCF-Q260AI videoscope used has an insertion diameter of 11.3 mm, an accessory channel diameter of 2.8 mm, a total length of 1335 mm, and a working length of 1030 mm.

Table 2 Relationship between age or sex and dose of propofol used in 2101 patients who underwent colonoscopy

Age (yr)	No. (M/F)	Propofol dose (mg)			<i>P</i> value
		Total (range)	Male	Female	
20-40 ¹	150 (89/61)	115.3 ± 32 (40-200)	113.4 ± 27	118.2 ± 24	0.43
41-60	563 (316/247)	107.7 ± 27 (40-200)	106.9 ± 28	108.7 ± 25	0.19
≥ 61	1388 (744/644)	89.7 ± 21 (40-200)	87.7 ± 23	92.1 ± 19	0.32
Total	2101 (1149/952)	96.4 ± 27	95.0 ± 26	98.1 ± 25	0.18

Values are mean ± SD except for number of patients and range of dose used of propofol. *P* value shows the difference between male and female patients. ¹There were significant differences in the doses of propofol in each group between age 20-40 and ≥ 61 years ($P < 0.001$).

Statistical analysis

Data are presented as mean ± SD. The χ^2 test, with Yates' correction for continuity where appropriate, was used for comparison of categorical data. Fisher's exact test was used when the numbers were small. For parametric data, Student's *t* test was used when two means were compared. Analysis of variance was used when the three groups were compared and positive results were confirmed using Tukey's Honestly Significantly Different procedure. A value of $P < 0.05$ was regarded as significant. Statistical analysis was performed by using JMP[®] 9.0.2 version software (SAS Institute Inc., Cary, NC, United States).

RESULTS

Between January 2010 and December 2010, 2101 consecutive patients received outpatient colonoscopy based on a standard protocol of age-adjusted doses of propofol (Table 1). All procedures were completed successfully. The patients' ages ranged from 20 to 94 years. The most common indications for colonoscopy were: colorectal cancer screening in 785 (37%), hemopositive stools in 538 (26%), and abdominal symptoms in 406 (19%). Mean procedure time was 14 min (range, 8-46 min). A biopsy and/or polypectomy was performed in 775 patients (37%). Oxygen desaturation requiring supplemental oxygen (1-3 L/min) occurred in 0.2% (five patients); mask ventilation or endotracheal intubation was not required in any case. In no case did respiratory event or laryngospasm occur. No other complications occurred (Table 1). Full recovery within 60 min after the procedure was present in 100%.

The mean dose of propofol used was 96.4 mg (Table 2). There were no significant differences in the dose of propofol used between men and women (95.0 ± 26 mg *vs* 98.1 ± 25 mg, $P = 0.18$), however younger patients required higher doses of propofol than older patients (20-40 years *vs* ≥ 61 years, 115.3 ± 32 mg *vs* 89.7 ± 21 mg, $P < 0.001$). Of the 2101 patients, 495 (24%) had at least an adenoma detected. Fifty-two (3%) had invasive colorectal cancer. Colorectal adenomas with high-grade dysplasia were found in 32 (2%) patients. The detection rate of adenoma in this study was 53 adenomas/100

Table 3 Demographic data and results of questionnaires in 1820 patients who underwent colonoscopy using propofol sedation

Sex: Male	954 (52)
Mean age (range) (yr)	65 (33-80)
Mean body weight (range) (kg)	58.7 (45-98)
Did you find propofol sedation for your colonoscopy satisfactory?	
Excellent	1456 (80%)
Good	309 (17%)
No	11 (0.6%)
NA	8 (2.4%)
Did you drive home or to the office after your colonoscopy?	
Yes	1183 (65%)
No	637 (35%)
Did you experience any accidents within 24 h after your colonoscopy?	
Yes	0 (0%)
No	1820 (100%)
Do you want to repeat the same procedure next time?	
Yes	1805 (99%)
No	15 (1%)

Values are numbers (percentages) of patients except for mean age and mean body weight. NA: Not available.

colonoscopies. Adenomas were distributed evenly in the colon and rectum.

The questionnaires were completed by 1820 (87%) of 2101 patients (Table 3). Their mean age and mean body weight were 65 years and 58.7 kg. The majority rated their overall satisfaction for this procedure as excellent (80%) or good (17%). The majority (65%) of subjects drove home or to their office after their colonoscopy. No associated incidents within 24 h after colonoscopy occurred. Most (99%) were willing to repeat the same procedure.

DISCUSSION

This study describes the safety and outcomes of propofol sedation given to 2101 patients for outpatient colonoscopy. Previous studies have involved propofol dosages ranging from 60 to 300 mg using an indwelling catheter for endoscopic sedation^[9-12,17-21]. In the present study, the mean dose of propofol used was 96.4 mg, which corresponded to that of a previous study in the Japanese population^[17]. On the basis of our previous experiences^[13,14], the protocol adopted here focused on safety with the initial dose of 40 or 60 mg propofol designed to minimize hypoxemia during the procedure; only 0.2% required oxygen, which is less than previous studies^[9-12]. No subject required mechanical ventilation during the procedures via either endotracheal intubation or a mask (Table 1). The routine use of supplemental oxygen during colonoscopy may mask respiratory depression^[22], therefore, we chose to administer supplemental oxygen only when needed. No subjects experienced bradycardia (heart rate < 50 beats/min). Other studies with low-dose propofol have reported rates of bradycardia up to 10%^[23]; the differences may relate to difference in the sedation method because studies with bradycardia have typically used a combination of propofol, midazolam

and meperidine. All procedures were done with pediatric variable stiffness colonoscope, thus, it is impossible to assess whether the pediatric variable stiffness colonoscope, or the lower dose of propofol required, was responsible for the low incidence of cardiopulmonary depression.

Generally, patients are not permitted to drive themselves within the first day after endoscopic sedation. Riphaut *et al.*^[24] have reported that current recommendations that patients should be refrain from driving and unescorted use of public transport for 24 h after sedation may need to be reconsidered in patients who receive propofol sedation. Based on our previous study using a driving simulator showing that driving ability recovered to the basal level within 60 min of low-dose propofol sedation^[13], patients have been permitted to drive themselves after colonoscopy as well as esophagogastroduodenoscopy at our endoscopy unit. In this study, the majority (65%) of patients drove home or to their office after their colonoscopy. No sedation-associated incidents within 24 h after colonoscopy occurred in the 1820 subjects responding.

Our recent study used a number connection test and a driving simulator test to assess psychomotor recovery before and 1 h and 2 h after colonoscopy^[25]. Psychomotor recovery was evident as early as 1 h after propofol sedation. Additional studies are needed before it can be routinely recommended that patients be permitted to drive home after endoscopy using only propofol sedation. Effective endoscopic sedation depends on the type of procedure and the procedure time. In most patients, an appropriate level of sedation can be reached through the use of a benzodiazepine combined with a narcotic. In contrast, we gave propofol by bolus titration with an initial dose of 40-60 mg followed by dose of 20 mg beginning 30-60 s later. The appropriateness of additional bolus doses was determined by the level of sedation and the respiratory effect. When moderate conscious sedation was not obtained or the patients were undersedated, additional injections of 20 mg propofol were given up to a maximum of 200 mg; 180-200 mg were required in only 25 patients (1.2%). The advantages of propofol using a butterfly needle include immediate onset of action and fast recovery, which likely contributed to the high level of acceptability and cost-effectiveness for outpatient colonoscopy. In our facility, a butterfly needle has been used instead of an indwelling cannula for about 60 000 patients. Even if a patient in conscious sedation moves his/her arm, the butterfly needle is usually placed stably in the vein. This butterfly needle method is thought to be a safe and practical way to administer propofol over 15-30 min.

The average procedure time was 14 min and the detection rate of adenoma was 24% as the proportion of patients in whom at least one adenoma was identified in this study. The adenoma detection rate that was reported to be an independent predictor of the risk of interval colorectal cancer after screening colonoscopy was compatible to our

data^[26]. Therefore, this sedation method is likely an important variable in ensuring quality in colonoscopy.

Unsedated colonoscopy is still the main type of procedure in many countries. Even if sedation is safe, it requires an extra nurse and a team educated for monitoring, resulting in higher costs. The requirement for an escort and time burden of recovery from sedation are both barriers to the acceptance of screening colonoscopy. Improved acceptance of colonoscopy is important to allow full use of colonoscopy in cancer screening and prevention. Colonoscopy can be completed without sedation in the majority of patients and undoubtedly there is a place for unsedated colonoscopy, especially in areas where cost containment is a primary concern^[27-29].

This study had some limitations. The sedation level was assessed using minimal, moderate, and deep sedation on the basis of the ASA level^[30], but this was not recorded for each patient during the study. Although the patients may have had amnesia after propofol sedation, the questionnaire in this study did not include any questions related to amnesia. Blood pressure monitoring was not routinely performed in this study. Blood pressure monitoring is generally a standard practice whenever administering propofol in the United States but not in Japan. We were therefore unable to comment on episodes of hypotension in our series. The monitoring used (e.g., continuous assessment of SpO₂ and heart rate) is the standard in Japan.

In conclusion, propofol sedation was associated with good acceptance of colonoscopy and willingness to repeat the procedure and enabled patients to drive home safely by themselves after colonoscopy. Propofol sedation using a dose < 200 mg was safe and practical. Increased use of this sedation method may improve the acceptability of colonoscopy, which may enable population wide screening and decrease colorectal cancer mortality.

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COMMENTS

Background

Despite the increasing usage of short-acting sedatives, the recommendations that patients should refrain from driving and the unescorted use of public transport for 24 h after sedation remain unchanged. The authors previously reported that recovery after low-dose propofol sedation for diagnostic esophagogastroduodenoscopy was such that the patients were able to drive home. In addition, the recent study using a number connection test and a driving simulator test demonstrated that psychomotor recovery was evident as early as 1 h after propofol sedation for outpatient colonoscopy.

Research frontiers

The authors report a prospective evaluation of the safety and effectiveness of propofol sedation with follow-up for 24 h after outpatient colonoscopy.

Innovations and breakthroughs

Propofol sedation was associated with good acceptance of colonoscopy and willingness to repeat the procedure and enabled patients to drive home safely by themselves after colonoscopy. Propofol sedation using a dose < 200 mg was safe and practical. Increased use of this sedation method may improve the

acceptability of colonoscopy, which may enable population-wide screening and decrease colorectal cancer mortality.

Applications

The findings in this study indicate that patients sedated with propofol may be capable of safely driving 1 h after colonoscopy. The ability to drive home after sedation reduces the need for an accompanying individual and thus lowers the costs and burden associated with endoscopy.

Peer review

This is a well-written paper providing new data on safety of propofol sedation and new information about patients checking out and driving home after propofol sedation.

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Efficacy of hepatic arterial infusion chemotherapy in advanced hepatocellular carcinoma

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Abstract

AIM: To investigate the efficacy of hepatic arterial infusion chemotherapy (HAIC) using floxuridine (FUDR) in patients with advanced hepatocellular carcinoma (HCC) confined to the liver.

METHODS: Thirty-four patients who had advanced HCC with unresectability or unsuccessful previous therapy in the absence of extrahepatic metastasis were treated with intra-arterial FUDR chemotherapy at our

hospital between March 2005 and May 2008. Among the 34 patients, 9 patients were classified as Child class C, and 18 patients had portal vein tumor thrombus (PVTT). One course of chemotherapy consisted of continuous infusion of FUDR (0.3 mg/kg during day 1-14) and dexamethasone (10 mg on day 1, 4, 7 and 11), and this treatment was repeated every 28 d.

RESULTS: Two patients (5.9%) displayed a complete response, and 12 patients (35.3%) had a partial response. The tumor control rate was 61.8%. The median overall survival times were 15.3 mo, 12.4 mo and 4.3 mo for the patients who were classified as Child class A, Child class B and Child class C, respectively ($P = 0.0392$). The progression-free survival was 12.9 mo, 7.7 mo and 2.6 mo for the patients who were classified as Child class A, Child class B and Child class C, respectively ($P = 0.0443$). The cumulative survival differed significantly according to the Child-Pugh classification and the presence of PVTT. In addition to hepatic reserve capacity and PVTT, the extent of HCC was an independent factor in determining a poor prognosis. The most common adverse reactions to HAIC were mucositis, diarrhea and peptic ulcer disease, but most of these complications were improved by medical treatment and/or a delay of HAIC.

CONCLUSION: The present study demonstrates that intra-arterial FUDR chemotherapy is a safe and effective treatment for advanced HCC that is recalcitrant to other therapeutic modalities, even in patients with advanced cirrhosis.

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Key words: Hepatic arterial infusion chemotherapy; Floxuridine; Advanced hepatocellular carcinoma; Child-Pugh classification; Portal vein tumor thrombus

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INTRODUCTION

Hepatocellular carcinoma (HCC), which is one of the most common malignancies worldwide, causes over 600 000 deaths per year and is the third most common cancer in South Korea^[1,2]. Although surgical resection or liver transplantation can be curative for HCC, most patients are not candidates for surgery at the time of diagnosis because it is difficult to detect HCC at an early stage. In addition, some patients have advanced cirrhosis by the time of diagnosis^[3].

Although recent advances in therapeutic modalities, such as hepatic resection, percutaneous ethanol injection, radiofrequency ablation (RFA), transarterial chemoembolization (TACE), radiotherapy and liver transplantation, have improved the treatment of HCC, the prognosis for patients with advanced HCC remains poor. In addition, these current therapies have many limitations, and recurrence and metastasis are relatively common^[4-7]. Among the currently available therapies, TACE is the current standard of care for patients who are not candidates for curative therapy. The survival benefit conferred by TACE was reported in a randomized controlled trial^[8], which showed that the median survival of the patients in the TACE group was approximately 14 mo. However, TACE is contraindicated in advanced HCC with main portal vein tumor thrombus (PVTT), massive or diffuse infiltration, poor liver function with Child class C, and severe hepatic arteriportal shunt.

Regional hepatic arterial infusion chemotherapy (HAIC) has also been used in patients with advanced HCC because liver cancers receive most of their blood supply from the hepatic artery, whereas normal liver tissue has a dual vascular supply (i.e., 20% of the blood supply comes from the hepatic artery, and the remaining 80% comes from the portal vein). Thus, HAIC may be used, albeit with caution, in cases in which TACE is not indicated or is ineffective^[9]. HAIC may provide higher concentrations of chemotherapeutic agents directly to the HCC and produces minimal systemic concentrations of chemotherapeutic agents, which can minimize systemic toxicity^[10].

Floxuridine (FUDR) is an active metabolite of 5-fluorouracil (5-FU) that has the advantage of being rapidly metabolized, with a 94%-99% extraction rate within the liver *via* first-pass metabolism. FUDR is maintained at an intrahepatic concentration that is more than tenfold

greater than that of 5-FU, cisplatin, mitomycin or doxorubicin, which permits maximal tumor cell death while preventing systemic toxicity^[11,12].

Most previous studies have reported the efficacy of HAIC using 5-FU and cisplatin in advanced HCC or HAIC using FUDR in patients with hepatic metastasis from colorectal cancer^[10,13,14].

In the present study, we evaluated the efficacy and toxicity profile of HAIC using FUDR in patients with advanced HCC confined to the liver who failed to respond to previous therapy or who were unable to receive other therapeutic modalities.

MATERIALS AND METHODS

Patient eligibility

Thirty-four patients with advanced HCC that was unresectable or resistant to previous therapy in the absence of extrahepatic metastasis were treated with intra-arterial FUDR chemotherapy at our hospital between March 2005 and May 2008. The criteria for unresectability included bilobar disease with 4 or more lesions, large tumors occupying more than 50% of the liver, and invasion of the tumor into major vascular structures. Previous therapies included RFA, TACE and radiotherapy. All of the patients belonged to tumor node metastasis stage IIIA or IIIB. To assess the eligibility for inclusion, each patient received a computed tomography (CT) scan of the abdomen and pelvis. Each patient provided a full medical history and underwent a physical examination, chest X-ray, and laboratory tests, including a test for alpha-fetoprotein. Additional examinations, such as magnetic resonance imaging (MRI) scans, liver biopsy and angiography, were performed if the CT scan and tumor marker analyses were insufficient for diagnosis. Among the 34 patients, disease was histologically confirmed in 5 patients. When metastasis was suspected, positron emission tomography scans and bone scans and/or CT scans of suspicious areas were conducted. Patients with distant metastases were excluded. Informed consent was obtained from all of the patients.

A pump catheter was inserted at the proper hepatic artery from the superior mesenteric artery. The distal gastroduodenal artery, the right gastric artery, the small branches supplying the stomach or duodenum, and all of the accessory hepatic arteries received ligations to prevent gastrointestinal toxicity. The patients with hepatitis B received prophylactic or therapeutic antiviral agents before HAIC. Some of the patients with hepatitis C had previously been treated with pegylated-interferon/interferon and ribavirin. The remaining hepatitis C patients were carefully monitored because antiviral treatment was deferred because of underlying bone marrow and immune suppression complications.

Assessment of responses

The patient responses were classified according to the Response Evaluation Criteria In Solid Tumors guidelines.

Table 1 Baseline characteristics *n* (%)

Characteristics	IA-FUDR (<i>n</i> = 34)
Age (yr)	62.2 ± 7.4
Sex	
Male	27 (79.4)
Female	7 (20.6)
Cause of HCC	
Hepatitis B virus	27 (79.4)
Hepatitis C virus	2 (5.9)
Alcoholism	5 (14.7)
Child-Pugh classification	
Child class A	7 (20.6)
Child class B	18 (52.9)
Child class C	9 (26.5)
Portal vein thrombosis	
Yes	18 (52.9)
No	16 (47.1)
Tumor morphology	
Multinodular	16 (47.1)
Huge, massive > 50% of liver	18 (52.9)
Bilirubin (mg/dL)	2.6 ± 2.3
Albumin (g/dL)	2.8 ± 1.1
Prothrombin time (INR)	2.0 ± 0.7
AFP (ng/mL)	5136.03 (median)
Treatment prior to chemotherapy or supportive care	
Surgery	0 (0)
RFA	3 (8.8)
TACE	20 (58.8)
RFA and TACE	1 (2.9)
Radiation	1 (2.9)
No treatment	9 (26.5)

HCC: Hepatocellular carcinoma; AFP: Alpha-fetoprotein; RFA: Radio-frequency ablation; TACE: Transarterial chemoembolization; IA-FUDR: Intra-arterial floxuridine; INR: International normalized ratio.

Complete response (CR) was defined as the disappearance of all evidence of disease and the normalization of tumor markers for at least 4 wk. Partial response (PR) was defined as a $\geq 30\%$ reduction in unidimensional tumor measurements without the appearance of any new lesions or the progression of any existing lesion. Progressive disease (PD) was defined as any of the following: a 20% increase in the sum of the diameters of five measurable lesions, the appearance of any new lesions, or the reappearance of any lesion that had previously disappeared. Stable disease (SD) was defined as a tumor response that did not fulfill the criteria for CR, PR or PD. CT scans or MRIs of the measurable lesions were carried out within 4 wk before the start of treatment and repeated every 2 cycles (2 mo). Responses were confirmed by subsequent CT or MRI scans after the documentation of the initial response.

Toxicity assessment

Toxicity was assessed according to the National Cancer Institute Common Toxicity Criteria. The FUDR dose was modified when grade 3–4 toxicity was observed.

Hepatic toxicity was defined as a significant increase over baseline values (3- to 4-fold for aspartate transaminase or alanine transaminase and greater than 1.5-fold for bilirubin), and the increases in hepatic enzyme levels

caused by the disease varied across patients. If a patient complained of epigastric pain, an evaluation that included an upper gastrointestinal endoscopy was performed. If an ulcer or gastroduodenitis was identified, then chemotherapy was stopped until recovery. If a patient had severe diarrhea or abdominal pain, chemotherapy was stopped until recovery. In addition, angiography was performed to block collateral vessels when extrahepatic perfusion was suspected.

Chemotherapy regimen

Local chemotherapy was started between 3 and 5 d after pump insertion. The patients received FUDR (0.3 mg/kg per day for 14 d) and dexamethasone (10 mg on day 1, 4, 7 and 11) *via* an intra-arterial pump. FUDR was synthesized by APP Pharmaceuticals, LLC (Schaumburg, IL, United states). After the 14 d of treatment with FUDR, the pump was emptied and refilled with 30 000 units of heparin in 0.9% saline for 14 d. This treatment was repeated every 28 d. FUDR was given indefinitely until the disease progressed or the therapy was discontinued due to toxicity or patient death.

Statistical analysis

The objective of the present study was to estimate the efficacy and toxicity of continuous HAIC with FUDR *via* an implantable pump. All of the analysis were performed using SPSS version 18.0.

Survival times were calculated from the start of the study treatment until patient death or the final follow-up. Progression-free survival (PFS) was calculated from the first day of chemotherapy until the date of progression. PFS and overall survival (OS) curves were obtained using the Kaplan-Meier method, and comparisons were made using the log rank test. Multivariate analysis to evaluate the influence of prognostic factors on survival was performed using Cox proportional hazard methods. Statistical significance was established as $P < 0.05$.

RESULTS

Patient characteristics

A total of 34 patients, 27 men and 7 women, with a median age of 62.2 years received intra-arterial FUDR chemotherapy between March 2005 and May 2008. The patient baseline characteristics are shown in Table 1. The major etiology of the patients' liver disease was hepatitis B virus (27 of 34, 79.4%). Eighteen patients had PVTT, whereas 16 patients did not have PVTT. The majority of patients had received TACE as the previous therapeutic modality (20 of 34, 58.8%).

Response to treatment

Patients received 2–10 (the median was 3.5) cycles of chemotherapy. All of the patients received at least 2 cycles of intra-arterial FUDR chemotherapy, and 17 (50%) patients received more than 4 cycles.

The patients' responses to treatment are summarized

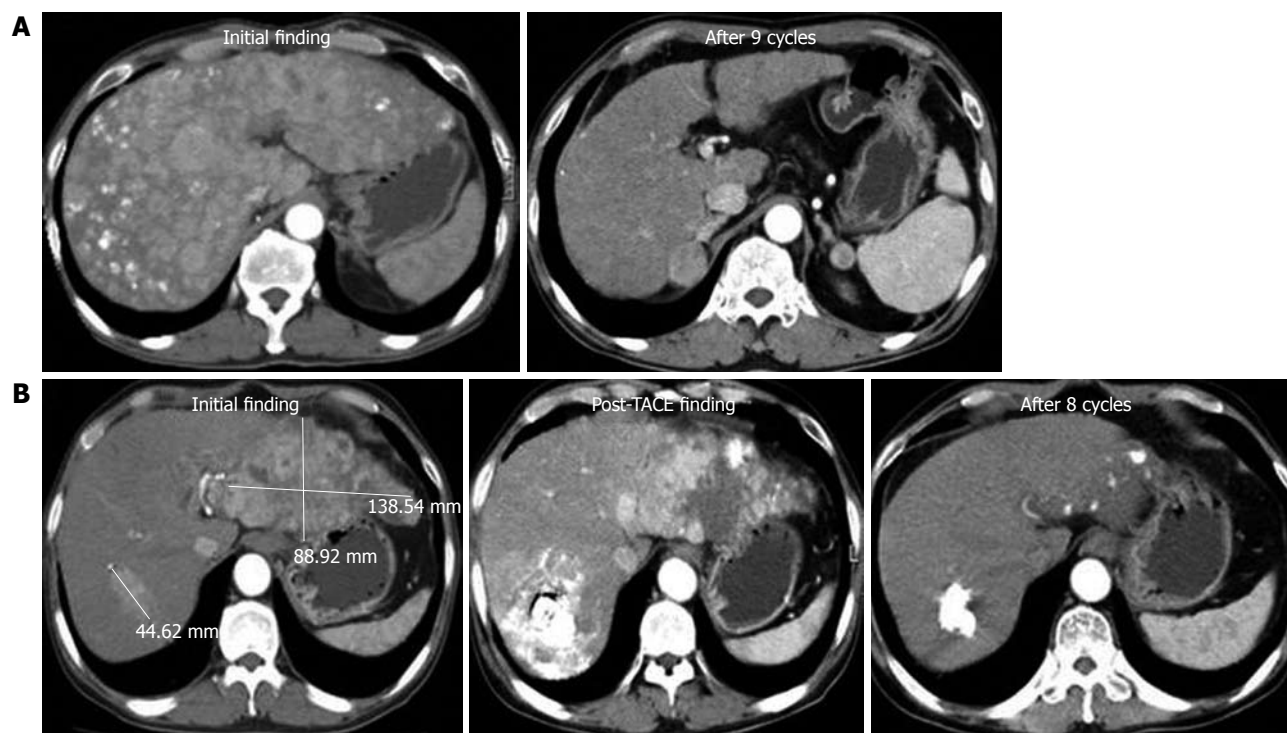


Figure 1 Two cases of nearly complete response. A: The first case was a 54-year-old patient with diffuse, multinodular hepatocellular carcinoma throughout the whole liver. After 9 cycles of hepatic arterial infusion chemotherapy (HAIC) with floxuridine, no enhancing nodular lesions were observed by dynamic contrast-enhanced computed tomography (CT); B: The second case was a 48-year-old patient with a large mass in the left lobe and intrahepatic metastasis in the right lobe. After transarterial chemoembolization treatment, viable masses were still observed in sequential CT images, and HAIC was started. After 8 cycles of chemotherapy, no viable masses were observed. TACE: Transarterial chemo-embolisation.

Table 2 Treatment response rate *n* (%)

Tumor response	Intra-arterial FUDR chemotherapy			
	Total	Child class A	Child class B	Child class C
CR	2 (5.9)	1 (14.3)	1 (5.6)	0 (0)
PR	12 (35.3)	2 (28.6)	9 (50)	1 (11.1)
SD	7 (20.6)	2 (28.6)	3 (16.7)	2 (22.2)
PD	13 (38.2)	2 (28.6)	5 (27.8)	6 (66.7)
Response rate	14 (41.2)	3 (42.9)	10 (55.6)	1 (11.1)
Disease control rate	21 (61.8)	5 (71.4)	13 (72.2)	3 (33.3)
Total	34 (100)	7 (100)	18 (100)	9 (100)

FUDR: Floxuridine; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

in Table 2. Two patients (5.9%) had CR, 12 (35.3%) had PR, seven (20.6%) had SD, and 13 (38.2%) had PD. The CT findings for the two patients with CR (Figure 1). Among the patients who were classified as Child class A or B, 2 (8%) had CR and 11 (44%) had PR. The patients who were classified as Child class A or B had a response rate of 52% and a disease control rate of 72%.

Survival

The cumulative survival of the 34 patients is presented in Figure 2A and B. The median OS was 8.9 mo, and the median PFS was 6.6 mo.

We assessed the cumulative survival according to the Child-Pugh classification and the presence of PVTT

(Figure 3). The median OS times were 15.3 mo, 12.4 mo and 4.3 mo in the patients who were classified as Child class A, Child class B and Child class C, respectively, and there were significant differences between the groups ($P = 0.0392$). Similarly, there were significant differences in the median PFS times, which were 12.9 mo, 7.7 mo and 2.6 mo in the patients who were classified as Child class A, Child class B and Child class C, respectively ($P = 0.0443$) (Figure 3A and B). These findings showed that the patients who were classified as Child class C had significantly shorter survival times compared with the patients who were classified as Child class A or Child class B.

Further differences were found in the OS and PFS according to the presence of PVTT, which indicated a negative impact of PVTT on survival time. The median OS and PFS of the patients without PVTT were 13.1 mo and 8.2 mo, respectively, compared with 8.2 mo and 3.4 mo, respectively, for the patients with PVTT (Figure 3C and D).

Prognostic factors

We conducted univariate and multivariate analyses of baseline characteristics, such as age, sex, Child-Pugh classification, PVTT, extent of HCC, bilirubin, albumin and PT international normalized ratio (INR), using a Cox proportional hazards model to evaluate the prognostic factors for survival (Table 3). The multivariate analysis showed that 4 variables (i.e., child classification, PVTT, HCC type and PT INR) were independent predictors of survival.

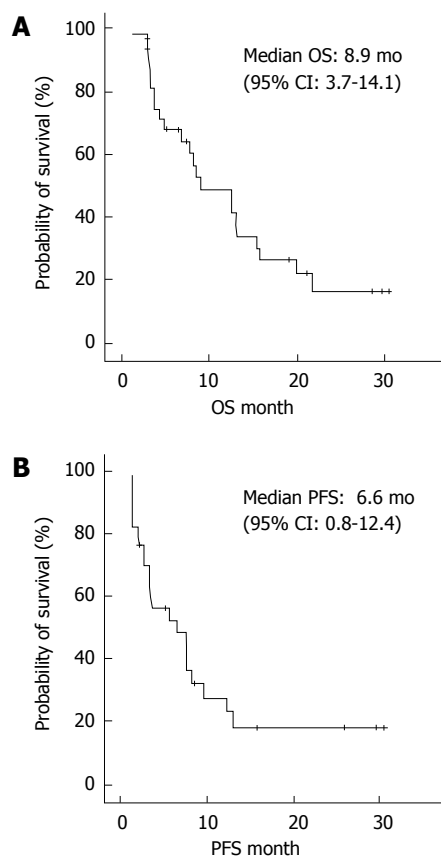


Figure 2 The overall survival and the progression-free survival determined by Kaplan-Meier analysis. A: The overall survival (OS) determined by Kaplan-Meier analysis; B: The progression-free survival (PFS) determined by Kaplan-Meier analysis.

Adverse reactions and complications

The treatment-related adverse reactions and complications that were observed in the 34 patients who were treated with HAIC (Table 4). Severe hematologic toxicity during HAIC was not noted in any of the patients. The most common grade 3-4 adverse reaction was gastric or duodenal ulcer (11.8%). Most of the adverse reactions were improved by medical treatment and/or delaying HAIC. One patient with hepatitis C experienced progressive hepatic failure during the third cycle of chemotherapy and eventually died. In addition, a major complication related to the indwelling catheter occurred in 1 patient. Indeed, an infection occurred around the catheter, but it was controlled by antibiotics and removal of the port. This patient continued HAIC after insertion of another catheter in the opposite site. Another major complication that has commonly been reported to be associated with FUDR treatment is biliary sclerosis; however, the patients in the present study received prophylactic dexamethasone on a regular schedule, and none of the present patients experienced biliary sclerosis.

Cause of death

During treatment and follow-up, 9 patients died from various causes, including tumor progression, hepatic failure, gastrointestinal bleeding and sepsis. Among the

Table 3 Prognostic significance of the clinical factors influencing survival

Multivariate	Hazard ratio	95% CI	P value
Age (≥ 60 yr, < 60 yr)	1.381	0.403-4.734	0.608
Sex (male, female)	1.462	0.433-4.937	0.541
Child-Pugh classification	3.710	1.490-9.238	0.005
Portal vein thrombosis (without, with)	0.086	0.019-0.387	0.001
Extent of HCC (multinodular, massive $> 50\%$ of liver)	0.185	0.051-0.679	0.011
Bilirubin (≥ 3 , < 3)	0.837	0.319-2.195	0.718
Albumin (< 3 , ≥ 3)	1.059	0.430-2.603	0.901
PT INR (< 2.3 , ≥ 2.3)	0.218	0.066-0.715	0.012

HCC: Hepatocellular carcinoma; PT INR: Prothrombin time international normalized ratio.

Table 4 Adverse reactions to floxuridine *n* (%)

Treatment group (toxicity)	NCI-CTC grade	
	1-2	3-4
Fever	2 (5.9)	0 (0)
Nausea/vomiting	0 (0)	0 (0)
Gastric or duodenal ulcer	3 (8.8)	4 (11.8)
Mucositis	4 (11.8)	3 (8.8)
Diarrhea	4 (11.8)	3 (8.8)
Leukopenia	2 (5.9)	1 (2.9)
Thrombocytopenia	2 (5.9)	1 (2.9)
Bilirubin elevation	4 (11.8)	1 (2.9)
AST/ALT elevation	5 (14.7)	1 (2.9)
BUN/Cr elevation	0 (0)	1 (2.9)
Catheter infection	1 (2.9)	
Total	19	9

NCI-CTC: National Cancer Institute Common Toxicity Criteria; AST: Aspartate transaminase; ALT: Alanine transaminase; BUN: Blood urea nitrogen; Cr: Creatinine.

patients who died, 4 (44.4%) died of hepatic failure related to an advanced cirrhotic condition. Only 1 of the 4 patients had hepatic failure in relation to therapy, and the remaining patients died after loss to follow-up or the incidence of another illness. Tumor progression and sepsis were the causes of death in 2 patients each (22.2%). One patient died of upper varix bleeding.

DISCUSSION

Many therapeutic modalities are available for the treatment of HCC, such as hepatic resection, percutaneous ethanol injection, RFA, TACE, radiotherapy and liver transplantation^[15]. Among these therapies, TACE has been the main treatment modality for the management of unresectable or recurrent HCC. A randomized controlled trial revealed that the median survival for patients undergoing TACE was approximately 14 mo^[8]. However, this treatment has not been useful in patients with PVTT or large infiltrative HCC because of the potential risk of hepatic failure resulting from ischemia^[16,17].

HAIC can be safely used in patients with impaired liver function due to advanced HCC or underlying liver

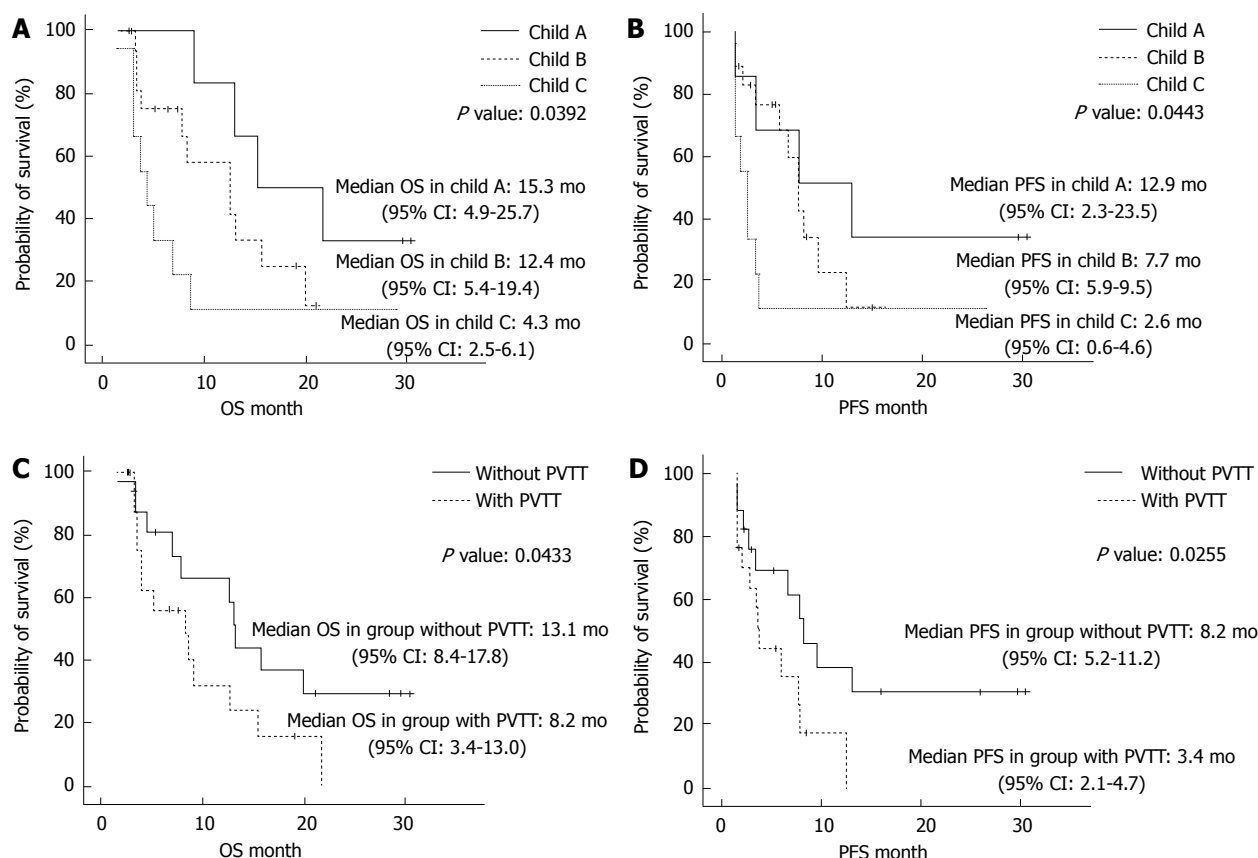


Figure 3 Kaplan Meier plot estimates of the overall survival and the progression-free survival according to the Child-Pugh classification and existence of portal vein tumor thrombus. A: Kaplan Meier plot estimates of the overall survival (OS) to the Child-Pugh classification; B: Kaplan Meier plot estimates of the progression-free survival (PFS) according to the Child-Pugh classification; C: Kaplan Meier plot estimates of the OS according to existence of portal vein tumor thrombus (PVTT); D: Kaplan Meier plot estimates of the PFS according to existence of PVTT.

cirrhosis because hepatic arterial blocking agents, such as lipiodol or gelatin sponges, are not used in HAIC^[18]. Thus, HAIC is not limited by tumor size, number, and/or proximity to major vasculature, all of which are common contraindications to resection and/or ablation. In addition, HAIC has several other advantages. For example, in most cases, there is no need for the administration of antiemetics or exogenous hydration, which can cause ascites or peripheral edema. Moreover, the higher first-pass hepatic extraction of infused drugs produces elevated local concentrations with lower systemic exposure, which results in fewer side effects than systemic chemotherapy^[10].

HAIC using 5-FU with cisplatin has been extensively studied as a treatment for advanced HCC^[9,19,20]. However, the pharmacokinetics of 5-FU are not linear over a hepatic extraction gradient of 19%-90%, and there are decreases in both systemic clearance and hepatic extraction at higher doses, which reduce the selective regional advantage^[21].

Intra-arterial FUDR, which is a metabolite of fluorouracil, is preferable because it is associated with an increased response rate due to its higher hepatic extraction (> 95%) and an intrahepatic concentration that is more than tenfold greater than that of 5-FU, cisplatin, mitomycin or doxorubicin^[11,12]. Therefore, FUDR is

associated with decreased toxicity and improved survival through maximal tumor cell death. Despite these advantages, regional chemotherapy using FUDR *via* an implantable pump has rarely been studied in HCC, although it has been studied extensively in patients with liver metastasis from colorectal cancers^[14,22-24]. Therefore, we hypothesized that the study of HAIC using FUDR in patients with advanced HCC could yield significant results.

In general, the prognosis is poor for patients with advanced HCC. Many studies have reported a median survival of 3-6 mo for unresectable and untreated HCC^[25]. Recently, a multinational phase III, randomized, double-blind, placebo controlled trial was conducted to assess the efficacy and safety of new therapeutic options in a group of Asian-Pacific patients with advanced HCC^[26]. The controlled trial reported a median OS and PFS of 4.2 mo and 1.4 mo, respectively, in the patients without treatment. Interestingly, most of the patients were classified as Child class A (Child class A: 220 and Child class B: 6). Many studies of HAIC have reported good results compared with those of untreated cases^[27,28]. In the present study, the response rate and the median survival time of the 34 patients who were treated with intra-arterial FUDR were 41.2% and 8.9 mo, respectively. Despite the inclusion of patients who were classified as Child class

C, the present outcomes are similar or better than those observed in other studies.

Studies have clearly shown that survival times are longer in patients with a good functional hepatic reserve. Many studies have shown that a patient's Child-Pugh status significantly influences survival, which is consistent with the present results^[29,30]. The OS times of the patients who were classified as Child class A, B and C were 15.3 mo, 12.4 mo and 4.3 mo, respectively. Similarly, the PFS times of the patients who were classified as Child class A, B and C were 12.9 mo, 7.7 mo and 2.6 mo, respectively. Most other studies excluded patients who were classified as Child class C because early reports demonstrated poor outcomes in these patients and no differences in survival between treated and untreated groups^[29]. The present study included patients who were classified as Child class C if they demonstrated a desire to receive therapy and had a relatively good performance status. The nine patients in the present study who were classified as Child class C had similar or slightly better outcomes compared with untreated patients. Interestingly, only 1 of the Child class C patients suffered from a serious adverse event (i.e., hepatic failure). Compared with other studies, the present study also showed relatively good results in the patients who were classified as Child class B.

Like the Child-Pugh classification, PVTT is a major independent factor in the determination of a poor prognosis in patients with advanced HCC^[30]. The median survival of untreated HCC with PVTT was reported to be 2.7 mo^[31]. One study reported a median survival time of 6 mo in patients with advanced HCC with PVTT (excluding Child class C patients) who received HAIC with 5-FU and cisplatin along with systemic chemotherapy^[32]. In the present study, the median OS in the groups with and without PVTT were 8.2 mo and 13.1 mo, respectively, and this difference was significant. In addition, the difference in the median OS times in the groups with and without PVTT also demonstrated the relatively good outcomes that were observed in the present study compared with those of other studies (despite the inclusion of Child class C patients with PVTT). The present study showed that the presence of PVTT, the extent of HCC, and hepatic function (as assessed by the Child classification) were major predictors of survival. Indeed, the present study demonstrated the significance of these factors using multivariate analysis.

Treatment tolerability and patient quality of life are also important when deciding therapies for advanced cancers. Only 1 of the 34 patients in the present study (2.9%) experienced progressive impaired hepatic function that justified the withdrawal of FUDR, and the majority of the patients demonstrated a relatively sustained quality of life during the HAIC.

Several studies have reported the outcomes of HAIC using FUDR in advanced HCC. One study investigated the efficacy of HAIC using FUDR for 5 patients with HCC and reported a response rate of 80% and a 1-year survival rate of 100%^[22]. In 2009, Jarnagin *et al.*^[33] report-

ed that HAIC with FUDR therapy could be effective and safe in patients with unresectable primary liver cancer. They reported a response rate of 25% and a tumor control rate of 62.5%. These two studies demonstrated positive results of HAIC with FUDR for HCC; however, they only included 5 and 8 patients, respectively. Compared with these two reports, the present study was significant in that it included more patients and demonstrated the efficacy and safety of HAIC with FUDR even in patients with advanced HCC.

Sorafenib was the first systemic agent to be approved for the treatment of advanced HCC. Both the Study of Heart and Renal Protection trial, which was conducted in Europe and North America, and an Asia-Pacific trial showed that sorafenib prolonged the time to progression by 1.4-2.7 mo and prolonged the OS by 2-3 mo^[26,34]. In the present study, the outcomes of HAIC with FUDR were superior to treatment with sorafenib. In addition, there are limitations to sorafenib therapy, namely its decreased efficacy over time (i.e., disease-stabilization for only a few months) and potential side effects. Moreover, the safety and efficacy of sorafenib in patients who are classified as having Child class B or C cirrhosis remain unclear. Many factors could eventually limit the potential advantages of anti-angiogenic sorafenib effects. Several mechanisms of resistance to vascular endothelial growth factor signaling-targeted therapy have been proposed, and other pre-existing or distinct oncogenic signaling pathways may begin to drive tumor growth during therapy^[35]. Recently, approaches to overcome resistance to anti-angiogenic sorafenib therapy in advanced HCC are being pursued. One of these approaches is the combination of anti-angiogenic therapy and metronomic chemotherapy to induce durable tumor shrinkage or disease stabilization in refractory cancer^[36,37]. Therefore, the combination of sorafenib and HAIC with FUDR may be a promising therapeutic approach for advanced HCC.

The present study has several limitations. For example, high doses of FUDR in HAIC can produce toxicity, which results in a fibrotic narrowing of the bile ducts that is similar to primary biliary sclerosis (up to 30% of patients)^[38]. The use of regional dexamethasone with FUDR, however, can reduce hepatic toxicity. In some studies, the biliary sclerosis rate was 3% or lower, and the patient response rate and survival also improved with the addition of dexamethasone^[39]. None of the patients in the present study had biliary sclerosis, which was likely due to the inclusion of dexamethasone in our treatment protocol.

Another limitation of the present study was that FUDR had to be administered for 14 d with continuous infusion, which might have been associated with poor patient compliance. Hepatic drug uptake and metabolic capacity can be saturated at high drug delivery rates^[40]. Therefore, continuous hepatic arterial infusion is regarded as the most effective means of delivery to maximize the regional advantage. In the present study, all but one patient continued therapy and showed relatively good compliance.

The final limitation of the present study was that we did not use a randomized design (i.e., HAIC was not compared with other therapeutic modalities). However, the present study was the first formal attempt to test HAIC using FUDR in patients with advanced HCC, including patients with advanced cirrhosis. Importantly, the present study demonstrated that survival was better in patients who received HAIC with FUDR compared with patients with unresectable tumors or in whom other therapeutic modalities had been used unsuccessfully.

In conclusion, the present study shows that intra-arterial FUDR chemotherapy is safe and effective for patients with severely advanced HCC confined to the liver for which other therapeutic modalities are ineffective, even in patients with advanced cirrhosis. Based on these results, additional large prospective randomized clinical trials should be performed to prove the efficacy and safety of HAIC using FUDR. Eventually, HAIC using FUDR could be widely applied for the treatment of advanced HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Despite recent advances in therapeutic modalities, the prognosis of advanced HCC remains poor. The current therapies have many limitations, and recurrence or metastasis is relatively frequent. Regional hepatic arterial infusion chemotherapy (HAIC) has been used in patients with advanced HCC. HAIC is believed to provide higher concentrations of chemotherapeutic agents directly to the HCC, which would minimize systemic concentrations of chemotherapeutic agents and potentially minimize systemic toxicity. However, previous studies of HAIC in HCC have shown various results.

Research frontiers

Floxuridine (FUDR) is an active metabolite of 5-fluorouracil (5-FU) that has the advantage of being rapidly metabolized (i.e., a 94%-99% extraction rate within the liver). Furthermore, FUDR is maintained at an exceptionally high intrahepatic concentration (i.e., more than tenfold greater than 5-FU, cisplatin, mitomycin or doxorubicin), which permits maximal tumor cell death while preventing systemic toxicity. In the present study, the authors demonstrated the efficacy and safety of HAIC using FUDR in patients with advanced HCC that was confined to the liver.

Innovations and breakthroughs

Most previous studies have reported the efficacy of HAIC using 5-FU and cisplatin in advanced HCC, whereas HAIC using FUDR in advanced HCC has rarely been reported. The present paper describes the results of HAIC using FUDR in 34 patients with advanced HCC.

Applications

HAIC with FUDR can be performed in patients with advanced HCC that is unresectable or has not responded to previous therapy.

Terminology

HAIC provides chemotherapeutic agents directly to the HCC *via* a pump catheter. The pump catheter is inserted at the proper hepatic artery from the superior mesenteric artery. The distal gastroduodenal artery, right gastric artery and small branches supplying the stomach or duodenum are ligated to prevent gastrointestinal toxicity.

Peer review

The importance of the present study is that HAIC with FUDR could be a promising therapeutic approach for patients with advanced HCC.

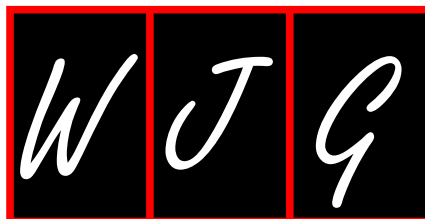
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Proteomic analysis of glutathione S-transferase isoforms in mouse liver mitochondria

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Abstract

AIM: To survey glutathione (GSH) S-transferase (GST) isoforms in mitochondria and to reveal the isoforms' biological significance in diabetic mice.

METHODS: The presence of GSTs in mouse liver mitochondria was systematically screened by two proteomic approaches, namely, GSH affinity chromatography/two dimensional electrophoresis (2DE/MALDI TOF/TOF MS) and SDS-PAGE/LC ESI MS/MS. The proteomic results were further confirmed by Western blotting using monoclonal antibodies against GSTs. To evaluate the liver mitochondrial GSTs quantitatively, calibration curves were generated by the loading amounts of individual recombinant GST protein *vs* the relative intensities elicited from the Western blotting. An extensive comparison of the liver mitochondrial GSTs was conducted between normal and db/db diabetic mice.

Student's *t* test was adopted for the estimation of regression and significant difference.

RESULTS: Using GSH affinity/2DE/MALDI TOF/TOF MS, three GSTs, namely, alpha3, mu1 and pi1, were identified; whereas five GSTs, alpha3, mu1, pi1, kappa1 and zeta1, were detected in mouse liver mitochondria using SDS-PAGE/LC ESI MS/MS, of these GSTs, GST kappa1 was reported as a specific mitochondrial GST. The R^2 values of regression ranged between values of about 0.86 and 0.98, which were acceptable for the quantification. Based on the measurement of the GST abundances in liver mitochondria of normal and diabetic mice, the four GSTs, alpha3, kappa1, mu1 and zeta1, were found to be almost comparable between the two sets of animals, whereas, lower GST pi1 was detected in the diabetic mice compared with normal ones, the signal of Western blotting in control and db/db diabetic mice liver mitochondria is 134.61 ± 53.84 *vs* 99.74 ± 46.2 , with $P < 0.05$.

CONCLUSION: Our results indicate that GSTs exist widely in mitochondria and its abundances of mitochondrial GSTs might be tissue-dependent and disease-related.

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Key words: Glutathione S-transferase; Mitochondria; Liver; Proteomics; Diabetes

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INTRODUCTION

The glutathione (GSH) S-transferase (GST, EC2.5.1.18) superfamily contains eight subclasses classified by their properties of sequence homology, immunology, substrate specificity and isoelectric point. GSTs catalyze the reactions between reduced GSH and unsaturated aldehydes, quinines, and many other substrates, especially under conditions of oxidative stress^[1-3]. These enzymes are involved in many physiological functions, such as the reduction of free radical damage, detoxification, metabolism, regulation of cell signaling and nitric oxide storage^[2-7]. The abundance of GSTs is closely related with the disease status of the organism^[8-12]. For instance, regarding a cancer biomarker, GST pi was found in higher abundance in several cancer cells and tissues and was believed to be involved in drug resistance^[13-15]. GST alpha has main functions in detoxification in the liver, and when hepatocytes are damaged, GST alpha enters the bloodstream. Therefore, GST alpha in blood and urine is an ideal marker indicating hepatocellular impairment^[16]. The accurate measurement of GSTs in tissues or body fluids is urgently required in biomedicine.

GSTs have been identified mainly in the cytoplasm, but they have also recently been detected in organelles, including the microsome, nucleus, mitochondria and peroxisomes^[17-20]. Mitochondria are the primary intracellular sites of oxygen consumption and reactive oxygen species (ROS) generation. GST kappa1 was identified in mitochondria and peroxisomes but is absent in the cytoplasm, and GST kappa was reported to participate in energetic and lipid metabolism in the mitochondria^[21]. In contrast to the cytoplasmic GSTs, the physiological functions of the mitochondrial GSTs have yet to be explored. Generally, it is accepted that GSTs are able to protect mitochondria from dysfunctions of catalase and superoxide dismutase by maintaining the redox balance^[22-24]. A high abundance of GST pi protected the decrease of the mitochondrial membrane potential induced by rotenone. Raza group observed that under oxidative stress, GST alpha was translocated from the cytoplasm into the mitochondria, but its functions in the mitochondria were not elucidated^[23]. To understand the functions of the mitochondrial GSTs fully, we must first define how many GST isoforms exist in the mitochondria. Although there were several reports regarding the distribution of GSTs in different tissue mitochondria, a systematic investigation in this field has not been undertaken.

Recently, proteomic approaches have provided a good opportunity to survey the members of a protein family, as they usually share similar properties, such as immunofluorescence, ligand binding sites, homology sequences and catalytic substrates. Using chromatography or electrophoresis based on these properties, protein family members could be separated from one another in a mixture. Moreover, mass spectrometry is able to identify the separated proteins. Such a proteomics strategy has been widely used in exploring protein isoforms^[25-27].

In this study, we propose a proteomic strategy to

define the GSTs in mouse tissue mitochondria. With combined separation based on size exclusion and affinity chromatography, we enriched the mitochondrial GSTs from a mitochondria preparation of mouse liver. Using MALDI TOF/TOF MS or ESI MS/MS, five GSTs were identified in the mitochondria. The presence of GSTs was further verified by Western blot using monoclonal antibodies. We constructed calibration curves of the GST quantification and employed a quantitative assay of the immunoblots to estimate the different abundances of the mitochondrial GSTs between normal and diabetic mice. For the first time, we found that GSTs are widely distributed in the mitochondria of many tissues and that mitochondrial GST pi1 is sensitive to the development of diabetes.

MATERIALS AND METHODS

Preparation of mitochondria from mouse tissue

The C57BLKS/J db/db and control mice were provided by Peking University Diabetes Center. The heart, kidney and liver tissues of the mice were minced and homogenized in buffer (25 mmol sucrose, 0.5% protease inhibitor cocktail and 10 mmol HEPES, pH 7.4). The crude mitochondria were prepared by differential centrifugation at $1000 \times g$ for 30 min and at $10\,000 \times g$ for 20 min at 4 °C. The purified mitochondria were extracted from a Nycodenz gradient at the interface of 25%-30% Nycodenz solution after centrifugation at $52\,000 \times g$ for 90 min. The purity and integrity of the mitochondria were determined by Western blotting and transmission electron microscopy (TEM). Mitochondrial proteins were extracted using lysis buffer [7 mol/L urea, 2 mol/L thio-urea, 4% CHAPS, 40 mmol/L Tris-HCl (pH 7.4) and protease inhibitor cocktail].

The animal experiments described in this article were approved by the Animal Care and Welfare Committee at the Beijing Institute of Genomics, Chinese Academy of Sciences.

GSH-affinity chromatography

We purified the GSTs using GSH-affinity chromatography with GSH-Sepharose 4B (Amersham Biosciences, United states). The GSH-Sepharose 4B was equilibrated with binding buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L ethylene glycol tetraacetic acid, and 0.1% Triton $\times$ 100]. The mitochondria were re-suspended in 500 μ L binding buffer and were sonicated. After centrifugation, the supernatant was mixed with the equilibrated resin and centrifuged for 30 min 3000 r/min at 4 °C. The affinity resin was washed 3 times with binding buffer, and the proteins were eluted from the resin using 30 mmol/L reduced GSH. A sample of the elution products was retained for two-dimensional electrophoresis (2-DE) separation.

2-DE

The first dimension separation was conducted using an

Ettan IPGphor IEF system with 7 cm (pH 6-11) IPG strips at 20 °C. The proteins isolated by GSH-affinity chromatography were loaded onto strips, and the strips were rehydrated without voltage for 4 h and with 50 V for 8 h. The isoelectric focusing was programmed for 1 h at 500, 1000 and 4000 V, respectively, and was subsequently focused at 4000 V up to a total of 30 kVh. The focused strips were equilibrated in buffer with 6 mol/L urea, 50 mmol/L Tris-HCl, 30% glycerol, 2% SDS and trace bromophenol blue and were subsequently reduced by dithiothreitol and alkylated by iodoacetamide. The treated strips were inserted into a 15% SDS-PAGE gel running in 2.5 W (each gel) for 30 min and 15 W (each gel) thereafter until the bromophenol blue dye reached the bottom of the gels. The gels were stained by silver staining.

Mass spectrometry for protein identification

The proteins were identified by two mass spectrometry methods: MALDI TOF/TOF and LC ESI MS/MS.

The proteins that were separated by GSH-affinity chromatography and 2D gel electrophoresis were excised and in-gel digested with trypsin overnight and identified by MALDI TOF/TOF MS. Briefly, the tryptic digests were co-crystallized with a matrix of α -cyn-4-hydroxycinnamic acid spotted onto the AnchorChip and desalted by 0.1% trifluoroacetic acid. The AnchorChip was analyzed using an Ultraflex TOF/TOF MS mass spectrometer (Bruker Dalton, Bremen, Germany) for protein identification. Positively charged ions were analyzed in the reflector mode. Typically, 100 shots were cumulated per spectrum in the MS mode and 400 shots in the MS/MS mode. The mass spectra and tandem mass spectra obtained were processed using the FlexAnalysis 2.2 and BioTools 2.2 software tools. The protein identification was performed using the Mascot software (<http://www.matrixscience.com>), and the NCBI database was searched using mouse as the taxonomy. The following parameters were used for the database searches: one incomplete cleavage, alkylation of cysteine by carbamidomethylation, oxidation of methionine, and pyro-Glu formation of the N-terminal Gln.

The 20-30 kDa proteins separated by SDS-PAGE were a mixture of many proteins, and the proteins were examined by LC ESI MS/MS after the in-gel trypsin digestion. Briefly, after capillary reversed-phase high-performance liquid chromatography, the separated peptides were analyzed using an ion-trap mass spectrometer LCQ DecaXP ion-trap mass spectrometer (Thermo Finnigan, Ringoes, NJ) with 3.2 kV of spray voltage and 150 °C at the heated desolvation capillary. A mass-to-charge (m/z) range from 400 to 2000 was scanned over 1.2 s, and the ions were detected with a high energy conversion dynode detector. The MS/MS data were converted into DTA-format files, which were further searched for proteins with Sequest software. All of the accepted results had a deltaCn of 0.1 or greater. Furthermore, a singly charged peptide must be tryptic, and the cross-correlation score (Xcorr) had to be more than 1.9. The tryptic peptides with a charge state of

+ 2 must have a Xcorr of more than 2.2. Triply charged tryptic peptides were accepted if the Xcorr was ≥ 3.0 .

Generation of monoclonal antibodies

To generate the specific antibodies against these five GST isoforms, we cloned these five *GST* genes and expressed the recombinant protein by inserting these genes into the prokaryotic expression vector, named pET-28a. The recombinant proteins expressed from the pET-GST plasmids were 6 $\times$ His-tagged. After expression in *E. coli* BL21 (DE3), the proteins were purified using Ni-NTA agarose resin (Qiagen, United States) and metal chelate chromatography.

Six-weeks-old female BALB/c mice were immunized subcutaneously with the recombinant protein in Freund's complete adjuvant. After booster injections, the mice with positive serum immunogenicity against the recombinant protein were used for the monoclonal fusion experiments. The mice's spleens were excised, and single-spleen cell suspensions were fused with Sp2/0 myeloma cells. After several days of fusion, the hybridomas were picked and cultured in complete medium supplemented with 1% HT. Ascitic fluids were collected from the mice after an intraperitoneal injection of the hybridomas, and the antibodies were purified by protein A/G-Sepharose affinity chromatography. The protein concentrations of the purified antibodies were determined using the Bradford method, and the antibodies were diluted to 1 mg/mL and stored in 50% glycerin at -20 °C.

Western blotting

Proteins were separated by 15% SDS-PAGE and were electro-transferred onto polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked with 5% non-fat milk dissolved in Tris-buffered saline with 0.05% Tween-20 (TTBS) at 37 °C for 90 min. The membranes were incubated with the primary antibodies at a dilution of 1:5000 in blocking reagent at room temperature for 2 h. The antibodies against the GSTs were generated by our laboratory, and the anti-ATP synthase β antibody was purchased from BD Biosciences. The membranes were incubated in goat anti-mouse/rabbit IgG conjugated with horseradish peroxidase at a 1:3000 dilution at room temperature for 1 h. The membranes were developed using the Super ECL Plus Detection Reagent kit, and the images were captured using ImageQuant ECL (GE Healthcare, United Kingdom).

Statistical analysis

To quantify the GSTs, calibration curves were constructed using the protein concentrations and immunosignal obtained from the Western blotting. To analyze the quantities of GSTs in the diabetes mouse model statistically, the relative intensity of the Western blotting signals in the samples was quantified using ImageQuant TL software. The three pairs of samples from the control and db/db mice were then randomly paired, and the relative abundance ratios of the five GST proteins were

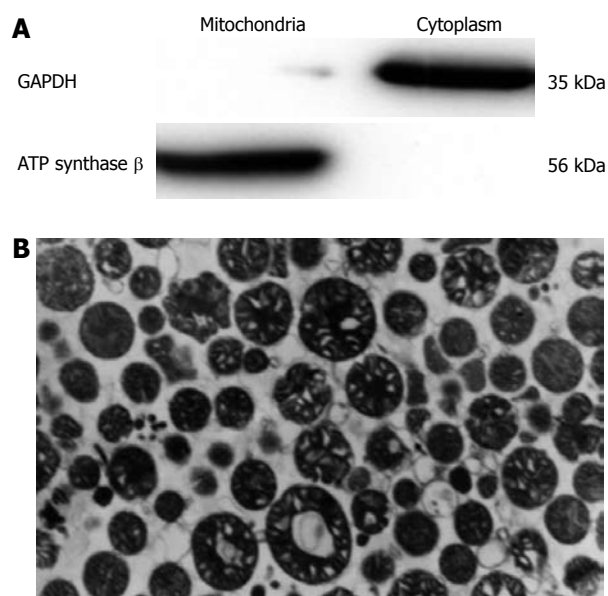


Figure 1 Measurement of mitochondrial purity by Western blotting and transmission electron microscopy. A: Glyceraldehyde-3-phosphate dehydrogenase is an indicator of cytoplasm and ATP synthase an indicator of mitochondria; B: Image of products purified by Nycodenz gradient centrifugation.

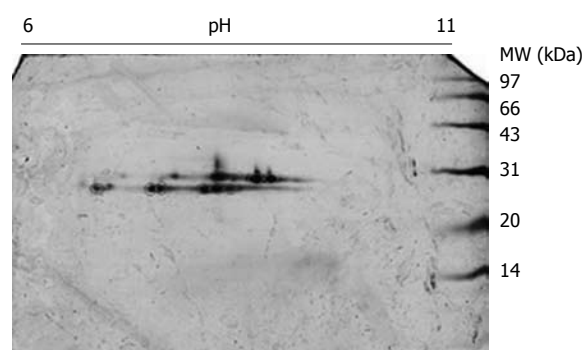


Figure 2 Two dimensional electrophoresis image of proteins purified by glutathione-affinity chromatography. The mitochondrial proteins enriched by glyceraldehyde affinity column were separated in two dimensional electrophoresis with pH 6-11 strips and 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The string spots around molecular weight (MW) 28 kDa appeared on the gels labeled by circles were identified.

statistically analyzed and were normalized using the levels detected in normal mice based on two parallel experiments. All of the values are expressed as the mean $\pm$ SD, and the statistical significance was set to a $P < 0.05$.

RESULTS

Screening of GST isoforms in liver mitochondria

Considering that the purity of the mitochondria is a key element in studying mitochondrial components, Nycodenz gradient centrifugation was employed for the preparation of the mouse liver mitochondria. The mitochondrial integrity and purity were examined by Western blot and TEM. Our data as shown in Figure 1 indicated that the purity and integrity of the mitochondria were satisfactory for further analysis.

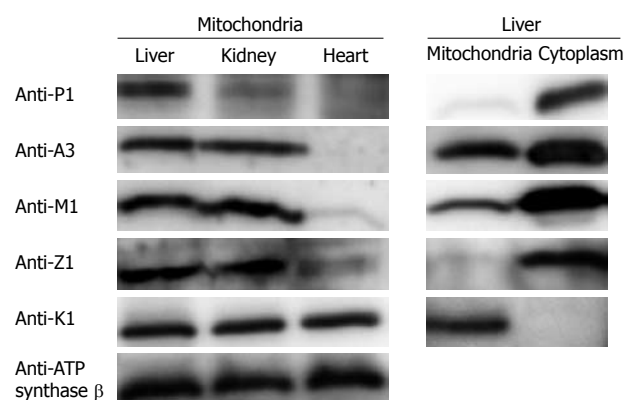


Figure 3 The distribution of glutathione S-transferases in mouse tissue mitochondria, as measured by Western blotting. A total of 20 μ g mitochondrial and cytoplasmic protein from mouse heart, kidney, and liver were loaded in each lane. Left: Glutathione S-transferases (GSTs) in mouse tissue mitochondria; Right: GSTs in liver mitochondria and cytoplasm.

The mitochondrial lysates were loaded onto GSH affinity columns, and the fractions eluted by GSH were collected and subjected to 2DE using pH 6-11 strips and 15% SDS-PAGE. After silver staining, a string spots of approximately 28 kDa and pI 7-10 appeared on the gels (Figure 2), whereas no spots were detected in other parts of the gel, indicating that the affinity enrichment was effective to remove non-GSH-binding proteins. The silver-stained spots were excised and digested with trypsin followed by protein identification using MALDI TOF/TOF MS. In total, three GSTs, GST alpha3, GST mu1 and GST pi1, were found in the liver mitochondria. Because all of the GSTs have similar MWs (about 28 kDa), we attempted to separate the mitochondrial GSTs from the other mitochondrial proteins through size-exclusion methods. The mitochondrial lysates were loaded onto a 15% SDS-PAGE gel, and the portion corresponding to 20-30 kDa was sliced into 19 fractions. These fractions were digested with trypsin, and the digested fractions were further analyzed using LC ESI MS/MS. All of the unique peptide sequences of GSTs are listed in Table 1, indicating five GSTs: GST pi1, GST alpha3, GST mu1, GST kappa1 and GST zeta1. All of the GSTs identified by GSH/2DE/MALDI TOF/TOF MS are included in the list of the identified GSTs by SDS-PAGE/LC ESI MS/MS; therefore, the SDS-PAGE/LC ESI MS/MS approach exhibits a better sensitivity for the detection of GSTs.

The existence of GSTs in the liver mitochondria was further confirmed by Western blotting using monoclonal antibodies against the individual GSTs (the specificity of detection is described below). As depicted in Figure 3, the five GST isoforms detected by the proteomic methods showed positive immunoreactivity; this evidence strongly supports the conclusion reached with the proteomic analysis. Furthermore, we prepared the tissue mitochondria from mouse kidney and heart and conducted Western blotting to evaluate the abundance of the GSTs in the mitochondria from different tissues. The left panel in Figure 3 reveals the wide distribution of GSTs in

Table 1 The unique peptides of glutathione S-transferases identified by LC ESI MS/MS

GST	Peptide sequence	MH +	Location	XC	DeltaCn	Ions
Mu1	MLLEYTDSSYDEK	1593.699	19-31	4.062	0.47	20 24
	ADIVENQVMDTR	1390.664	97-108	4.1055	0.5208	17 22
	MLIMLCYNPDFEK	1801.811	109-122	4.8593	0.5987	20 26
Zeta1	GIDYEIVPINLIK	1486.852	28-40	3.0229	0.3943	15 24
	VITSGFNALEK	1178.642	134-144	2.6217	0.4564	16 20
Kappa1	FLTTVSMEQPEMLEK	1782.866	102-116	4.944	0.5723	22 36
	AGMSTAQAQHFLEK	1518.737	145-158	2.4224	0.2908	13 16
Alpha3	SDGSLMFQQVPMVEIDGMK	2111.982	46-64	3.9127	0.5378	21 28
	AILNYIASK	992.578	70-78	3.0611	0.3416	17 26
	YFPAFEK	901.446	132-138	2.5832	0.3767	8 12
Pi1	EAAQMDMVNDGVEDLR	1792.785	86-101	3.9673	0.5391	22 30
	YVTLIYTNYENGK	1577.785	104-116	3.6743	0.4268	18 24

MH+ stands for the mass of ions detected by LC ESI MS/MS. XC stands for the cross-correlation score. GST: Glutathione S-transferase.

the mitochondria from the three tissues with relatively different abundances. As a specific GST only located in mitochondria, GST kappa1 exists in all three tissues with similar abundances. GST pi1 is clearly detected in the mitochondria of all three of the tissues, whereas GST pi1 in the liver mitochondria is in the highest abundance. The three GSTs, GST alpha1, GST mu1, and GST zeta1, are clearly observed in the liver and kidney mitochondria, whereas they are almost undetected in the heart. Furthermore, we examined the cytoplasmic GSTs and the mitochondrial GSTs in the mouse liver using Western blotting. The data illustrated in the right panel in Figure 3 indicate that GST kappa1 is the only isoform undetectable in the cytoplasm, whereas the other GST isoforms are readily detected in the two subcellular fractions. When equal amounts of proteins, either cytoplasmic or mitochondrial, are loaded onto an SDS-PAGE gel, the analysis of the relative intensities demonstrate that the abundances of GSTs in the cytoplasm are commonly higher than those in the mitochondria.

Quantitative analysis of the mitochondrial GST using Western blotting

The five GSTs in liver mitochondria were first discovered by proteomic approach. As a routine assay in laboratory, mass spectrometry is not a common instrument and easy in use. Western blotting is still a widely acceptable and enough sensitive approach to detect proteins. We therefore developed a quantitative assay for the mitochondria GSTs based on immuno-blot. It is well known that specificity and sensitivity of antibody are a key factor for a successful Western blotting. To have the qualified antibodies against GSTs, we generated a set of monoclonal antibodies of GSTs, and performed a strict screening. A qualified antibody should match to criteria: (1) It can recognize 2 ng of the correspondent recombinant protein; and (2) It cannot pick up any signals to 20 ng of unrelated GSTs. The qualified GST antibodies are illustrated in the upper panel of Figure 4.

Selection of the concentration ranges for the recombinant GSTs is an important consideration for quantitative calibration. In our routine experiments, the

maximum loading of mitochondrial proteins are approximately 20 μ g; therefore, we loaded 20 μ g of proteins and different amounts of recombinant GSTs on a relatively large scale onto the same gels and performed the Western blotting analysis. After determining the band intensities, we were able to estimate the proper amounts of the recombinant GSTs for the generation of calibration curves. As shown in Figure 4, five calibration curves are generated corresponding to the multiple assays of our Western blotting. However, Western blotting has a disadvantage in quantitative measurements because the reproducibility of the band intensities is relatively poor. The data shown in Figure 4 is in agreement with the observation, although some spots indeed display a large deviation. However, the regression calculation revealed that the values of R^2 ranged from 0.85-0.98, which are generally accepted in quantitative measurements using Western blot. We further estimated the possible dynamics of the GST abundances in the liver mitochondria. With the exception of GST pi1, measuring approximately 50 ng to 300 ng, the other four GSTs were between 10 ng to 40 ng, indicating that GST pi has the highest abundance in liver mitochondria. According to the regression curves in Figure 4, the mitochondrial GST abundances were quantitatively estimated as follows: $134.61 + 53.84$, $38.83 + 2.33$, $29.25 + 0.29$, $27.28 + 0.27$, $15.84 + 0.16$ ng per μ g mitochondria protein for GST pi1, mu1, alpha3, kappa1, and zeta1, respectively.

Comparison of the GSTs' abundances in liver mitochondria between normal and diabetes mice

The db/db mouse is a well-accepted type II diabetes model, and previous studies have shown that free radicals were increased in most of the tissues in this model. After carefully examining the mice's blood glucose and body weight to ensure diabetes development, we collected the livers from three normal and three db/db mice; the prepared mitochondria were analyzed by Western blot to quantify the contents of the GSTs. Figure 5A shows such a typical Western blotting, suggesting that the immunoreactivity of GST pi1 is significantly attenuated in the mitochondria of the diabetic mice, the signal of Western

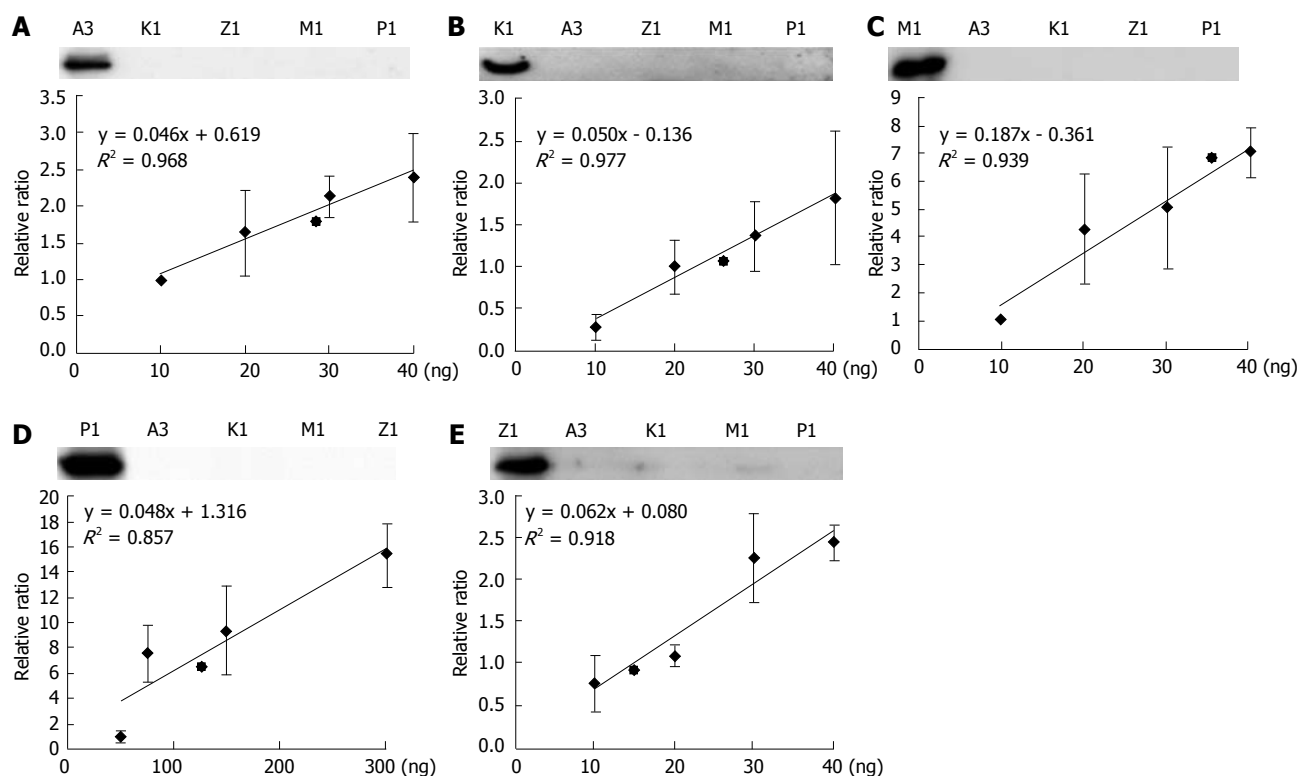


Figure 4 The calibration curves of mitochondrial glutathione S-transferase proteins. Upper panel in A, B, C, D, E: Examination of the specificity of the five glutathione S-transferase (GST) antibodies, 2 ng of the corresponding GST was loaded in all of the left lanes, and 20 ng of the other unrelated GSTs was loaded in the other four lanes; Lower panel in A, B, C, D, E: The X-axis represents the amounts of recombinant GSTs loaded (ng), and the y-axis represents the relative intensity of the Western blotting, taking the signals generated from the lowest GST amount as the reference. The calibration curves were generated by regression. Insert: The linear equations and R^2 were generated by regression.

blotting in control and db/db diabetic mice liver mitochondria is 134.61 ± 53.84 vs 99.74 ± 46.2 , with $P < 0.05$, whereas the other four GST isoforms are nearly comparable between the normal and diabetic mice. Statistically, we performed pairwise comparisons and estimations of the significant differences based on multiple Western blotting. The comparison data depicted in Figure 5B support the conclusion drawn from Figure 5A that the abundance of mitochondrial GST pi1 in diabetic mice is lower than in normal mice, whereas the other mitochondrial GSTs did not exhibit significant changes.

We screened the response of the GSTs to diabetes in the db/db liver mitochondria. As depicted in Figure 5A, which shows representative images of mitochondrial tissue from one pair of mice (control vs db/db), GST pi1 showed a decrease in the db/db mouse when we compared three pairs of mice. In contrast, similar expression levels of GST alpha3, mu1, and zeta1 were observed in the liver mitochondria of the db/db mice and control mice. The statistical analysis indicated in Figure 5B supported these observations.

DISCUSSION

GSTs have been detected as a group of oxidative stress proteins in mitochondria, and they were characterized as associated with the maintenance of mitochondria functions^[22,28]. For example, alpha-class isozymes of

GST translocated into the mitochondria under oxidative stress, and the isozymes showed glutathione peroxidase activity toward phospholipid hydroperoxide in the rat liver cytosol. Chemicals that generate reactive oxygen species, such as rotenone and antimycin A, reduced the cell viability and mitochondrial membrane potential, and the overexpression of GST pi1 diminished these changes.

In general, GSTs were determined by their enzymatic activities and immunological features; however, these methods had limitations in evaluating the presence of GST. The substrate specificity of purified rat liver GSTs has been investigated by a series of gamma-glutamyl-modified GSH analogues, and GST had a different ability to conjugate with the substrates. Furthermore, a product of the purification using GSH could not provide the status of GST *in vivo*. Therefore, the uncertainty of the type of GSTs localized in the mitochondria and the antibody specificity led to the uncertainty of an increase or decrease of GSTs under oxidative stress or in a pathological condition.

In this study, we have taken multiple approaches to identify the GSTs in mouse liver mitochondria. The mitochondrial proteins were separated by SDS-PAGE, and the 20-30 kDa proteins were analyzed by liquid chromatography and subsequently identified by ESI MS/MS. Five GST isoforms were detected in the liver mitochondria. Our strategy has two advantages: more proteins could be resolved regardless of the solubility by SDS-

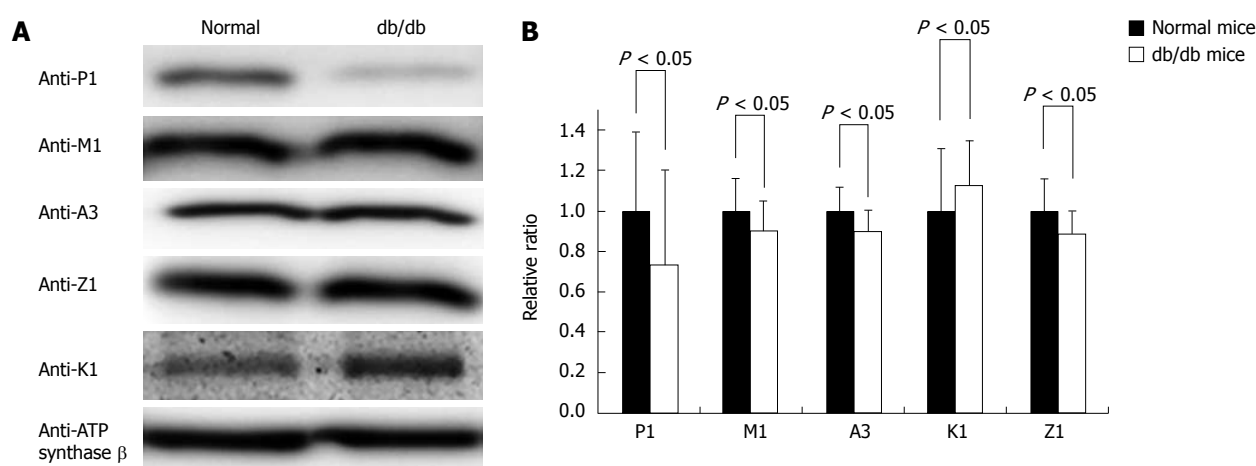


Figure 5 Comparison of mitochondrial glutathione S-transferase abundances between normal and diabetic mice by Western blotting. A: A typical image of a Western blotting comparing a pair of mouse liver mitochondria from normal and diabetic mice. Approximately 20 μ g mitochondrial proteins were loaded in each lane. Anti-ATP synthase β acted as a control to normalize the loading; B: The statistical comparison of the relative ratio of mitochondrial glutathione S-transferases (GSTs) between normal and diabetic mouse liver when normalized by the GSTs in normal mice ($n = 3$).

PAGE, and the sample complexity was reduced when the proteins distributed in the 20-30 kDa range were divided into 19 fractions for digestion and separation by liquid chromatography. Although we could not dismiss the possibility of other GSTs in the mitochondria, our multiple proteomic techniques provided new insights into analyzing GST isoforms, especially for those of low abundance.

GSH is not generated in mitochondria and is instead transferred from the cytoplasm; a GSH concentration of 5-10 mmol is maintained in mitochondria^[29]. GSH is a major defense molecule against ROS, and mitochondrial GST may play an important role in responding to chemical and oxidative stress because GST is the main phase II enzyme that catalyzes the conjugation of GSH with numerous reactive electrophiles. The identification of GSTs would influence the study of the functions of mitochondrial GST.

We discovered GST zeta1 in the mitochondria. GST zeta1 is localized in the cytoplasm and plays a significant role in the catabolism of phenylalanine and tyrosine^[5]. When GST zeta1 is lacking in mice, the inductions of the class alpha, mu, and pi GSTs could be detected by Western blotting and high-performance liquid chromatography analysis of glutathione affinity-purified proteins^[30]. The role of GST zeta1 in the mitochondria will be investigated further.

The expression of GSTs is different in distinct tissues. Our data indicate that mitochondrial GST also has tissue-specific expression. GSTs are abundant in the liver but are scarce in heart tissue. The abundance of GST kappa1 in the heart, kidney, and liver is similar, but this GST is only detected in the mitochondria, indicating that it might perform a conserved function in the mitochondria of different cell types.

We screened these mitochondrial GSTs in the diabetes mouse model and found that the GST response to

oxidative stress varies: only GST pi1 is decreased in the db/db mouse compared to normal mice, and the levels of the other four isoforms did not change. This result indicated that a decrease of GST pi1 might accelerate the pathological progress of diabetes.

COMMENTS

Background

The glutathione (GSH) S-transferase (GST) involve in many physiological functions. They are located mainly in the cytoplasm, but they have also been detected in suborganelles. Because mitochondria are the primary intracellular sites of oxygen consumption and reactive oxygen species generation, the mitochondrial GSTs abundances may relate with disease status. The goal was to survey GST isoforms in mitochondria and to reveal their biological significance in diabetic mice.

Research frontiers

It is reported that GSTs are able to protect mitochondria from oxidation by maintaining the redox balance, but their functions in the mitochondria were not elucidated. To fully understand the functions of the mitochondrial GSTs, new method to monitor the mitochondrial GSTs is urgently required.

Innovations and breakthroughs

The mitochondrial GSTs are measured by the combined quantitative proteomic strategies based on mass spectrum and antibodies. The authors discovered that GSTs are widely distributed in tissue mitochondria and their responses to the diabetes physiology. It is noted the mitochondrial GST pi1 is sensitive to diabetic development.

Applications

This research showed how the proteomics produces meaningful information and offered a relatively easy and simple workflow to detect GSTs in liver mitochondria and to reveal their biological significance of normal and diabetes mouse. At the same time, the application of this strategy provides an alternative tool to analyze how isoforms of protein family response to disease in complex biological systems.

Terminology

Proteomics is the protein map of a biological system and involves the systematic study of proteins in order to provide a comprehensive view of the function and regulation of proteins.

Peer review

This paper provides solid data on systematic analysis of GST isoforms in mouse liver mitochondria.

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Anticoagulation therapy prevents portal-splenic vein thrombosis after splenectomy with gastroesophageal devascularization

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Abstract

AIM: To compare the incidence of early portal or splenic vein thrombosis (PSVT) in patients treated with irregular and regular anticoagulation after splenectomy with gastroesophageal devascularization.

METHODS: We retrospectively analyzed 301 patients who underwent splenectomy with gastroesophageal devascularization for portal hypertension due to cirrhosis between April 2004 and July 2010. Patients were categorized into group A with irregular anticoagulation and group B with regular anticoagulation, respectively. Group A (153 patients) received anticoagulant monotherapy for an undesignated time period or with aspirin or warfarin without low-molecular-weight heparin (LMWH) irregularly. Group B (148 patients) received subcutaneous injection of LMWH routinely within the

first 5 d after surgery, followed by oral warfarin and aspirin for one month regularly. The target prothrombin time/international normalized ratio (PT/INR) was 1.25-1.50. Platelet and PT/INR were monitored. Color Doppler imaging was performed to monitor PSVT as well as the effectiveness of thrombolytic therapy.

RESULTS: The patients' data were collected and analyzed retrospectively. Among the patients, 94 developed early postoperative mural PSVT, including 63 patients in group A (63/153, 41.17%) and 31 patients in group B (31/148, 20.94%). There were 50 (32.67%) patients in group A and 27 (18.24%) in group B with mural PSVT in the main trunk of portal vein. After the administration of thrombolytic, anticoagulant and anti-aggregation therapy, complete or partial thrombus dissolution achieved in 50 (79.37%) in group A and 26 (83.87%) in group B.

CONCLUSION: Regular anticoagulation therapy can reduce the incidence of PSVT in patients who undergo splenectomy with gastroesophageal devascularization, and regular anticoagulant therapy is safer and more effective than irregular anticoagulant therapy. Early and timely thrombolytic therapy is imperative and feasible for the prevention of PSVT.

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Key words: Portal vein hypertension; Splenectomy with gastroesophageal devascularization; Portal or splenic vein thrombosis; Anticoagulation regimen; Thrombolytic therapy

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INTRODUCTION

Although endoscopic surgery has been widely used to treat esophagogastric variceal bleeding (EGVB), splenectomy with gastroesophageal devascularization is still the primary method to treat and prevent recurrent EGVB in East Asia^[1,2]. Portal or splenic vein thrombosis (PSVT) is a common and potentially life-threatening complication of splenectomy with gastroesophageal devascularization for portal hypertension due to cirrhosis. Severe PSVT can reduce hepatopetal blood flow in the portal system and elevate the blood pressure in the visceral side of portal vein, leading to further deterioration of liver function and the recurrence of upper gastrointestinal (GI) bleeding^[3,4]. In serious cases, PSVT can significantly affect patient's life expectancy.

A prospective study with contrast-enhanced computed tomography scan showed that PSVT occurred in 12 (55%) patients of the laparoscopic splenectomy (LS) group, but in only 4 (19%) of the open splenectomy (OS) group, and the difference was significant^[5]. A recent clinical study performed by the same group of authors has emphasized that the incidence of PSVT still reached 51.52% in 33 cases after LS without any anticoagulation therapy^[6]. The different incidences of PSVT between laparoscopic and open splenectomy may be caused by the operative technique of pneumoperitoneum and ligation of splenic hilar vessels. In the OS group, splenic hilar vessels were ligated conventionally, while these vessels were divided with an endoscopic vascular stapler in the LS group^[5]. But in some other studies, the incidence of PSVT varied after splenectomy with gastroesophageal devascularization^[7-9].

The literatures mentioned above focused on splenectomy to treat hematologic and metabolic disorders. The reported incidence of PVST related to these diseases may be different from that related to liver cirrhosis because of the different disease spectrum. Kawanaka *et al*^[10] analyzed 50 consecutive cirrhotic patients who underwent splenectomy, the PVST incidence was 36.0% (9/25) up to postoperative day (POD) 7 without any prophylactic anticoagulation therapy, the PVST incidence of the other 25 patients was only 4.0% (1/25) up to POD 30 who received antithrombin III (AT-III) therapy in the first three POD. Ushitora *et al*^[11] retrospectively examined 38 consecutive cirrhotic patients who underwent splenectomy, the total incidence of PSVT detected by postoperative dynamic computed tomography was 34.2% (13/38) without any prophylactic anticoagulation therapy. Deng *et al*^[12] reported a 33.69% incidence of PVT 7 to 14 d after portal hypertension surgery (splenectomy with gastroesophageal devascularization) in 52 surgically treated patients with portal hypertension due

to hepatitis B virus (HBV)-related cirrhosis. The 10-year survival rate among adults with PSVT was 38%-60%, the mortality rate from variceal bleeding in patients with PSVT with cirrhosis was 30%-70%, significantly higher than 5% from variceal bleeding in patients with PSVT without cirrhosis^[13].

Therefore, PSVT is indeed a common complication of splenectomy with gastroesophageal devascularization with a high incidence and morbidity even though with different disease spectrum. PSVT was considered contraindicated for liver transplantation in the past because of technical difficulties^[14] and currently it still remains as a risk factor^[15]. It makes the surgical procedure more cumbersome, resulting in a higher morbidity and mortality in the PSVT patients^[16,17]. Englesbe *et al*^[18] found that mortality of patients with previous portal vein thrombosis (PVT) after liver transplantation was higher than that of patients without PVT (at 30 d 17.7% *vs* 4.4%, $P = 0.07$; at 1 year 33.0% *vs* 25.0%, $P = 0.354$; and at follow-up 36.7% *vs* 28.4%, $P = 0.371$), even these differences were not statistically significant. And patients with cirrhosis complicated with PVT have significantly increased risks of death after liver transplantation (hazard ratio 7.389, 95% CI: 2.392-22.827).

So, it is very important to prevent the occurrence of PSVT after splenectomy with gastroesophageal devascularization for the better long-term clinical outcomes and following possible liver transplantation. Prophylactic anticoagulation therapy is a prime method to prevent PSVT after splenectomy with gastroesophageal devascularization. The most frequently used drugs are low molecular weight heparin (LMWH), vitamin K antagonist such as warfarin^[5], and aspirin^[19-21]. But the coarse and dosage of these drugs were variable according to different literatures, there was no generally acceptable PSVT prophylactic regimen for all patients after splenectomy with gastroesophageal devascularization^[22,23].

We studied retrospectively the efficacy of regular anticoagulant therapy on preventing early postoperative PSVT in the cirrhotic patients who received splenectomy with gastroesophageal devascularization in Beijing You-An Hospital of Capital Medical University from December 2004 to July 2010.

MATERIALS AND METHODS

Inclusion and exclusion criteria

Inclusion criteria: Patients with liver cirrhosis due to any causes; liver function grade: Child-Pugh A-B; splenomegaly and hypersplenism; severe esophageal varicose confirmed by gastroscopy; and previous histories of recurrent upper gastrointestinal bleeding. All patients signed the informed consent and the study was approved by the Ethics Committee of the hospital. These patients' data within one month after splenectomy with gastroesophageal devascularization were collected and analyzed.

Exclusion criteria: Patients who did not fulfill the inclusion criteria and could not tolerate surgical treatment were excluded.

Clinical examinations

After fasting for 8 h, all patients lied in the supine or left lateral position, and color Doppler ultrasound examination was performed with ACUSON Sequola 512 SIE-MENS Ultrasound system with a probe frequency of 3.5 MHz. The inner diameter of the portal vein and the splenic vein were measured in the sagittal position. Platelets/international normalized ratio (PLT/INR) was also measured before surgery and at day 1, day 3, day 7 and 1 mo after surgery.

Surgical procedure and portal venous pressure measurement

Selective gastroesophageal devascularization was performed using intraoperative free portal venous pressure (FPVP) as guidance^[24]. A 20G antithrombotic catheter (BD Insyte, Becton Dickinson Medical Devices Co, Ltd. Suzhou, China) was inserted into the right gastro-omental vein to test FPVP after laparotomy and splenectomy, respectively. Gastroesophageal devascularization was performed according to the FPVP.

Treatment and grouping

The patients were classified into two groups according to whether regular anticoagulation was administered. In group A, the patients received irregular anticoagulant aspirin or warfarin monotherapy for an undesignated time period without LMWH due to poor blood clotting after surgery and perioperative abdominal or GI bleeding.

Patients in group B had nearly normal blood clotting before and after surgery; 24 h after surgery, they received subcutaneous injection of LMWH routinely, 0.3 mL per 12 h for 5 d and then maintained by oral therapy with warfarin for one month to keep the target prothrombin time/international normalized ratio (PT/INR) at a level between 1.25 and 1.5. If the postoperative platelet level was increased to $100 \times 10^9/\text{L}$ or above, aspirin 100 mg daily was added for one month. If the postoperative platelet level was increased to $300 \times 10^9/\text{L}$ or above, ticlopidine was added with the dose of 0.25 g daily for one month.

Color Doppler ultrasound examination and PLT/INR measurement were repeated. Once PSVT was confirmed after splenectomy with gastroesophageal devascularization, the patients would receive a thrombolytic therapy. Urokinase was administered *via* the peripheral venous route with a bonus dose of 200 000 units within 30 min, followed by continuous infusion of 20 000-50 000 units/h for 3-5 d *via* a micro-infusion pump. During the thrombolytic treatment, PT/INR and PLT were measured daily. Following the thrombolytic treatment, the patients were administrated with oral warfarin (2.5 mg, 1-2 times daily) and aspirin (100 mg daily). The drug doses were adjusted according to PT/INR and PLT levels. If repeated color

Doppler ultrasound examinations showed complete or partial dissolution of target thrombus, the treatment was considered effective and switched to oral warfarin monotherapy (2.5 mg, 1-2 times daily) for one month. If repeated color Doppler ultrasound examinations showed little change or even enlargement of the target thrombus, the thrombolytic therapy was defined as ineffective. The patients would continue to receive oral warfarin and aspirin and followed up regularly.

Statistical analysis

SPSS version 11.5 software (SPSS Inc., Chicago, IL, United States) was used for statistical analyses. Continuous data were presented as mean $\pm$ SD and compared with two-tailed nonpaired Student's *t* test. Categorical data were analyzed with Chi-square or Fisher exact test. Chronologic changes in the laboratory data were analyzed by the analysis of variance for repeated measures. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 301 cirrhotic patients undergoing splenectomy with gastroesophageal devascularization in our department was included in the study, including 202 males and 99 females with an average age of 45.96 years (range, 14-77 years). They were divided into two groups: Group A, 153 patients, including 103 men and 50 women with a mean age of 46.14 ± 10.39 years; and Group B, 148 patients, including 99 men and 49 women with a mean age of 46.47 ± 9.58 years. There were 254 cases of HBV related cirrhosis, 19 cases of hepatitis C virus related cirrhosis, 22 cases of alcoholic cirrhosis, 3 cases of idiopathic portal hypertension and 3 cases of primary biliary cirrhosis.

As for liver function grade, Child-Pugh A was found in 246 patients and Child-Pugh B in 55 patients. Of the 301 patients, 236 patients (78.40%) had a history of upper GI bleeding before surgery. All major clinical parameters were not significantly different between the two groups (Table 1).

The FPVP before splenectomy was 37.79 ± 5.03 cm H₂O in group A and 36.66 ± 5.24 cm H₂O in group B ($P = 0.179$). The FPVP after splenectomy with gastroesophageal devascularization was 30.01 ± 4.58 cm H₂O in group A and 29.59 ± 4.37 cm H₂O in group B ($P = 0.559$).

Sixteen patients (5.31%) had preoperative spontaneous mural PSVT, with an incidence of 3.92 % (6/153) in group A and 6.76 % (10/148) in group B ($\chi^2 = 1.201$, $P = 0.273$).

The total incidence of postoperative mural PSVT was 31.22 % (94/301), 41.17% (63/153) in group A and 20.94 % (31/148) in group B ($\chi^2 = 15.009$, $P = 0.002$). There were 50 (32.67%) cases of mural thrombi in the main trunk of the portal vein in group A and 27 (18.24%) cases in group B ($P = 0.004$). As shown in Table 2, there was no difference in terms of the location of thrombi between the two groups. No single case of thrombosis involved whole portal system. The incidence of throm-

Table 1 Comparison of preoperative data between two groups

	Irregular anticoagulation (group A)	Regular anticoagulation (group B)	P value
Sex (M/F)	103/50	99/49	0.937
Age (yr), mean $\pm$ SD	46.14 $\pm$ 10.39	46.47 $\pm$ 9.58	0.814
Type of disease			
Hepatitis B viral cirrhosis	128	126	0.816
Hepatitis C viral cirrhosis	11	8	
Alcoholic cirrhosis	11	11	
Idiopathic portal hypertension	1	2	
Biliary cirrhosis	2	1	
Child-Pugh classification (grade A/grade B)	121/32	125/23	0.228
MELD index: mean $\pm$ SD, median	7.68 $\pm$ 3.24, 7.70	6.62 $\pm$ 2.76, 6.51	0.055
History of upper GI bleeding	125 (81.69%)	111 (75%)	0.158
History of GI ulcer	18 (11.76%)	23 (15.54%)	0.340
Preoperative portal vein diameter (mm): mean $\pm$ SD, median	12.38 $\pm$ 1.17, 13	12.90 $\pm$ 1.20, 12	0.083
Preoperative splenic vein diameter (mm): mean $\pm$ SD, median	9.85 $\pm$ 1.69, 10	10.10 $\pm$ 1.41, 10	0.551

MELD: Model for end-stage disease; GI: Gastrointestinal.

Table 2 Distribution of thrombi in two groups, *n* (%)

Location	Irregular anticoagulation (group A)	Regular anticoagulation (group B)
Simple PV main trunk mural thrombosis	31 (20.26)	18 (12.16)
Simple splenic vein mural thrombosis	12 (7.84)	4 (2.70)
Complicated portal and splenic vein thrombosis	19 (12.41)	7 (4.72)
Simple SMV thrombosis	1 (0.65)	0
Complicated portal PV and SMV thrombosis	0	2 (1.35)
Total	63	31
Incidence of thrombosis	(41.17)	(20.94)

$\chi^2 = 15.009$, $P = 0.002$. PV: Portal vein; SMV: Superior mesenteric vein.

bosis in the portal vein main trunk was significantly different between the two groups ($\chi^2 = 8.236$, $P = 0.004$).

During the anticoagulant therapy, mild GI bleeding occurred in 3 patients, including 1 in group A and 2 in group B. The anticoagulant therapy was terminated immediately and hemostatic agents were administered. Bleeding was successfully controlled, and the patients recovered well.

All 94 patients with postoperative PSVT were treated with thrombolytic drug, urokinase, anticoagulant and antiaggregation agents. Among the 94 patients, 76 achieved complete or partial thrombus dissolution, including 50 (79.37%, 50/63) patients in group A and 26 (83.87%, 26/31) patients in group B ($\chi^2 = 0.272$, $P = 0.602$). No thrombolytic therapy-related complications such as bleeding were noted.

As shown in Figure 1, platelet count was not significantly different between the two groups ($P = 0.981$). However, the PT/INR in group B was gradually increased from day 7 after surgery, which was statistically different from that in group A ($P = 0.020$). We also compared the patients with and without PSVT and found that the PT/INR in patients without PSVT was greater than those with PSVT at day 14 after surgery (1.30 ± 0.21 vs 1.23 ± 0.17 , $P = 0.037$). Similar results were found in patients with and without PSVT in group B (1.28 ± 0.21 without PSVT vs 1.18 ± 0.14 with PSVT, $P = 0.017$) (Figure 2).

DISCUSSION

Selection of thrombosis prevention regimens after splenectomy with gastroesophageal devascularization

Currently, a number of studies have suggested possible mechanisms of PSVT formation following splenectomy with gastroesophageal devascularization, including elevated postoperative platelet count, hemodynamic changes in splenic and portal veins, endothelial damage, spleen size, postoperative release of procoagulant factors, the reduction of anticoagulant factors, and the postoperative use of hemostatic drugs^[10,25,26]. PSVT may lead to severe clinical adverse events or poor outcomes^[3,4]. Therefore, the importance of PSVT prevention has been gradually recognized and emphasized^[19-21,27].

Various prevention protocols have been proposed^[25,28,29], but the effectiveness of these protocols varied^[4,22,30] because the duration and doses of these drugs were variable. Therefore, there was no generally acceptable PSVT prophylactic regimen for all patients^[22,23]. There are many blood coagulation factors involved in the formation of thrombosis^[31]. Xa is a major factor in the procedure of thrombosis^[32,33]. LMWH can suppress factor Xa by combining with ATIII to depress the activation of thrombin and formation of thrombosis^[34]. LMWH is safer than heparin because of its lower molecular weight, weaker inhibition of factor IIa and longer impact on co-

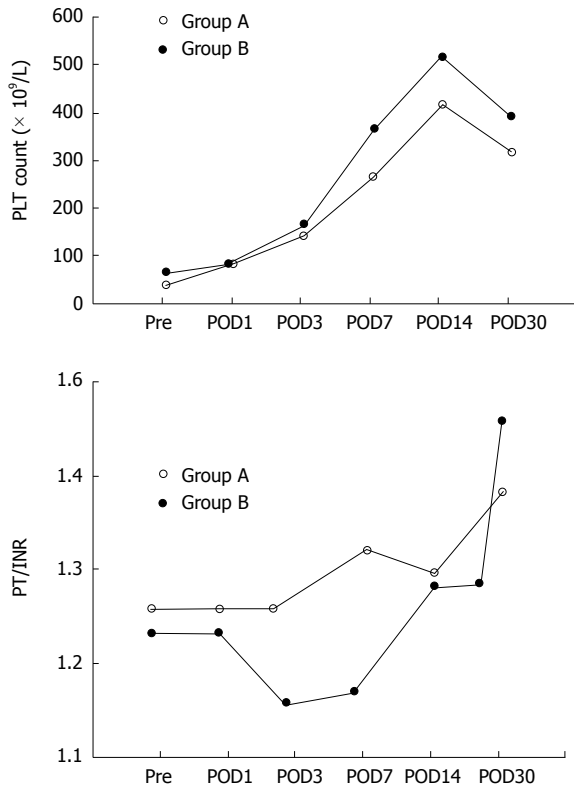


Figure 1 Changes in platelet count and prothrombin time/international normalized ratio in group A and group B. It was not different in platelet (PLT) between the two groups ($P = 0.981$), but it was statistically different in prothrombin time/international normalized ratio (PT/INR) between the two groups ($P = 0.020$). PT/INR in group A had no sequential changes pre- and post-operatively ($P = 0.479$) and PT/INR in group B was increasing gradually from postoperative day 7 with statistical difference ($P = 0.003$). POD: Postoperative day.

agulation system^[35,36].

The increased count and augmented aggregation competence of PLT after operation were important factors related to PSVT^[25]. These factors should be taken into account when selecting anticoagulation drugs. Aspirin has been applied in prevention and treatment of thrombotic diseases with satisfied safety because of its anti-PLT aggregation competence^[37,38]. Also, warfarin is an important antagonist of vitamin K (VK) with powerful anticoagulation effects by inhibiting VK-dependent coagulation factors such as II, VII, IX, X^[39,40]. We selected LMWH, warfarin and aspirin as a regular PSVT prevention regimen, which targets the major factors of mechanisms of PSVT. It was reported that the median interval between splenectomy and PSVT was 1-2 wk after surgery^[5,22,23,41].

Therefore, we used LMWH for 2-5 d followed by oral warfarin and aspirin after surgery in group B. Oral warfarin or aspirin was merely applied to those postoperative patients who were not suitable for LMWH in early postoperative phase as an irregular PSVT prevention regimen in group A.

In this study, we collected and analyzed retrospectively the data about the thrombosis prevention regimens for cirrhotic patients after splenectomy with gastroesophageal devascularization who were treated at our de-

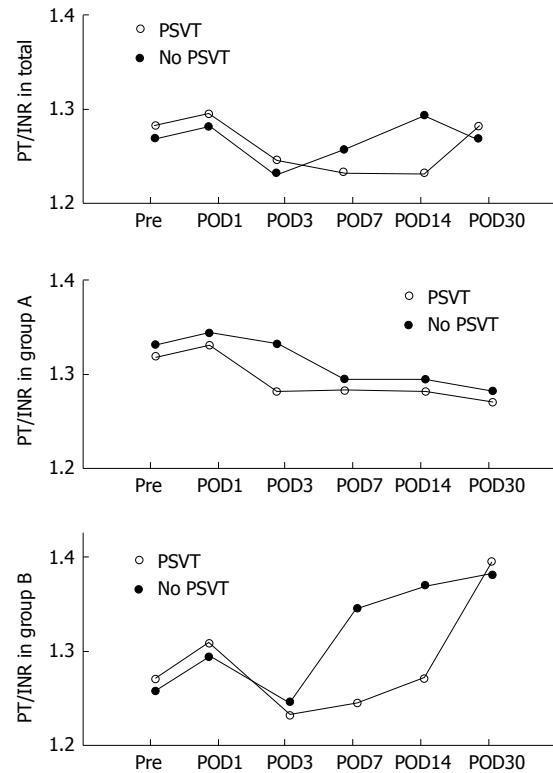


Figure 2 Changes in prothrombin time/international normalized ratio in patients without portal or splenic vein thrombosis and portal or splenic vein thrombosis. In group A, patients with or without portal or splenic vein thrombosis (PSVT) had similar prothrombin time/international normalized ratio (PT/INR), and the difference was not significant between the two subgroups. Patients with or without PSVT presented different PT/INR only at day 14 after surgery, with statistical significance (1.23 ± 0.17 vs 1.30 ± 0.21 , $P = 0.037$) by Student's *t* test. In group B, patients with or without PSVT had different PT/INR only at day 14 after surgery, with statistical significance (1.18 ± 0.14 vs 1.28 ± 0.21 , $P = 0.017$) by Student's *t* test. POD: Postoperative day.

partment over the past six years. Our results showed that the early use of LMWH followed by a long-term maintenance therapy with warfarin and aspirin could reduce the incidence of PSVT in group B (20.94%) without causing major bleeding complications, as compared with group A (41.17%) and other studies (51.5%-55%)^[5,6]. We also compared the patients with and without PSVT and found that the PT/INR in patients without PSVT was greater than those with PSVT at day 14 after surgery. Similar results were found in patients with and without PSVT in group B, suggesting that our anticoagulant regimen with the PT/INR target value set at 1.25-1.5 was reasonable and effective.

Classification and treatment of PSVT

PSVT in patients with portal hypertension due to cirrhosis following splenectomy with gastroesophageal devascularization can have various clinical manifestations, from no symptoms in mild cases to life-threatening recurrence of upper GI bleeding or small bowel necrosis in serious cases^[29]. The difference in clinical consequences stems from the location of PSVT, degree of obstruction, and its impact on portal vein (PV) hemodynamics. In fact, grade IV PSVT (Yerdel's classification^[42]), which involved

PV and superior mesenteric vein (SMV), would result in significant reduction in venous blood return to the liver and elevation in PVP, leading to esophageal and gastric variceal rupture and bleeding. SMV thrombosis can impede venous blood return from the bowels and cause small bowel necrosis, which could be vital for patients.

In this study, all 94 PSVT cases were grade I, which led to almost no clinical manifestations, such as variceal bleeding recurrence and bowel necrosis, and were easy to treat. Thrombolytic therapy has been proven to be effective for acute PSVT. Previous studies have recommended early and timely thrombolytic anticoagulant therapy^[43,44] and commonly used drugs, including urokinase, recombinant tissue plasminogen activator (rt-PA), LMWH, warfarin and dipyridamole. Intervention can be categorized by the administration route, i.e., systemic, portal system, and intravascular interventional treatment^[45,46]. Krauth *et al.*^[23] reported that immediate thrombolytic anticoagulant therapy in PSVT patients can achieve a complete dissolution rate of 63.3% and a partial dissolution rate of 13.3%. In our study, thrombolytic therapy *via* the peripheral venous route was administered in all 94 patients with PSVT, among whom 18 patients showed no sign of thrombus dissolution, but 61 patients (64.89%) achieved complete dissolution and 15 patients (15.95%) had partial dissolution. The overall thrombolytic effectiveness in our study was similar to other studies mentioned above. These phenomena were related to the benefit of regular anticoagulant therapy, which confirmed the clinical value of the anticoagulant regimen.

Our findings suggested that regular anticoagulation and early thrombolytic therapy are safe, effective and rational for PSVT patients who had portal hypertension and underwent splenectomy with gastroesophageal devascularization. At present, splenectomy with gastroesophageal devascularization may only be used as a bridge to liver transplantation in cirrhotic patients, timely prevention and thrombolytic treatment for PSVT offers a significant clinical value in terms of facilitating the portal venous reconstruction of the recipients for liver transplantation^[47].

Dilemma between anticoagulation and bleeding

Theoretically, preventive use of anticoagulants against PSVT in cirrhotic portal hypertensive patients early after surgery would counter the dilemma of bleeding. But in fact, previous studies have demonstrated that early anticoagulant treatment in these patients is a safe and effective protocol to prevent PSVT^[48,49]. Based on our study, the regular monitoring during anticoagulant treatment is necessary, which can guarantee the safety and maintain the PT/INR level between 1.25 and 1.5. Because most of our subjects had end-stage HBV cirrhosis, our suggestion differs from previous studies on non-HBV cirrhotic patients, mostly with hematologic and metabolic disorders and alcoholic cirrhosis, for which, PT/INR value of 2-3 is recommended^[50]. In this study, only three patients presented with mild GI bleeding during the anticoagulant treatment. We immediately terminated it and

shifted to symptomatic treatment such as hemostatic agents. Bleeding was successfully controlled, and the patients were discharged uneventfully. Therefore, our anticoagulant therapy has been proven safe.

On the other hand, there is a lack of large-scale controlled and long-term studies on the prevention of PSVT in cirrhosis patients receiving splenectomy with gastroesophageal devascularization for splenomegaly and hypersplenism. Since a randomized prospective controlled study is difficult to perform, we choose to conduct this retrospective controlled study. However, randomized controlled trial is necessary in the future.

Complications of splenectomy with gastroesophageal devascularization

Overwhelming postsplenectomy infection (OPSI) syndrome is associated with a high mortality, even it is a rare condition. Major risk factors include the age of the patients with splenectomy, the time after splenectomy, the reason for splenectomy, and the overall immune status of the patients. Splenectomy performed for hematological disorders, including thalassemia, hereditary spherocytosis, autoimmune hemolysis, immune thrombocytopenic purpura, or lymphoma, appears to carry a higher OPSI risk than splenectomy performed as a result of other diseases. Treatment of OPSI is generally aggressive due to the serious nature of the condition and associated mortality. The major preventive strategy is the vaccination using the 23-valent pneumococcal polysaccharide vaccine, a 7-valent proteinconjugated pneumococcal vaccine, the hemophilus influenzae type B vaccine, and the meningococcal vaccine^[51]. Fortunately, there was no OPSI occurrence in our study. The reasons may be that all the patients are adults and no splenectomy was performed for hematological disorders, the antibiotic agents were administered for 3-5 d after surgery, and the follow-up period was too short for OPSI.

In summary, the early and regular initiation of anticoagulant treatment using LMWH followed by warfarin and aspirin has been proven safe and effective in early prevention of PSVT in patients with cirrhotic portal hypertension, undergoing splenectomy with gastroesophageal devascularization. The treatment can reduce the incidence of PSVT in the early stage after splenectomy with gastroesophageal devascularization, early and timely thrombolytic therapy is imperative and feasible for the prevention and treatment of PSVT. The protocol presented in our study may benefit the patients not only for approximate and long-term clinical outcome, but also for potential liver transplant candidates in the future. But a better designed randomized prospective study with a longer follow-up period is still needed to clarify our conclusions.

COMMENTS

Background

Portal or splenic vein thrombosis (PSVT) is a common and potentially life-threatening complication of splenectomy with gastroesophageal devascularization for portal hypertension due to cirrhosis, which may lead to further deteriora-

tion of liver function and the recurrence of upper gastrointestinal (GI) bleeding and significantly affects patient's life expectancy. In some severe cases, PSVT may be contraindicated for liver transplantation. It is therefore very important to prevent the occurrence of PSVT after splenectomy with gastroesophageal devascularization to achieve better long-term outcomes and following possible liver transplantation if required.

Research frontiers

Prophylactic anticoagulation therapy using a combination protocol of low molecular weight heparin (LMWH), vitamin K antagonists, such as warfarin and aspirin, is a prime method to prevent PSVT. But the duration and doses of these drugs were variable according to the literature, there was no generally acceptable PSVT prophylactic regimen for all patients after splenectomy with gastroesophageal devascularization. So, a safe and effective prophylactic anticoagulation protocol is needed.

Innovations and breakthroughs

In order to reduce the incidence of PSVT after splenectomy with gastroesophageal devascularization, a regular prophylactic anticoagulation protocol was established with a confirmed monitoring index. This study used the combined and sequential application of LMWH, warfarin, aspirin and ticlopidine according to the regular coagulating function test and color Doppler flow imaging. The incidence and severity of PSVT caused by anticoagulation therapy were reduced, without causing major bleeding complications.

Applications

The study results suggest that prophylactic anticoagulation therapy using LMWH, warfarin and aspirin regularly is a safer and more effective method for PSVT prevention.

Terminology

PSVT is a sort of clinical disease caused by thrombus development in the portal vein system. The major causes of PSVT included the reduced blood flow, the increased platelet count, the injured vessel endothelium and enhanced coagulation function. PSVT is a common complication of abdominal surgery, especially after splenectomy with gastroesophageal devascularization with a high incidence and morbidity.

Peer review

This is a good descriptive study in which authors analyze the preventive effect of prophylactic anticoagulation therapy using low molecular weight heparin, warfarin and aspirin for PSVT. The results are interesting and imply that a regular prophylactic anticoagulation protocol is a safer and more effective method that could be used in preventing PSVT after splenectomy with gastroesophageal devascularization.

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Uncoupling protein 2 regulates glucagon-like peptide-1 secretion in L-cells

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Abstract

AIM: To investigate whether uncoupling protein 2 (UCP2) affects oleic acid-induced secretion of glucagon-like peptide-1 (GLP-1) in L-cells.

METHODS: mRNA and protein expression of UCP2 were analyzed in human NCI-H716 cells, which serve as a model for enteroendocrine L-cells, by quantitative reverse transcription-polymerase chain reaction and Western blotting before and after treatment with oleic acid. Localization of UCP2 and GLP-1 in NCI-H716 cells was assessed by immunofluorescence labeling. NCI-H716 cells were transiently transfected with a small interfering RNA (siRNA) that targets UCP2 (siUCP2) or with a non-specific siRNA using Lipofectamine 2000. The concentrations of bioactive GLP-1 in the medium were measured by enzyme linked immunosorbent assay.

RESULTS: Both GLP-1 and UCP2 granules were expressed mainly in the cytoplasm of NCI-H716 cells. NCI-H716 cells that secreted GLP-1 also expressed UCP2. Time-course experiments revealed that release

of GLP-1 from NCI-H716 cells into the medium reached a maximum at 120 min and remained stable until at least 180 min after treatment with oleic acid (the level of GLP-1 increased about 2.3-fold as compared with the level of GLP-1 in the control cells, $P < 0.05$). In an experiment to determine dose dependence, stimulation of NCI-H716 cells with ≤ 8 mmol oleic acid led to a concentration-dependent release of GLP-1 into the medium; 10 mmol oleic acid diminished the release of GLP-1. Furthermore, GLP-1 secretion induced by oleic acid from NCI-H716 cells that were transfected with si-UCP2 decreased to 41.8%, as compared with NCI-H716 cells that were transfected with a non-specific siRNA ($P < 0.01$).

CONCLUSION: UCP2 affected GLP-1 secretion induced by oleic acid. UCP2 plays an important role in L-cell secretion that is induced by free fatty acids.

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Key words: Glucagon-like peptide-1; L-cell; NCI-H716 cells; Oleic acid; Uncoupling protein 2

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INTRODUCTION

Glucagon-like peptide-1 (GLP-1) which is released from L-cells in the intestine plays an important role in post-prandial glucose homeostasis^[1]. With the recent advent

of anti-diabetic drugs aimed at either mimicking GLP-1 or preventing its degradation, researchers have turned their attention toward the L-cell and have focused on determining whether it would be both possible and beneficial to stimulate the endogenous release of GLP-1. This approach requires an understanding of the mechanisms underlying GLP-1 release from L-cells.

GLP-1 is involved in the regulation of nutrient homeostasis through insulinotropic effects on the β -cell as well as inhibition of glucagon release and gastric emptying and induction of satiety^[1,2]. Most L-cells that secrete GLP-1 are located in the distal small intestine and colon^[3]. GLP-1 release from L-cells is regulated by nutrient ingestion and demonstrates a biphasic pattern of secretion in response to either mixed meals or carbohydrate or fat alone^[4,5]. The first phase of GLP-1 secretion is regulated by neural signals originating in the proximal intestine, within minutes after a meal^[6-8]. In contrast, the second phase is induced by direct nutrient stimulation of the L-cells, resulting in secretion of GLP-1^[9-11]. In addition, *in vitro* studies using primary rat L-cells in culture have shown that the GLP-1 response to fat is highly specific, requiring mono-unsaturated fatty acids (MUFAs) with a chain length of 16 or more carbons (e.g., palmitoleic acid, 16:1 or oleic acid, 18:1). Mono-unsaturated long-chain fatty acids, such as oleic acid, are strong stimulators of GLP-1 secretion from L-cells^[9].

Uncoupling protein 2 (UCP2), a member of the UCP family, is located in the inner mitochondrial membrane and induces proton leakage and regulates the production of reactive oxygen species (ROS)^[12-14]. UCP2 plays an important role in α -cell dysfunction that is induced by free fatty acids (FFAs) *in vitro*, which may be related to its effects on oxidative stress and the insulin signaling pathway^[15]. UCP2 is also involved in the effect of FFAs on β cells, as FFAs stimulate both the expression and activity of UCP2 in α cells^[15-17]. When FFA stimulates UCP2 over-expression, the negative effects of UCP2 on ATP production result in the suppression of insulin secretion in β cells^[18]. Long-term exposure to FFAs also stimulates the release of glucagon and GLPs in a time- and dose-dependent manner in α cell lines^[19]. UCP2 is also believed to be involved in this effect in α cells. A chronic high concentration of FFAs impairs the function of both β and α cells, and this impairment involves UCP2^[16]. It is not, however, known whether UCP2 has similar effects on L-cells. In this study, we investigated the effect of oleic acid on the expression of UCP2 in human NCI-H716 cells, an L-cell model, and the effect of UCP2 down-regulation on GLP-1 secretion in these cells.

MATERIALS AND METHODS

NCI-H716 cell culture and secretion studies

Human enteroendocrine NCI-H716 cells were maintained in suspension culture as described by the American Type Culture Collection. Two days before an experiment, cells were seeded into 12-well culture plates precoated

with Matrigel as described^[20]. On the day of the experiment, the medium was replaced by PBS containing 1 mmol CaCl₂ and dipeptidyl peptidase IV inhibitor, adjusted to pH 7.2 (Millipore Corporation, Bedford, MA, United States). Cells were incubated at 37 °C for 2 h with or without different oleic acid concentrations (0, 2, 4, 6, 8, 10 mmol) and RNA interference targeting UCP2 (see below). GLP-1 was measured by enzyme linked immunosorbent assay (ELISA) (see below).

Small interfering RNA preparation and NCI-H716 cell transfection

The siRNA sequences targeting UCP2 (siUCP2) and a non-specific control small interfering RNA (siRNA) were purchased from Shanghai Shinegene Molecular Biotechnology Co., Ltd. (Shanghai, China). NCI-H716 cells were 70% confluent in 250 μ L of complete RPMI 1640 medium. Transfections of siRNA (at a final concentration of 300 nmol) were performed in NCI-H716 cells using Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, United States). Cells were usually examined 48 h after transfection.

Localization of uncoupling protein-2 and GLP-1 in NCI-H716 cells

The antibodies used for immunofluorescence staining in NCI-H716 cells were anti-GLP-1 (C-17; Santa Cruz) and anti-UCP2 (LS-B1911; LifeSpan Bio-Sciences). Cells were grown on coverslips, fixed in 4% paraformaldehyde in PBS for 10 min, washed with PBS, and cooled in 100% methanol at -20 °C for 20 min. Cells were then washed with PBS and permeabilized with 0.1% Triton X-100 for 10 min. After blocking with Dako blocking solution, primary antibody (anti-GLP-1; 1:100) was added and incubated at 4 °C overnight. For the secondary antibody, Cy3-conjugated donkey anti-goat (1:200; red, Beyotime Institute of Biotechnology) was incubated for 30 min at room temperature in the dark. The cells were washed with phosphate buffer saline (PBS), and anti-UCP2 (1:50) was added and incubated at 4 °C overnight. For the secondary antibody, fluorescein isothiocyanate-conjugated goat anti-rabbit (1:200; green, Beyotime Institute of Biotechnology) was added for 30 min at room temperature in the dark. Cells were then incubated for 45 min with Hoechst stain to counterstain nuclei. After a final wash with PBS, the coverslips were mounted with Slow-Fade Antifade kit (Molecular Probes).

RNA isolation and quantitative reverse transcription-polymerase chain reaction

Reagents used for RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) included TRIzol Reagent, oligo(dT)₁₈ primers, and AMV reverse transcriptase (all from Invitrogen, Carlsbad, CA, United States) and primers and TaqMan probes (Shanghai Shinegene Molecular Biotechnology). Total cellular RNA was extracted with TRIzol Reagent. The purity and concentration of RNA was determined by measuring

the absorbance at 260 and 280 nm, with $A_{260}/A_{280} > 1.7$ considered sufficient purity^[21]. RNA (1 µg) was reverse-transcribed into cDNA using oligo(dT)₁₈ primers at 42 °C for 1 h. Quantitative RT-PCR was performed with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, United States) using TaqMan probes; threshold cycle numbers were obtained by ABI Prism 7000 SDS software, version 1.0. The primer and probe sequences used in this study were as follows: UCP2 gene (sense primer, 5'-CCAATGTTGCCCGWAATG-3'; antisense primer, 5'-TGAGGTTGGCTTTCAGGAG-3'; probe, 5'-FAM+CTGGTGACCTATGACCTCATCAAAG-3'); β -actin gene (sense primer, 5'-GGCACCACACYTTCCTACAATG-3'; antisense primer, 5'-GGGGGTGTGAAGGTCTCAAAAC-3'; probe, 5'-FAM+TGTGGCCCTGAGGAGCACCC-3').

Amplification conditions were one cycle at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 1 min, and one final cycle at 72 °C for 4 min.

Western blot analysis

Mitochondrial proteins from NCI-H716 cells were isolated with 1 mL of extraction buffer (250 mmol sucrose; 1 mmol ethylenediaminetetraacetic acid; 10 mmol Tris, pH 7.4) supplemented with protease inhibitors (Sigma). The mixture was centrifuged at $800 \times g$ for 10 min. The supernatant was centrifuged at $10\,000 \times g$ for 10 min, and the mitochondrial pellet was resuspended in 25 µL of N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer. Mitochondrial protein concentration was determined colorimetrically with the BCA Protein Assay (Pierce, Rockford, IL, United States). Mitochondrial proteins (15 µg) were mixed with $3 \times$ sample buffer [0.5 mol phosphate buffer, pH 7.0; 30% (w/v) glycerol; 7.5% (w/v) SDS; 0.75 mmol bromophenol blue], boiled for 5 min, and electrophoresed on an SDS-PAGE gel (12.5% acrylamide). Proteins were then transferred to Immobilon PVDF membranes (Millipore). UCP2 proteins were detected with polyclonal anti-UCP2 (LifeSpan Bio-Sciences) at a dilution of 1:600 followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz) at a dilution of 1:2000 and detection with enhanced chemiluminescence (ECL detection system; NEN, Boston, MA, United States). To validate equal protein loading across various lanes, PVDF membranes were stripped and re-probed with polyclonal anti-cytochrome c (Santa Cruz Biotechnology) at a dilution of 1:1000.

GLP-1 measurement

The level of active GLP-1, GLP-1 (7-36) amide, was measured with an ELISA kit (Linco). This assay relies on a monoclonal antibody fixed in a coated micro-well plate that binds to the N-terminal region of active GLP-1. The concentration of active GLP-1 is proportional to the fluorescence generated by umbelliferone, which is produced by alkaline phosphatase-catalyzed hydrolysis of methyl umbelliferyl phosphate (conjugated to GLP-1 monoclonal

antibodies). Samples of the cell culture medium were collected, and dipeptidyl peptidase IV inhibitor (10 µL/mL) was added to prevent GLP-1 degradation. Samples (100 µL each) were added to individual assay wells. The ELISA has a working range of 2 to 100 pmol/L (according to Linco GLP-1 ELISA kit). Each sample was replicated three times.

Statistical analysis

Data are presented as the mean $\pm$ SE. Statistical significance was calculated by a one-way ANOVA and unpaired two-tailed *t* test. *P* values < 0.05 were regarded as significant.

RESULTS

Localization of uncoupling protein-2 and GLP-1 in NCI-H716 cells

The localization of UCP2 and GLP-1 was studied in the human cell line NCI-H716, which serves as a model for intestinal L-cells. Immunofluorescence staining for GLP-1 was observed in the cytoplasm in NCI-H716 cells (Figure 1A1 and A2). Immunofluorescence staining for UCP2 was also observed in the cytoplasm in human NCI-H716 cells (Figure 1B1 and B2). Both GLP-1 and UCP2 granules were expressed mainly in the cytoplasm. Thus L-cells that expressed GLP-1 were also able to express UCP2. Merged picture, yellow staining shows co-expression of GLP-1 and UCP2 (Figure 1C1 and C2).

Oleic acid increases GLP-1 secretion in NCI-H716 cells

The possible effect of UCP2 on the secretion of GLP-1 which is induced by oleic acid in the intestinal enteroendocrine L-cell was investigated. Time-course experiments revealed that GLP-1 release from NCI-H716 cells into the medium peaked 120 min after treatment with oleic acid (the release of GLP-1 increased approximately 2.3-fold of the baseline value by treatment with oleic acid; $P < 0.05$) and maintained a stable plateau until at least 180 min after treatment (Figure 2A). In a dose-dependent experiment, oleic acid significantly increased GLP-1 secretion to a maximum of 2.5 ± 0.3 -fold of baseline levels at 8 mmol ($P < 0.01$); 10 mmol oleic acid led to a less robust increase in GLP-1 expression (Figure 2B). These results indicated that oleic acid led to a concentration-dependent release of GLP-1 from NCI-H716 cells (final concentration of oleic acid ≤ 8 mmol).

Oleic acid increases the expression of uncoupling protein-2 in NCI-H716 cells

We then determined the effect of oleic acid on the expression of UCP2 in NCI-H716 cells. Based on our time-course and dose-dependency results for GLP-1 expression, we pretreated NCI-H716 cells with 8 mmol oleic acid for 2 h and then collected the cells. qRT-PCR for UCP2 mRNA transcripts and western blot analysis for UCP2 protein were carried out on the isolated total RNA and mitochondrial proteins from these cells, respectively. The UCP2 mRNA level in NCI-H716 cells increased 3-fold after

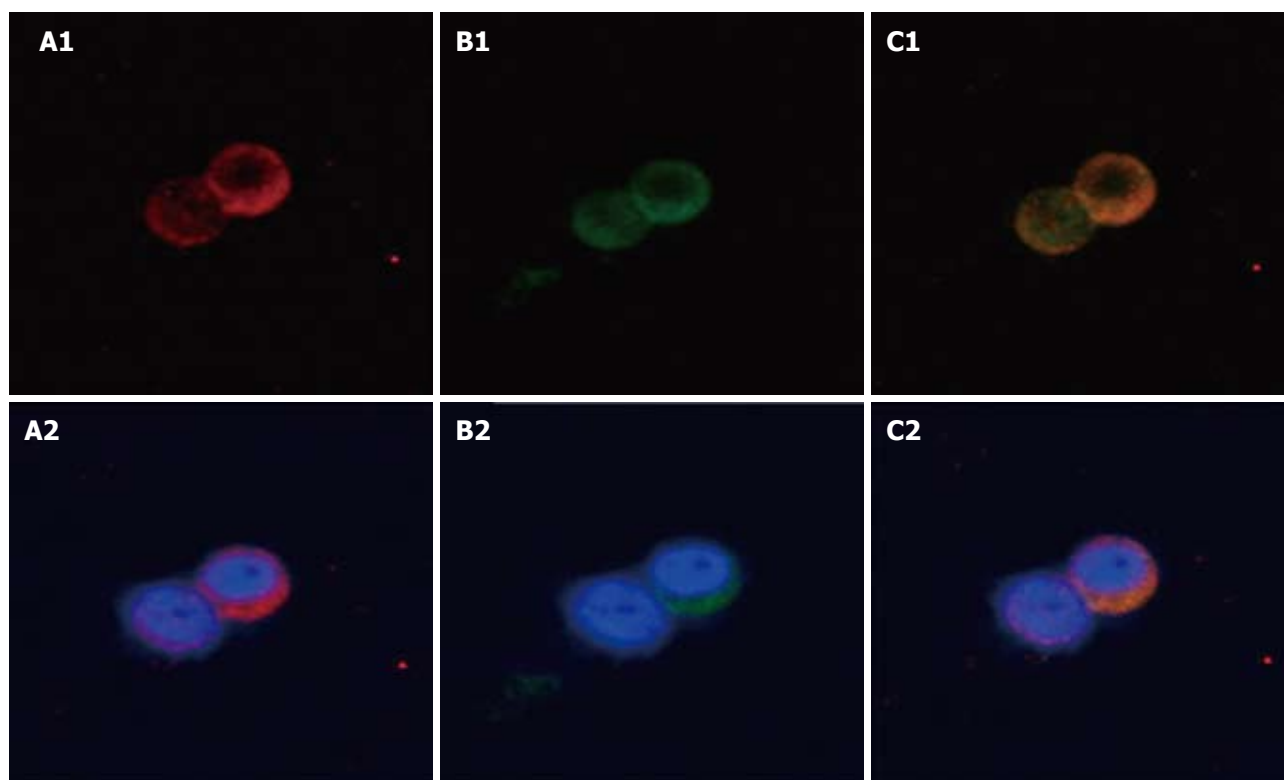


Figure 1 Localization of uncoupling protein-2 and glucagon-like peptide-1 in NCI-H716 cells. Immunofluorescence staining in human NCI-H716 cells which serve as a model for L-cells. A1, A2: Glucagon-like peptide-1 (GLP-1) antibody staining (red); B1, B2: Uncoupling protein-2 (UCP2) antibody staining (green); C1, C2: Merged image shows co-expression of UCP2 and GLP-1. Blue (Hoechst) staining indicates the nuclei. Original magnification, $\times 400$.

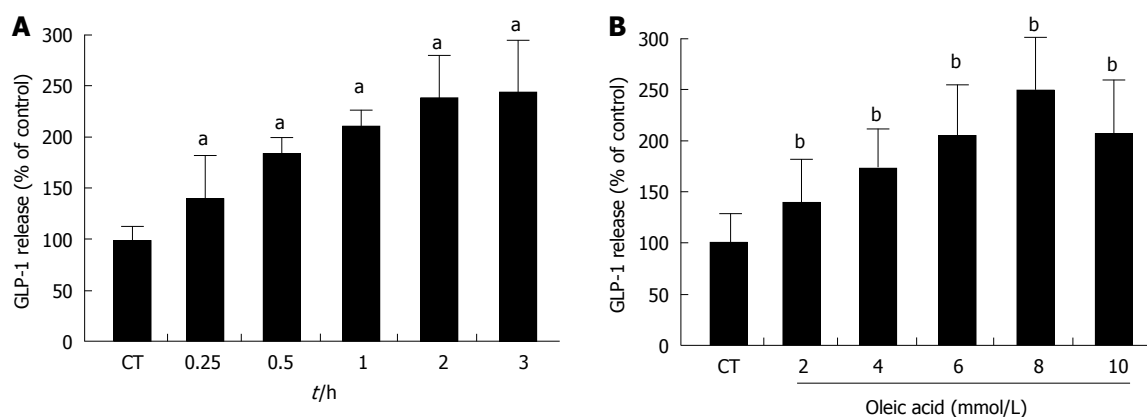


Figure 2 Glucagon-like peptide-1 secretion in NCI-H716 cells after treatment with oleic acid. A: Time course of oleic acid-induced release of glucagon-like peptide-1 (GLP-1) from NCI-H716 cells. NCI-H716 cells were incubated with 8 mmol oleic acid, and the culture medium was collected at 0.25, 0.5, 1, 2, and 3 h to detect GLP-1 levels; B: Dose-dependent oleic acid-induced release of GLP-1 from NCI-H716 cells. NCI-H716 cells were incubated with 2, 4, 6, 8, and 10 mmol oleic acid for 2 h. The culture medium was then analyzed for GLP-1 concentration. NCI-H716 cells without oleic acid exposure. ^a $P < 0.05$, ^b $P < 0.01$ vs baseline value.

treatment with 8 mmol oleic acid (Figure 3A). The UCP2 protein level also increased 1.8-fold *vs* the control amount (Figure 3B). These results indicated that oleic acid increased the expression of UCP2 in NCI-H716 cells at the transcriptional level.

Knocking down uncoupling protein-2 expression decreases oleic acid-induced secretion of GLP-1

To study the function of UCP2, a siRNA targeting UCP2 was used to specifically suppress UCP2 mRNA transla-

tion. Human NCI-H716 cells were left untransfected (blank), were mock transfected (no siRNA), or were transfected with siUCP2. After 48 h, mRNA levels of UCP2 were determined by qRT-PCR. The UCP2 mRNA level was reduced approximately 50% by siUCP2 as compared with the control level (Figure 4, up panel). There were no observable changes in the UCP2 mRNA level in mock-transfected or untransfected cells. The result from the western blot analysis also demonstrated that UCP2 protein was markedly reduced (Figure 4, up panel). Next,

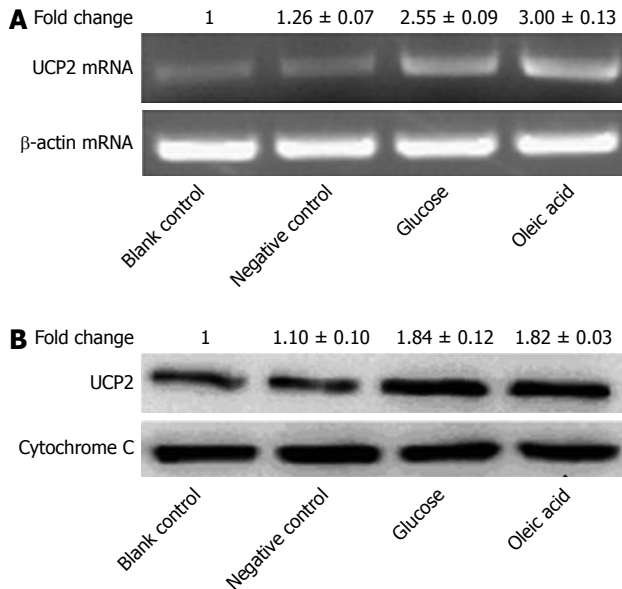


Figure 3 Expression of uncoupling protein-2 in NCI-H716 cells after treatment with oleic acid. A: Total RNA was extracted, and relative mRNA levels of uncoupling protein-2 (UCP2) were determined by quantitative reverse transcriptase polymerase chain reaction with normalization to β-actin ($P < 0.05$); B: UCP2 protein was detected by western blotting. Cytochrome C was used as the loading control ($P < 0.01$); Individual data points in this figure represent the mean ± SE of 12 determinants from four independently prepared samples each with three measurements. NCI-H716 cells were incubated with medium alone (blank control), medium containing 1% dimethyl sulfoxide (negative control), medium with 30 mmol glucose (glucose), and medium with 8 mmol oleic acid (oleic acid).

we treated NCI-H716 cells transfected with siUCP2 with 8 mmol oleic acid for 2 h. Cells that were incubated with medium alone were used as the blank control to determine baseline GLP-1 expression. Oleic acid-stimulated GLP-1 secretion decreased to 41.8% in NCI-H716 cells with siUCP2 compared to NCI-H716 cells with a non-specific siRNA ($P < 0.01$) (Figure 4, down panel).

DISCUSSION

The “incretin effect” of GLP-1 has been known for many years, and its role in the overall regulation of insulin release *in vivo* is well established. The regulation of GLP-1 secretion itself is, however, not well understood. GLP-1 is secreted from isolated perfused ileum preparations when glucose or fat is infused through the gut lumen^[22,23]. Nutrients that infuse directly into the ileal lumen can lead to the release of GLP-1^[24], indicating that direct stimulation of L-cell secretion is possible in humans. Oral glucose^[25-29], sucrose^[30], triglycerides^[27], and mixed meals also lead to an increase in plasma GLP-1^[31-34].

The mechanism of GLP-1 release is believed to involve a proximal-distal loop as well as a glucose sensor. Holst *et al.*^[35] proposed a proximal-distal loop to explain the phenomenon of GLP-1 release. This regulatory pathway involves nutrient detectors located in the upper regions of the gastrointestinal tract, which would control the release of GLP-1 from distal L-cells by neural or hormonal circuitry^[36]. Theodorakis *et al.*^[37] reported that the density

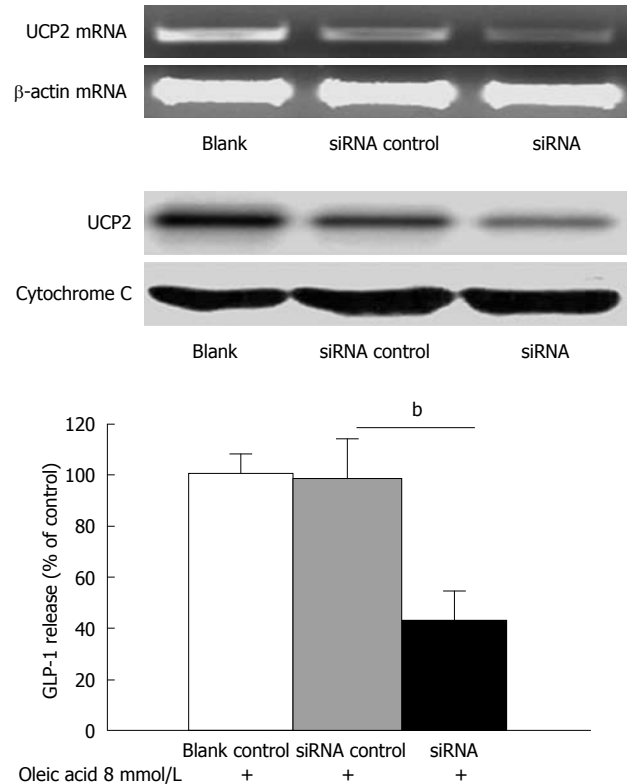


Figure 4 Knockdown of uncoupling protein-2 expression decreased oleic acid-induced secretion of glucagon-like peptide-1 in NCI-H716 cells. Up panel: The knock-down efficiency of small interfering RNA which targets UCP2 (siUCP2) in NCI-H716 cells. NCI-H716 cells were left untransfected (blank), were mock transfected (no siRNA), or were transfected with siUCP2. After 48 h, the mRNA levels of uncoupling protein-2 were determined as in Figure 4. Protein levels were determined as in Figure 4; Down panel: Effects of oleic acid on levels of secreted glucagon-like peptide-1 (GLP-1). NCI-H716 cells were transfected as described above and were then incubated with 8 mmol oleic acid for 2 h. GLP-1 concentrations were determined as in Figure 4. ^b $P < 0.01$ vs siRNA control.

of L-cells in the human duodenum may be sufficiently high to account for the early phase of GLP-1 secretion. With respect to the glucose sensor, a variety of signaling mechanisms have been proposed to explain how L-cells might sense glucose, including ATP-sensitive potassium channel closure, sodium glucose cotransporter activity, and activation of sweet taste receptors^[19,36]. Fats and protein are other well-known stimuli of GLP-1 release *in vivo*^[5,27].

G protein-coupled receptors (GPCRs) are regarded as potentially important components of signaling pathways in L-cells, as they may underlie the regulation of L-cells by certain neurotransmitters and hormones^[23,37,38], as well as by some luminal nutrients. One potential mechanism by which lipids, fatty acids, and bile acids might stimulate GLP-1 release is *via* activation of specific GPCRs on L-cells, such as GPR40, GPR120, GPR119, and TGR5. GPRs 40 and 120 are responsive to long-chain unsaturated fatty acids^[39,40]. GPR119 and TGR5 are responsive to oleoylethanolamide and bile acids, respectively^[41,42]. There is also convincing evidence that activation of protein kinase C- ζ contributes to the stimulatory action of fatty acids on GLP-1 release^[43]. These do not, however, explain the mechanism of GLP-1 release completely. Thus,

there may be other fatty acid-responsive pathways.

Chronic exposure of pancreatic islet cells to FFAs blunts glucose-stimulated insulin secretion and is accompanied by elevated levels of UCP2^[12]. UCP2 expression is regulated in tandem with the level of circulating FFAs^[44]. FFAs that increase as a result of fasting or a high-fat diet lead to UCP2 expression in adipose tissue and muscle^[45-47]. In isolated rat islets and INS-1 pancreatic β cells, long-term treatment with FFAs can increase UCP2 mRNA^[48,49].

We found that the human L-cell model, NCI-H716 cells, also expressed UCP2 in the cytoplasm. Oleic acid stimulated GLP-1 secretion in NCI-H716 cells. Time-course experiments revealed that release of GLP-1 from NCI-H716 cells into the medium peaked at 120 min after treatment with oleic acid and maintained a stable plateau at least until 180 min after treatment (Figure 2A).

Whereas the stimulation of NCI-H716 cells with ≤ 8 mmol oleic acid led to a concentration-dependent increase in GLP-1 secretion into the medium, this increase was not maintained at 10 mmol (Figure 2B). It seemed that the higher concentration of oleic acid inhibited GLP-1 release from NCI-H716 cells. Lauffer *et al.*^[50] indicated that higher concentrations of oleoylethanolamide are associated with diminished GLP-1 release in all L-cell models which is suggestive of desensitization. Our results may also be related to desensitization.

Oleic acid also increased UCP2 mRNA levels in NCI-H716 cells. Furthermore, reduced UCP2 expression as a result of RNA interference decreased oleic acid-induced GLP-1 secretion (Figure 4). These results suggest that UCP2 is intimately involved in oleic acid-stimulated secretion of GLP-1 from intestinal endocrine L-cells. Understanding the mechanism responsible for this effect of UCP2 on GLP-1 secretion will require further investigation.

COMMENTS

Background

A chronic high concentration of free fatty acids (FFAs) impairs β cell function, as well as α cell function. This impairment involves uncoupling protein-2 (UCP2). Whether UCP2 has similar effects on intestinal L-cells is not clear. In this study, we investigated the effect of oleic acid, a FFA, on the expression of UCP2 in human NCI-H716 cells, which are a model for L-cells. We also analyzed the effect of UCP2 down-regulation on glucagon-like peptide-1 (GLP-1) secretion in these cells.

Research frontiers

Recent therapeutic successes of antidiabetic drugs aimed at either mimicking GLP-1 or preventing its degradation have focused attention on the L-cell and on addressing whether it would be both possible and beneficial to stimulate the endogenous release of GLP-1. However, the mechanism underlying GLP-1 secretion *in vivo* is still not fully understood.

Innovations and breakthroughs

To our knowledge, this is the first study to investigate the effect of oleic acid on the expression of UCP2 and the effect of UCP2 down-regulation on GLP-1 secretion in a model for human L-cells.

Applications

We have shown that UCP2 might be involved in a novel mechanism for regulating the secretion of GLP-1. This finding has potential benefit in finding new therapies for diabetes.

Terminology

UCP2 played an important role in α cell and β cell dysfunction. A chronic high

concentration of FFAs impairs the function of both β and α cells, and this impairment involves UCP2. The pancreatic endocrine β -cells are glucose-sensing cells and the intestinal endocrine L cells secreting GLP-1 are also glucose-sensing cells.

Peer review

The manuscript is a very interesting topic. The article is experimentally well prepared and effectively addresses the relationship between oleic acid and UCP2 and GLP-1. The research is very well described; the conclusions are applicable.

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Phosphoinositide-3-kinase, catalytic, alpha polypeptide RNA interference inhibits growth of colon cancer cell SW948

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Abstract

AIM: To investigate the gene knock-down effect by the phosphoinositide-3-kinase, catalytic, alpha polypeptide (*PIK3CA*)-targeted double-stranded RNA (dsRNA) and its effect on cell proliferation and cycle distribution in SW948.

METHODS: Two *PIK3CA*-targeted dsRNAs were constructed and transfected into SW948 cells. Transfections were performed using lipofectamine™ 2000. The transfection effectiveness was calculated basing on the rate of fluorescence cell of SW948 at 6 h after transfection. Total messenger RNA was extracted from these cells using the RNeasy kit, and semiquantitative reverse transcription polymerase chain reaction was performed

to detect the down-regulation of *PIK3CA*, *AKT1*, *MYC*, and *CCND1* gene expression. Cells were harvested, proteins were resolved, and western blot was employed to detect the expression levels of *PIK3CA*, *AKT1*, *MYC*, and *CCND1* gene. Cell proliferation was assessed by 3-(4,5)-dimethylthiazoliazol-2-yl-5-(3,4-dimethyl-5-phenyl-1,3,4-oxadiazol-2-yl)tetrazolium bromide assay and the inhibition rate was calculated. Soft agar colony formation assay was performed basing on colonies greater than 60 μ m in diameter at $\times 100$ magnification. The effect on cell cycle distribution and apoptosis was assessed by flow cytometry. All experiments were performed in triplicate.

RESULTS: Green fluorescence was observed in SW948 cell transfected with plasmid Pgenesil-1, and the transfection effectiveness was about 65%. Forty-eight hours post-transfection, mRNA expression of *PIK3CA* in SW948 cells was 0.51 ± 0.04 vs 0.49 ± 0.03 vs 0.92 ± 0.01 vs 0.93 ± 0.03 ($P = 0.001$) in Pgenesil-CA1, Pgenesil-CA2, negative and blank group respectively. mRNA expression of *AKT1* was 0.50 ± 0.03 vs 0.48 ± 0.01 vs 0.93 ± 0.04 vs 0.92 ± 0.02 ($P = 0.000$) in Pgenesil-CA1, Pgenesil-CA2, negative and blank group respectively. mRNA expression of *MYC* was 0.49 ± 0.01 vs 0.50 ± 0.04 vs 0.90 ± 0.02 vs 0.91 ± 0.03 ($P = 0.001$) in the four groups respectively. mRNA expression of *CCND1* was 0.45 ± 0.02 vs 0.51 ± 0.01 vs 0.96 ± 0.03 vs 0.98 ± 0.01 ($P = 0.001$) in the four groups respectively. The protein level of *PIK3CA* was 0.53 ± 0.01 vs 0.54 ± 0.02 vs 0.92 ± 0.03 vs 0.91 ± 0.02 ($P = 0.001$) in Pgenesil-CA1, Pgenesil-CA2, negative and blank group respectively. The protein level of *AKT1* in the four groups was 0.49 ± 0.02 vs 0.55 ± 0.03 vs 0.94 ± 0.03 vs 0.95 ± 0.04 , ($P = 0.000$). The protein level of *MYC* in the four groups was 0.51 ± 0.03 vs 0.52 ± 0.04 vs 0.92 ± 0.02 vs 0.95 ± 0.01 ($P = 0.000$). The protein level of *CCND1* in the four groups was 0.54 ± 0.04 vs 0.56 ± 0.03 vs 0.93 ± 0.01 vs 0.93 ± 0.03 ($P = 0.000$). Both Pgenesil-CA1 and Pgenesil-CA2 plasmids significantly suppressed the growth of SW948 cells when compared with the negative or blank group at 48 h after transfection.

tion (29% *vs* 25% *vs* 17% *vs* 14%, $P = 0.001$), 60 h after transfection (38% *vs* 34% *vs* 19% *vs* 16%, $P = 0.001$), and 72 h after transfection (53% *vs* 48% *vs* 20% *vs* 17%, $P = 0.000$). Numbers of colonies in negative, blank, CA1, and CA2 groups were 42 ± 4 , 45 ± 5 , 8 ± 2 , and 10 ± 3 , respectively ($P = 0.000$). There were more than 4.5 times colonies in the blank and negative control groups as there were in the CA1 and CA2 groups. In addition, the colonies in blank and negative control groups were also larger than those in the CA1 and CA2 groups. The percentage of cells in the CA1 and CA2 groups was significantly higher in G₀/G₁ phase, but lower in S and G₂/M phase when compared with the negative and control groups. Moreover, cell apoptosis rates in the CA1 and CA2 groups were 5.11 ± 0.32 and 4.73 ± 0.32 , which were significantly higher than those in negative (0.95 ± 0.11 , $P = 0.000$) and blank groups (0.86 ± 0.13 , $P = 0.001$). No significant difference was found between CA1 and CA2 groups in cell cycle distribution and apoptosis.

CONCLUSION: *PIK3CA*-targeted short hairpin RNAs can block the phosphoinositide 3-kinase-Akt signaling pathway and inhibit cell growth, increase apoptosis, and induce cell cycle arrest in the *PIK3CA*-mutant colon cancer SW948 cells.

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Key words: Phosphoinositide-3-kinase, catalytic, alpha polypeptide; RNA interference; Colon cancer; Phosphoinositide-3-kinase pathway

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INTRODUCTION

Colon cancer is the fifth most common cancer in the United States, and the third leading cause of cancer-related death in the Western world. The phosphoinositide 3-kinase (PI3K)/Akt signaling transduction pathway is believed to play an important role in carcinogenesis. PI3K is a major signaling component downstream of growth factor receptor tyrosine kinases (RTKs), which may serve as a potential target for colon cancer therapy^[1,2]. PI3K is a heterodimer consisting of a regulatory subunit (p85) and a catalytic subunit (p110). The subtype p110a encoded by the gene phosphoinositide-3-kinase, catalytic, alpha

polypeptide (*PIK3CA*) is very important in phosphorylating phosphatidylinositol (4,5)-bisphosphate (PIP₂) to the lipid second messenger PIP₃, and PIP₃ in turn functions in the recruitment and activation of a wide range of downstream targets, including the serine-threonine protein kinase Akt. Gene reactions distal to the PI3K-Akt signaling pathway contribute to tumor cell proliferation, cell cycle progression, energy metabolism, and resistance to apoptosis^[3-6]. Troxell *et al*^[7] found *PIK3CA* mutations were identified in 13/24 breast columnar cell lesions (54%) and 3/8 invasive carcinomas (37%). The high prevalence of *PIK3CA* mutations in breast cancer is an emerging tumor marker which might become used in treatment-choosing process^[8]. Higgins *et al*^[9] detected the *PIK3CA* status in metastatic breast cancer using peripheral blood, and found plasma level of *PIK3CA* presented predictive biomarkers of response to targeted therapies. *PIK3CA* mutations were detected in 18% patients with breast and gynecologic malignancies, and cases with *PIK3CA* mutations treated with its inhibitors demonstrated a higher response rate than patients without mutations^[10]. Studies demonstrated *PIK3CA* mutations were not common, but its amplification was very common in gastric cancer, Akt (p-Akt) was often functionally linked to tumor progression and metastasis in gastric cancer, and double-stranded RNA (dsRNA) mediated targeting of *PIK3CA* may specifically knockdown the expression of *PIK3CA*, providing a potential implication for therapy of cancer^[11-13].

RNA interference (RNAi) is a potent gene-silencing tool, which is triggered by the introduction of dsRNA. Degradation of mRNA homologous to dsRNA can cause a post-transcriptional gene-silencing effect. Recently, the vector-based RNAi has been developed in order to achieve long-term and stable effects. Short hairpin RNA (shRNA) is formed by hairpin structures and stretches of dsRNA. After being cleaved by a ribonuclease dicer, it becomes mature microRNA (miRNA) inside the targeted cells^[14-17].

In our study, two shRNA plasmid vectors were constructed against the gene *PIK3CA*, and were transfected into the colon cancer cell line SW948. Their effects on cell proliferation and cycle distribution in this cell line were investigated.

MATERIALS AND METHODS

Cell line and culture

The human colon carcinoma cell line SW948 was routinely grown in Leibowitz's L-15 medium supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37 °C in a humidified 5% CO₂/95% air atmosphere.

RNA silencing

Two small interference RNA (siRNA) for *PIK3CA* were designed according to the consensus sequence of the *PIK3CA* gene (GeneBank NM_006218) obtained from the online database of the National Center for Biotech-

nology Information, and then were cloned separately into the vector plasmid Pgenesil-1. One sequence, 5'-GCTATCATCTGAACAATTA-3' was designated Pgenesil-CA1, and the other 5'-GGATAGAGGCCAAATAATA-3' was designated Pgenesil-CA2. Both were verified in a basic local alignment search tool search of the database. An siRNA scrambled sequence 5'-GACTTCATAAGGCGCATGC-3', designated Pgenesil-Neg, was used as a negative control. All siRNA sequences were synthesized by Invitrogen (Carlsbad, CA). The multicloning sites of plasmid Pgenesil-1 containing enhanced green fluorescent protein gene were as follows: *Hind*III-*ShRNA-Bam*H I -U6Promotor-*Eco*R I -*Sal*I -*Xba*I -*Dra*III. All the above RNAi sequences were transcribed with DNA polymerase III U6 promoter. Cells at 80%-90% confluency were transfected with the three shRNA vectors CA1, CA2, and Neg group, described above. Transfections were performed using lipofectamine TM2000 (Invitrogen, United State) according to the manufacture's instructions. The Blank group was also treated with only LipofectamineTM2000, but without vector. Six hours post-transfection, 500 μ L of fetal bovine serum (FBS) was added per well. Twenty-six hours after transfection, the medium was replaced by normal medium containing 10% FBS and antibiotics up to 72 h post-transfection. The transfection effectiveness was calculated basing on the rate of fluorescence cell of SW948 at 6 h after transfection.

RNA isolation and semiquantitative reverse transcription polymerase chain reaction

Cultured cells (described above) were harvested 48 h post-transfection. Total messenger RNA was extracted from these cells using the RNeasy kit. Reverse transcription polymerase chain reaction was performed using 500 ng of total RNA samples with oligo dT primers (Fermentas, United States). β -actin mRNA was included as an internal standard for quantitative analysis. The primer pairs used in this study are listed in Table 1. Samples in each group were run in triplicate. The photodensity of the goal gene product was normalized with respect to β -actin content.

Western blotting

Cells were harvested 48 h post-transfection, and were washed twice in ice-cold 1 $\times$ phosphate-buffered saline (PBS). Cell pellets were treated with the lysis buffer, and whole cell extracts were isolated. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%), then transferred onto a polyvinylidene fluoride membrane, and subjected to immunohybridization analysis using a monoclonal antibody against the targeted proteins (Santa Cruz Biotechnology, Inc., United States). Peroxidase-conjugated secondary antibody was added later (Bolster, China). Hybridized protein bands were displayed *via* chemiluminescence reagents (Santa Cruz Biotechnology, Inc., United State) according to the manufacturer's instructions. β -actin staining was used as an internal standard. All experiments were performed in triplicate.

3- (4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

The transfected cells were seeded in 96-well plates (1 $\times$ 10⁴/mL), and were allowed to attach for 24 h. Thiazolyl blue tetrazolium bromide (MTT, Sigma, United States) was dissolved in 1 $\times$ PBS at a concentration of 5 mg/mL, and filtered as a stock solution. Ten microlitres of stock solution were added to 100 μ L of medium in each well. The plates were incubated for 4 h at 37 $^{\circ}$ C. After loading of 3- (4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), medium was replaced with 100 μ L dimethyl sulfoxide, and was incubated for 30 min at room temperature for color development. Results were read by an enzyme-linked immunosorbent assay reader (570 nm, DG-3022A, United States) to determine absorbance values (*A*). In each group, time points for detection of absorbance values were 36, 48, 60 and 72 h after transfection. The inhibition rate was calculated as follows: Inhibition rate (IR) = $[1 - A1/A2] \times 100\%$, where *A1* is the absorbance value of the observation group, and *A2* is the absorbance value of the control group.

Soft-agar colony-formation assays

Different groups of cells were mixed with culture medium containing agar at a final concentration of 0.4% 24 h after transfection. One milliliter of cell suspension was seeded onto 6-well plates coated with 0.5% agar in culture medium. Colonies greater than 60 μ m in diameter at $\times 100$ magnification were counted in each plate to measure the colony efficiency after 10 d incubation. The assays were performed in triplicate.

Flow cytometry

Flow cytometry was used to assess cell cycle and apoptosis. Cells were harvested 72 h after incubation, and then were washed with cold 1 $\times$ PBS and fixed with 80% ethanol overnight at -20 $^{\circ}$ C. Next, cells were treated with RNase A (Sigma, United States), and were stained with propidium iodide (PI, Sigma, United States). All samples underwent analysis using flow cytometry (Becton Dickinson, United States). The experiments were performed three times.

Statistical analysis

Results of experimental data are reported as mean $\pm$ SE. Significance levels were determined by one-way analysis of variance and Student's *t* test using SPSS 11.5 Statistics software. A statistically significant result is indicated by a *P* < 0.05.

RESULTS

PIK3CA-specific shRNA inhibited mRNA and protein expression of target genes

SW948 transfected with plasmid Pgenesil-1 presented green fluorescence, and the transfection effectiveness was about 65% (Figure 1). Forty-eight hours post-transfection, the expression of mRNA and protein from targeted genes was tested in SW948 cells. The results are shown in Figure 2, Tables 1 and 2. All mRNA and protein expres-

Table 1 Oligonucleotides sequences of primer pairs

Goal gene	Upstream primer	Downstream primer	PCR frag (bp)
<i>β-actin</i>	5'-TCCTGTGGATCCACGAAACT-3'	5'-GAAGCATTTGCGGTGGACGAT-3'	330-bp
<i>PIK3CA</i>	5'-CCCTGCTCATCAACTAGGAAACC-3'	5'-TTGCCGTAAATCATCCCCATT-3'	160-bp
<i>Akt</i>	5'-GGACAACGCCATCCAGACT-3'	5'-GCCAGGGACACCTCCATCTC-3'	121-bp
<i>c-myc</i>	5'-TACCCTCTCAACGACAGCAG-3'	5'-TCTTGACA TTCTCCTCGGTG-3'	477-bp
<i>cyclinD1</i>	5'-GCCAACCTCCTCAACGACCGG-3'	5'-GTCCATGTTCTGCTGGGCCTG-3'	744-bp

PCR: Polymerase chain reaction; *PIK3CA*: Phosphoinositide-3-kinase, catalytic, alpha polypeptide.

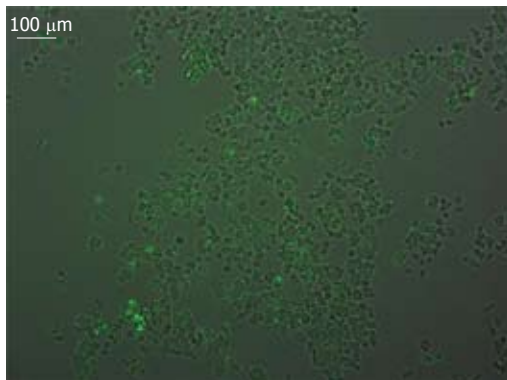


Figure 1 Fluorescence of SW948 cell transfected with plasmid Pgenesil-1 (×100).

Table 2 Levels of mRNA expression and protein expression of target genes in different groups normalized by *β-actin*

Goal gene	CA1	CA2	Negative	Blank
mRNA expression				
<i>PIK3CA</i>	0.51 ± 0.04 ^a	0.49 ± 0.03 ^a	0.92 ± 0.01	0.93 ± 0.03
<i>Akt</i>	0.50 ± 0.03 ^b	0.48 ± 0.01 ^b	0.93 ± 0.04	0.92 ± 0.02
<i>c-myc</i>	0.49 ± 0.01 ^a	0.50 ± 0.04 ^a	0.90 ± 0.02	0.91 ± 0.03
<i>cyclinD1</i>	0.45 ± 0.02 ^a	0.51 ± 0.01 ^a	0.96 ± 0.03	0.98 ± 0.01
Protein expression				
<i>PIK3CA</i>	0.53 ± 0.01 ^a	0.54 ± 0.02 ^a	0.92 ± 0.03	0.91 ± 0.02
<i>Akt</i>	0.49 ± 0.02 ^b	0.55 ± 0.03 ^b	0.94 ± 0.03	0.95 ± 0.04
<i>c-myc</i>	0.51 ± 0.03 ^b	0.52 ± 0.04 ^b	0.92 ± 0.02	0.95 ± 0.01
<i>cyclinD1</i>	0.54 ± 0.04 ^b	0.56 ± 0.03 ^b	0.93 ± 0.01	0.93 ± 0.03

PIK3CA: Phosphoinositide-3-kinase, catalytic, alpha polypeptide. ^a*P* = 0.001, ^b*P* = 0.000 *vs* negative or blank group.

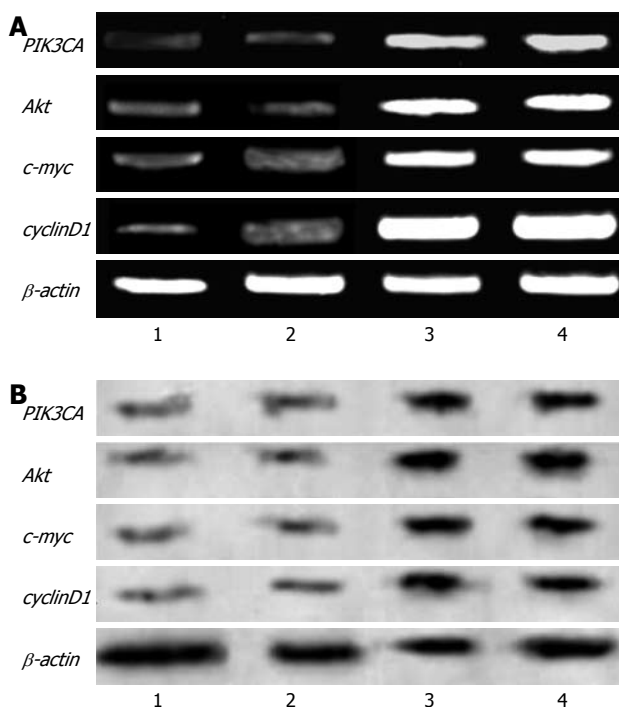


Figure 2 Phosphoinositide-3-kinase, catalytic, alpha polypeptide specific short hairpin RNA inhibited target genes mRNA expressions (A) and protein expressions (B) in SW948 cells. Lane 1: CA1 group; Lane 2: CA2 group; Lane 3: Negative group; Lane 4: Blank group. *PIK3CA*: Phosphoinositide-3-kinase, catalytic, alpha polypeptide.

sion from targeted genes in SW948 cells was down-regulated significantly after transfection with either plasmids

Table 3 Phosphoinositide-3-kinase, catalytic, alpha polypeptide short hairpin RNA suppressed SW948 cell proliferation (%)

	Blank	Negative	CA1	CA2
36 h	13	14	17	15
48 h	14	17	29 ^a	25 ^a
60 h	16	19	38 ^a	34 ^a
72 h	17	20	53 ^b	48 ^b

^a*P* = 0.001, ^b*P* = 0.000 *vs* negative or blank group.

Pgenesil-CA1 or Pgenesil-CA2 (*P* < 0.001, *vs* negative or blank group). No significant difference was found between the CA1 and CA2 groups. Pgenesil-Neg plasmid had no significant inhibitory effect on the expression of mRNA or protein.

PIK3CA RNA silencing inhibited SW948 cell proliferation

The effect of *PIK3CA* silencing on proliferation of SW948 cells was analyzed by the MTT assay. As demonstrated in Table 3, both Pgenesil-CA1 and Pgenesil-CA2 plasmids significantly suppressed the growth of SW948 cells when compared with the negative or blank group.

Soft-agar colony-formation assays

Soft-agar colony-formation assays were used to analyze the anchorage-independent proliferation of SW948 cells. Numbers of colonies in negative, blank, CA1, and CA2 group were 42 ± 4, 45 ± 5, 8 ± 2, and 10 ± 3, respectively. There were more than 4.5 times more than colonies in the blank and negative control groups as

Table 4 Effect of short hairpin RNA interference on cell cycle distribution and apoptosis (%)

Group	G ₀ /G ₁	S	G ₂ /M	Apoptosis
Blank	35.41 ± 2.63	47.54 ± 3.87	16.74 ± 1.59	0.86 ± 0.13
Negative	40.12 ± 2.54	41.71 ± 2.16	17.57 ± 1.26	0.95 ± 0.11
CA1	79.72 ± 4.63 ^a	15.24 ± 1.28 ^a	4.37 ± 0.43 ^b	5.11 ± 0.32 ^a
CA2	77.93 ± 4.31 ^a	16.71 ± 0.89 ^a	5.17 ± 0.29 ^a	4.73 ± 0.32 ^b

^aP = 0.001, ^bP = 0.000 *vs* negative or blank group.

there were in the CA1 and CA2 groups. In addition, the colonies in blank and negative control groups were also larger than those in the CA1 and CA2 groups.

Effect of PIK3CA shRNA on cell cycle distribution and apoptosis

As shown in Table 4, the percentage of cells in the CA1 and CA2 groups was significantly higher in G₀/G₁ phase, but lower in S and G₂/M phase when compared with the negative and control groups. Moreover, cell apoptosis rates in the CA1 and CA2 groups were around 5%, which was significantly higher than those in negative and blank groups. No significant difference was found between CA1 and CA2 groups in cell cycle distribution and apoptosis.

DISCUSSION

One of the areas of greatest interest in cancer research concerns the coordination of cancer cell proliferation and apoptosis. The balance between cell proliferation and apoptosis, and the distribution of cell cycles play very important roles in the process of carcinogenesis in colon cancer^[18]. Abnormal activation of the PI3K signaling pathway can cause hyper-proliferation of intestinal crypt progenitors, and promote the transformation of a normal cell to a cancer cell. This process involves a series of complicated molecular activities. The PI3K signaling cascade begins with the phosphorylation of PIP₂ to PIP₃, which is largely regulated by the balance of activity of PTEN and PI3Ks. Many investigators have reported that mutations of *PI3Ks* contribute to the process of carcinogenesis in colon cancer^[2,5]. Liao *et al.*^[19] reported that co-existence of PIK3CA exon 9 and 20 mutations, but not PIK3CA mutation in either exon 9 or 20 alone, was associated with poor prognosis of colorectal cancer patients. Thus, *PI3K* has become the focus of considerable attention for research into the treatment of colon cancer. Small-molecule inhibitors targeting *PI3K* are important to investigate, but their toxicity, drug resistance, and side-effects have prevented widespread use of these compounds^[20]. Therefore, more efficient and safer new methods have been developed to solve those problems.

RNA interference is a ubiquitous mechanism of eukaryotic gene regulation, and an excellent strategy for specific gene silencing. Recently the vector-based approach of shRNA interference has been developed in order to achieve highly specific suppression of gene ex-

pression in mammalian cells. ShRNA is formed by hairpin structures and stretches of double-stranded RNA, which determine the specificity of RNA interference. Previous reports about ShRNA interference have demonstrated their easy and stable introduction into cells, and more importantly, their consistently efficacy, which has potential as a new method for cancer therapy^[15-17,21].

PI3K is a heterodimer composed of a regulatory subunit (p85) and a catalytic subunit (p110), which has three isoforms. Among them, p110α (PIK3CA), is an important catalytic subunit that is implicated in a wide range of cancers including colon cancer. Beuers *et al.*^[22] reported strong associations were found between KRAS, PIK3CA mutations and colorectal cancers. Dukes' staging, and KRAS and PIK3CA bi-mutations were more likely to develop into liver metastasis. It naturally serves as a potent potential target for colon cancer therapy^[2-6]; however, the use of an RNA interference technique for PIK3CA gene silencing in established colon cancer cell lines has rarely been reported. In a previous study, Wee *et al.*^[23] reported successful PIK3CA gene depletion by RNA interference and consequential inhibition of proliferation in the colon cancer cell lines HCT116 and DLD1; however, the down-regulation of PIK3CA gene expression by RNAi in other colon cancer cell line has not been reported, due to the limited availability of colorectal carcinoma cell lines with PIK3CA gene mutation. Herein we describe our experience with the effects of two PIK3CA-specific shRNAs on cell proliferation and apoptosis in the colon cancer cell line SW948, which harbors mutation in the PIK3CA gene.

In this study we used plasmid Pgenesil-1 as vector into which two PIK3CA-specific interference sequences were successfully inserted. The vector Pgenesil-1 could transcribe and generate interfering RNAs continually under the control of U6 promoter. Thus, a persistent gene knock-down effect could be achieved by successfully transfecting the vectors into SW948 cells. Our results demonstrated that these two PIK3C-specific shRNAs both showed evident effects on silencing the PIK3CA gene either at the mRNA or at the protein level. At the same time, the expression of downstream genes, namely *AKT1*, *MYC*, and *CCND1* was also significantly suppressed. MTT assays demonstrated that cell proliferation in the PIK3CA knockdown groups was significantly inhibited from 48 to 72 h after transfection. Similar results were achieved in detecting anchorage-independent cell proliferation by soft agar colony formation assays, in which significant reduction in either the number or the size of colonies was found in both CA1 and CA2 groups. These results suggest that PIK3CA knockdown by specific shRNAs decreased the abilities of SW948 cells to form colonies in soft agar, which was consistent with Wee's report^[23]. Our results revealed that these two PIK3CA-specific shRNAs expressed an anti-proliferation function in the PIK3CA-mutant cancer cells, and suggest that they may have therapeutic potential for colon cancer.

Our flow cytometry results showed cell cycle arrest

at G₁/S, and an increase in apoptosis in these two *PIK3CA*-specific shRNAs-transfected SW948 cells, which may have caused the inhibition of cell growth. This finding is consistent with previously published results in the HCT116 cell line^[23]. Akt, a major downstream effector of PI3K, is activated by phosphoinositide-dependent protein kinase 1, and plays an important role in cell proliferation, apoptosis, cell motility and invasion^[12]. Lots of studies have shown that Akt is activated in a variety of malignances, and is often functionally linked to cancer progression and metastasis^[13]. Another possible mechanism is that *AKT* down-regulation induced by *PIK3CA* gene silencing resulted in the degradation of cyclinD1 and down-regulation of c-myc expression *via* GSK3 β activity. CyclinD1 is a regulatory kinase that is critical in modulating the cell cycle through G₁ to S phase. C-myc is a positive regulator of G₁-specific cyclin-dependent kinases. Over-expression of these two genes may stimulate cells to overcome the cell cycle checkpoints, and thus enhance cell proliferation^[24,25]. Inhibition of these two genes may result in cell cycle arrest and increased apoptosis, and thus inhibit cell growth. Migliardi *et al*^[26] reported that PI3K/mTor inhibitor BEZ235 could produce prevalent growth-suppressive effects in patient-derived xenografts of RAS-mutant colorectal carcinomas.

In conclusion, our experiment showed that the shRNA targeted against *PIK3CA* could specifically silence the *PIK3CA* gene and consequentially suppress the expression of its downstream genes including, *AKT1*, *MYC*, and *CCND1*. This silencing effect on these genes could efficiently inhibit the growth of *PIK3CA*-mutant colon cancer SW948 cells, and might be a potential approach for treating human *PIK3CA*-mutant colon cancer.

COMMENTS

Background

The phosphoinositide-3-kinase (PI3K)/Akt signaling transduction pathway is believed to play an important role in carcinogenesis. PI3K is a major signaling component, which is a heterodimer consisting of a regulatory subunit (p85) and a catalytic subunit (p110). The subtype p110a encoded by the gene phosphoinositide-3-kinase, catalytic, alpha polypeptide (*PIK3CA*) is very important in phosphorylating, and may serve as a potential target for colon cancer therapy.

Research frontiers

The coordination of cell proliferation and apoptosis in cells is one of the hotspots in cancer research. The balance between cell proliferation and apoptosis, and the distribution of cell cycles play very important roles in the carcinogenesis process of many cancers. *PIK3CA* is the key component in this process. Thus *PIK3CA* might be a gene therapy target for lots of cancers include colon carcinoma.

Innovations and breakthroughs

In this study, two *PIK3CA* specific shRNAs showed their evident effect on silencing the *PIK3CA* gene. The results also indicated that *PIK3CA* shRNA inhibits cell growth and induces apoptosis of SW948 cell.

Applications

The shRNA interference targeted against *PIK3CA* may have potential therapeutic utility in human *PIK3CA*-mutant colon cancer.

Terminology

The official name of *PIK3CA* is "phosphoinositide-3-kinase, catalytic, alpha polypeptide". It is located on the long(q) arm of chromosome 3 at position 26.3. Phosphatidylinositol 3-kinase is composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. The protein encoded by *PIK3CA* gene represents the catalytic subunit. The *PIK3CA* gene has been found to be oncogenic and is

mutated in a range of human cancers. Due to the association between *PIK3CA* and cancer, it is believed to be a promising drug target.

Peer review

The *PIK3CA* knock-down effect on cells was investigated in the present study. The results showed that shRNAs transfection down-regulated *PIK3CA* and its downstream genes such as *Akt1*, *MYC* and *CCND1*, and inhibited cell growth. Further, it was demonstrated that cell cycle arrest and apoptosis were induced in *PIK3CA* shRNAs-transfected SW948 cells. The study appears to be interesting and may have therapeutic implication.

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Endoscopic therapy for gastric stromal tumors originating from the muscularis propria

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Abstract

AIM: To explore endoscopic therapy methods for gastric stromal tumors originating from the muscularis propria.

METHODS: For 69 cases diagnosed as gastric stromal tumors originating from the muscularis propria, three types of endoscopic therapy were selected, based on the size of the tumor. These methods included endoscopic ligation and resection (ELR), endoscopic submucosal excavation (ESE) and endoscopic full-thickness resection (EFR). The wound surface and the perforation of the gastric wall were closed with metal clips. Immunohistostaining for CD34, CD117, Dog-1, S-100 and smooth muscle actin (SMA) was performed on the resected tumors.

RESULTS: A total of 38 cases in which the tumor size was less than 1.2 cm were treated with ELR; three cases were complicated by perforation, and the perforations were closed with metal clips. Additionally, 18 cases in which the tumor size was more than 1.5 cm were treated with ESE, and no perforation occurred. Finally, 13 cases in which the tumor size was more

than 2.0 cm were treated with EFR; all of the cases were complicated by artificial perforation, and all of the perforations were closed with metal clips. All of the 69 cases recovered with medical treatment, and none required surgical operation. Immunohistostaining demonstrated that among all of the 69 gastric stromal tumors diagnosed by gastroscopy, 12 cases were gastric leiomyomas (SMA-positive), and the other 57 cases were gastric stromal tumors.

CONCLUSION: Gastric stromal tumors originating from the muscularis propria can be treated successfully with endoscopic techniques, which could replace certain surgical operations and should be considered for further application.

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Key words: Gastrointestinal stromal tumors; Therapy; Endoscopy; Muscularis propria; Resection

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INTRODUCTION

Gastric stromal tumors are the most common mesenchymal tissue-originated tumors in the digestive tract; these tumors are often found at the gastric fundus, the front wall of the gastric corpus and the antrum and can be sorted based on tissue of origin as originating from the muscularis mucosae or the muscularis propria. As the location of the tumors originating from the muscularis

mucosae is superficial, endoscopic resection or ligation has been widely performed to treat these tumors. In contrast, the location of the tumors originating from the muscularis propria is deep, especially when the tumors grow outside the gastric wall, so perforation often occurs during endoscopic therapy and the tumor cannot be resected easily and thoroughly. Therefore, this tissue of origin has often been considered to be a contraindication for endoscopic therapy, and the traditional treatment for such tumors is a surgical or laparoscopic operation^[1-5]. In recent years, on the basis of sufficient clinical practice, we treated gastric stromal tumors originating from the muscularis propria with endoscopic techniques and obtained satisfactory therapeutic effects, which are reported as follows.

MATERIALS AND METHODS

Patients

A total of 69 patients (36 males and 33 females, age range 24-68 years, mean age 42.5 years) were diagnosed with gastric stromal tumors originating from the muscularis propria by gastroscopy and endoscopic ultrasonography from January 2008 to March 2011. Among the 69 cases, 19 tumors were located at the gastric antrum, 24 tumors were located at the gastric corpus, and 26 tumors were located at the gastric fundus. Each patient exhibited only one tumor, and no metastasis was detected *via* computed tomography (CT) examination. Routine blood tests, tests of blood coagulation function and hepatic and renal function, electrocardiograms and abdominal CTs were performed before the endoscopic therapy, and each patient's written informed consent was obtained.

Instruments

The following instruments were used: Electronic gastroscopy (Olympus GIF-Q260J, Olympus company, Japan), hyaline cap (D-201-11304, Olympus company, Japan), spiculiform cutting knife (KD-1 L-1, Olympus company, Japan), IT knife (KD-611 L, Olympus company, Japan), hook knife (KD-620 LR, Olympus company, Japan), injection needle (NM-200 L-0525, Olympus company, Japan), snare (AS-1-S, ASJ-1-S, COOK company, United States), hot biopsy forceps (FD-410 LR, Olympus company, Japan), hemostatic clip (HX-610-90, Olympus company, Japan; HX-600-135, Olympus company, Japan; Boston ResolutionTM, Boston company, United States), high frequency electric knife (ERBE VIO 200S, ERBE company, Germany) and Argon Plasma Coagulation instrument (ERBE APC₂, ERBE company, Germany).

Endoscopic therapeutic methods

Endoscopic ligation and resection: Endoscopic ligation and resection (ELR) was used for gastric stromal tumors of which the size was less than 1.2 cm. A COOK ligator was assembled on the tip of the gastroscopy, and the gastroscopy was inserted into the stomach. The ligator was aimed at the tumor, and sufficient aspiration

was applied to the tumor that the whole tumor entered the ligator, at which time the rubber band on the ligator was released to ligate the tumor. The purpose of the ligation was to manipulate the gastric stromal tumor into the shape of a polypoid, after which a snare was used to hitch the tumor, and it was resected. The resected tumor was removed for pathologic diagnosis, and the stump was closed with metal clips. If the procedure was complicated by perforation, the perforation was generally small and could be closed with metal clips (Figure 1).

Endoscopic submucosal excavation: Endoscopic submucosal excavation (ESE) was performed for gastric stromal tumors originating from the muscularis propria by the following steps. (1) Marking: Argon plasma coagulation (APC) was used to mark the border of the gastric stromal tumor; (2) Submucosal injection: A mixture of indocarmine, adrenalin and physiological saline (2-3 mL of indocarmine, 1 mL of adrenalin and 100 mL of physiological saline) was injected at the lateral of the mark; (3) Dissection: the mucosa was dissected with a needle knife; (4) Excavation: the submucosa was dissected with a needle knife; when the tumor was exposed, dissection was performed around the margin of the tumor, and when the dissection was finished, the tumor could be resected wholly with a snare; and (5) Wound surface handling: for small vessels bleeding on the surface, hot biopsy forceps or APC could be used for hemostasis, and if necessary, metal clips could be used (Figure 2).

Endoscopic full-thickness resection: A hyaline cap was assembled on the tip of the gastroscopy before the endoscopic full-thickness resection (EFR) procedure, and intravenous anesthesia was performed using propofol for patients. EFR was then performed using the following steps. (1) Pre-cutting the mucosa and submucosa around the gastric stromal tumor: APC was used to mark the margin of the tumor; a mixture of indocarmine, adrenalin and physiological saline (2-3 mL of indocarmine, 1 mL of adrenalin and 100 mL of physiological saline) was injected at the lateral portion of the mark, using approximately 2-3 mL of the mixture for each injected point; the mucosa and submucosa around the gastric stromal tumor were pre-cut with a hook knife, and thus the tumor was exposed; (2) Dissection of the muscularis propria around the gastric stromal tumor: a hook knife or IT knife was used to dissect the tumor from the muscularis propria to the serous membrane; (3) Dissection of the serous membrane around the tumor: typically, the tumor was in compact adhesions with the serous membrane; thus the tumor could not be directly dissected with an IT knife, so instead, the serous membrane was cut around the tumor with a needle knife or hook knife, and an "artificial" perforation was made; (4) Full resection of the tumor: the liquid in the stomach was aspirated completely, and an IT knife or hook knife was used to cut the serous membrane around the tumor, after which the tumor was completely resected; and (5)

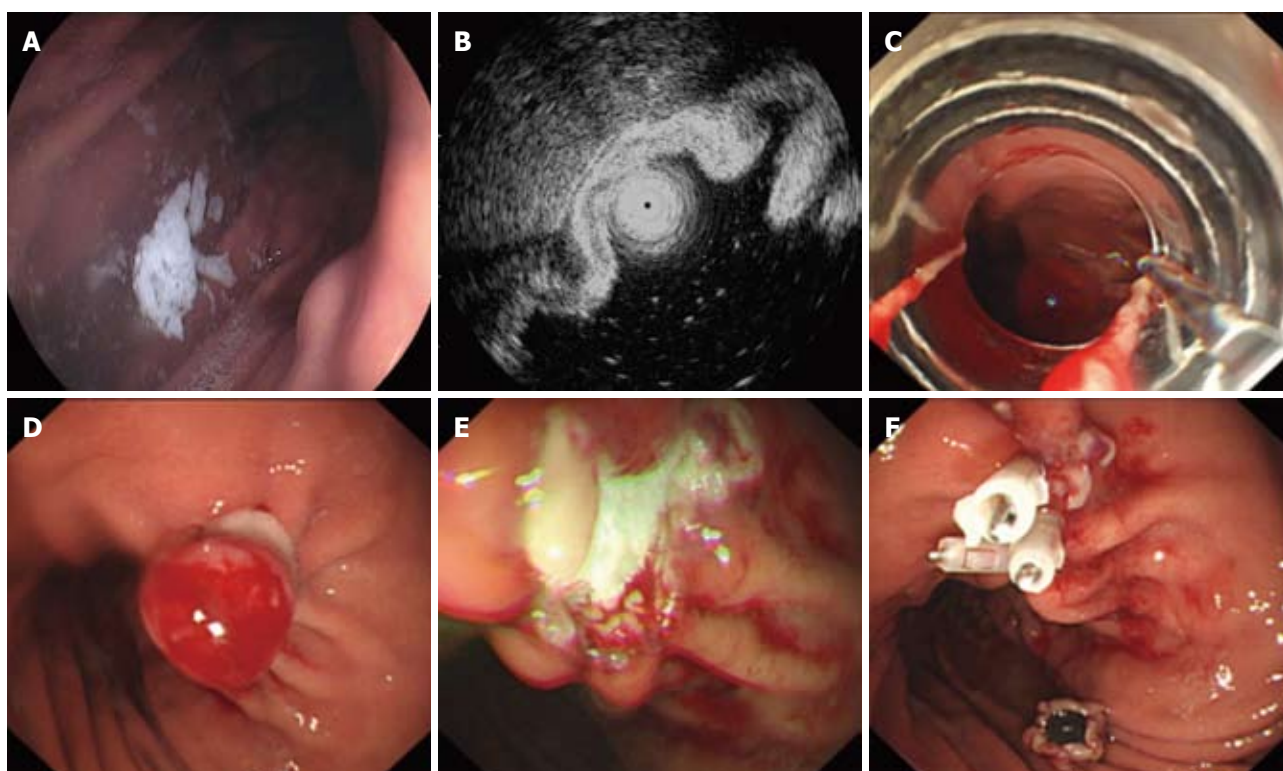


Figure 1 Endoscopic ligation and resection treatment for a gastric stromal tumor less than 1.2 cm in size originating from the muscularis propria. A: Submucosa lesion at the posterior wall of the gastric corpus; B: Endoscopic ultrasound shows that the lesion originates from the muscularis propria; C: COOK ligator aimed at the lesion, ready to ligate; D: The ligated stromal tumor was in the shape of a polypoid with deuto-stem; E: A snare was used to cut the tumor above the rubber band; F: The wound surface was closed with metal clips.

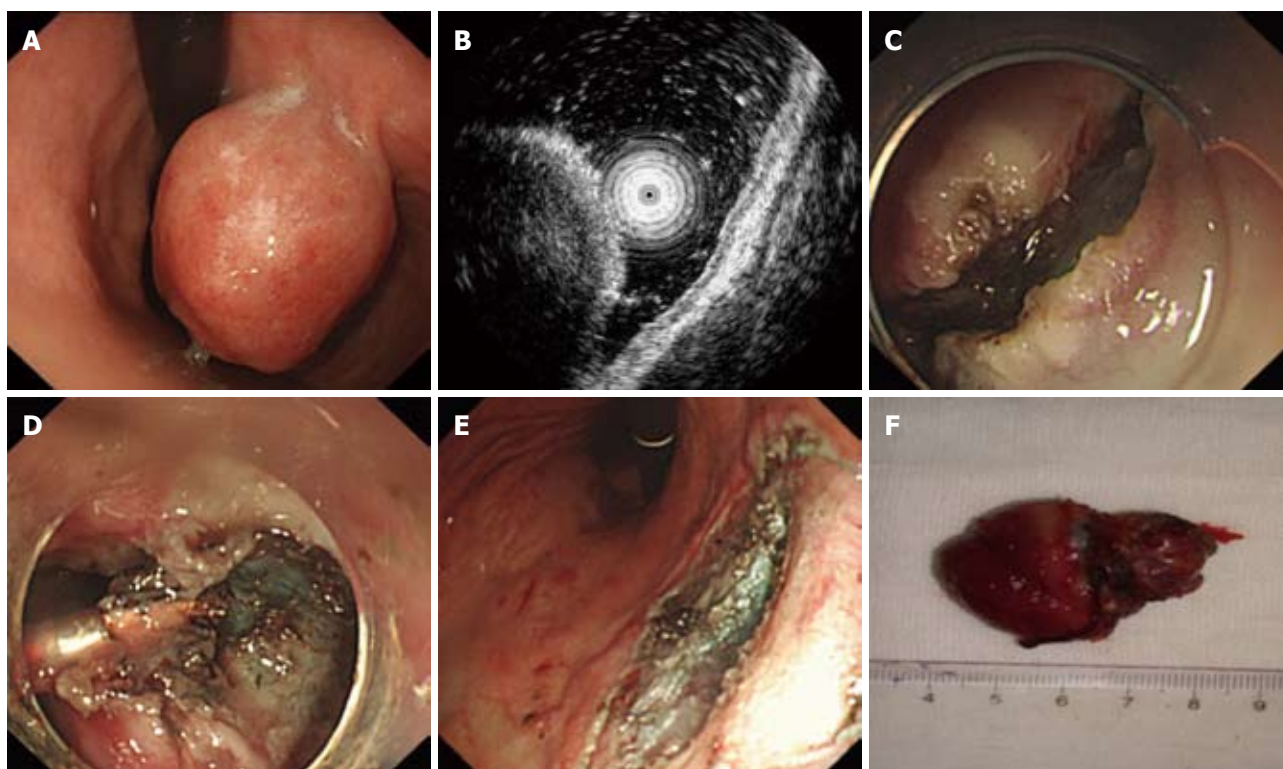


Figure 2 Endoscopic submucosal excavation treatment for a gastric stromal tumor originating from the muscularis propria that is larger than 1.2 cm. A: Submucosa lesion at the gastric corpus; B: Endoscopic ultrasound shows that the lesion originates from the muscularis propria; C: The mucosa of the stromal tumor was cut after submucosal injection; D: Dissection with an IT knife; E: The excavated wound surface, showing that no perforation occurred; F: The resected stromal tumor (4 cm in size).

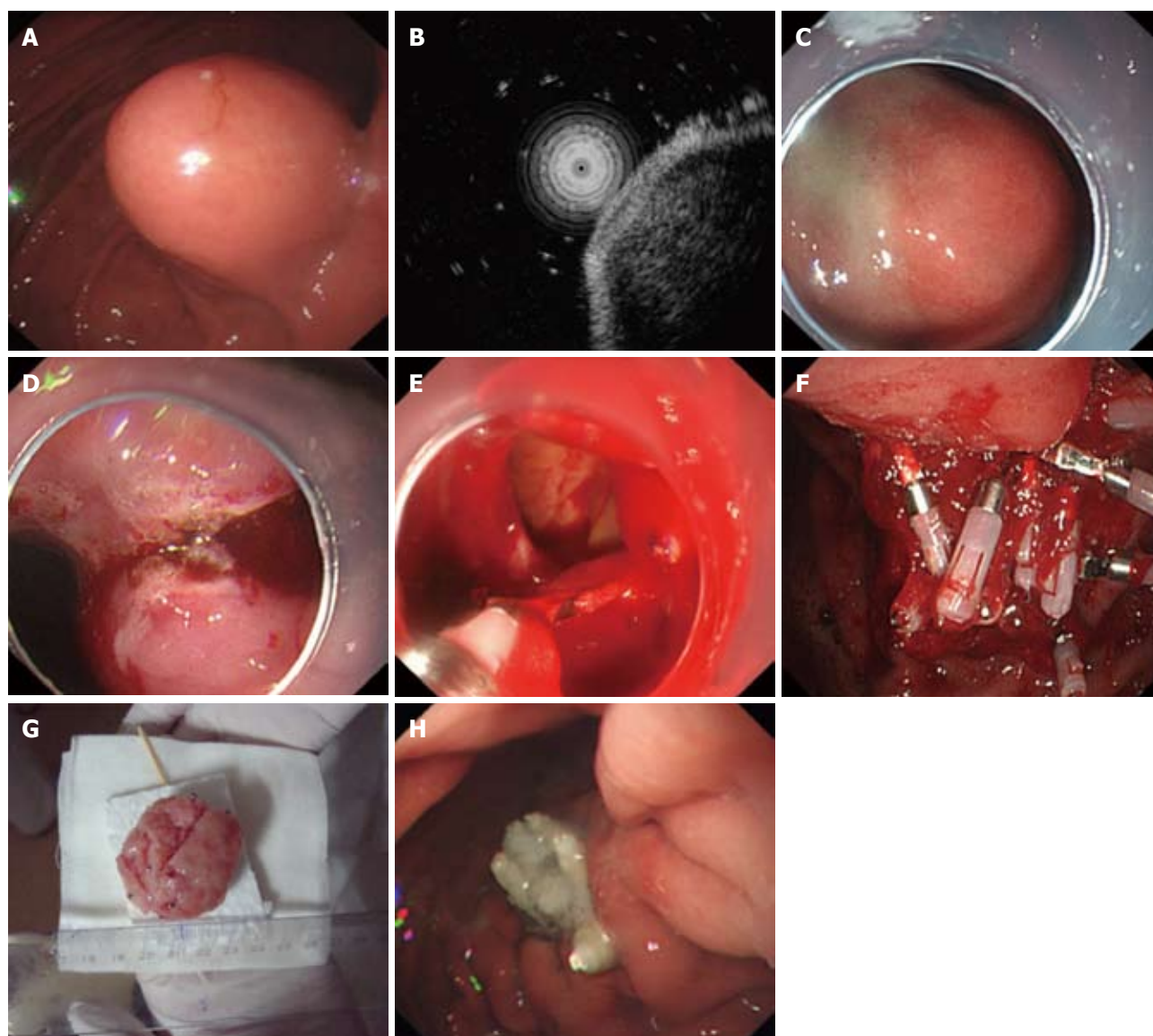


Figure 3 Endoscopic full-thickness resection treatment for a gastric stromal tumor originating from the muscularis propria larger than 1.2 cm. A: Submucosa lesion at the gastric corpus; B: Endoscopic ultrasound shows that the lesion originates from the muscularis propria; C: Submucosal injection of the mixture of indigocarmine, adrenaline and physiological saline; D: Dissection with an IT knife; E: “Artificial” perforation after gastric stromal tumor resection, closed with metal clips; F: Many clips used to close the wound defect; G: The resected tumor without mucosa (5 cm in size); H: The perforation healed 9 d after endoscopic full-thickness resection.

Closure of the wound surface of the stomach: a metal clip was used to close the wound surface from the rim to the center. If the wound surface defect was too large to be closed with a metal clip directly, negative pressure aspiration was used to aspirate the omentum majus into the stomach, and then a metal clip was used to close the wound surface defect (Figure 3).

Specimen handling

The resected specimens were fixed in neutral formalin for pathologic diagnosis. Immunohistostaining of CD34, CD117, Dog-1, S-100 and smooth muscle actin (SMA) was performed for specimens suspected of being gastric stromal tumors^[6-10].

Postoperative handling

For patients without perforation, the measures of fast-

ing, infection prevention and limiting acid with proton pump inhibitors were sufficient. For patients with “artificial” perforation, if pneumoperitoneum was serious, evacuation was performed during and after the operation. The evacuating needle was inserted into the lower right quadrant to release abdominal distension. A semi-reclining position was recommended after the operation, along with the use of fasting and gastrointestinal decompression, to allow the perforation to heal. Abdominal pain, abdominal distension and signs of peritoneal irritation after the operation were carefully observed. Meglumine diatrizoate was recommended to be taken orally three days after the operation. To confirm whether there was extravasation of the contrast media and to observe stomach motivation, ultrasonic examination was conducted to observe whether there was hydrops in the abdominal cavity and pelvic cavity. Gastroscopy was

performed one month after the operation to observe the conditions of the wound surface and whether there was residual or recurrence.

RESULTS

Effects of endoscopic therapy

ELR: Thirty-eight patients with gastric stromal tumors with a size of less than 1.2 cm underwent ELR, in which the tumor was ligated wholly and then resected. The procedure was complicated by perforations in 3 patients, and all of the wound surfaces and perforations were closed with metal clips. The patients recovered with medical treatment, and no patient needed surgical operation.

ESE: Eighteen patients with gastric stromal tumors in which the size of the specimen resected was more than 1.5 cm underwent ESE. The tumors were resected successfully, and no perforations complicated the procedure.

EFR: Thirteen patients with gastric stromal tumors, for which the size of the specimen resected was more than 2.0 cm, underwent EFR. The tumors were resected successfully, but “artificial” perforation occurred in all of the patients. All of the perforations were closed with metal clips, and meglumine diatrizoate upper gastroenterography three days after the operation showed that there was no extravasation of the contrast media. Gastroscopy showed that the wound surface of the stomach was nearly healed on the 9th day after the operation (Figure 3H).

Immunohistostaining

The positive expression rates of CD34, CD117, Dog-1, S-100 and SMA in the 69 patients who were diagnosed with gastric stromal tumors by gastroscopy were 81.2% (56/69), 82.6% (57/69), 82.6% (57/69), 0% and 17.4% (12/69), respectively, which demonstrated that, among the 69 patients, there were 12 leiomyomas, which express SMA; the other 57 patients had gastric stromal tumors.

Average stay

The average stay for all of the patients was 8.4 ± 2.4 d.

Residual or recurrence of the tumor

Gastroscopy and abdominal CT scans were performed one month after the operation; these procedures showed that the wound surfaces had healed, and there was no residual or recurrence.

DISCUSSION

Stromal tumor is the most common mesenchyme-originated tumor; as it has potential cancerous tendencies, gastric stromal tumor is recommended to be resected^[11-15]. For gastric stromal tumors originating from the muscularis propria in which the size was less than 1.2 cm, ELR was the ideal method^[16]. After ligation, each

tumor was in the shape of a polypoid with a deuto-stem, which made snare resection easy; additionally, a pathologic diagnosis could be obtained with the resected tumor. Because the wound surface in the stomach was not large, perforation would not generally occur; however, if perforation did occur, it could be closed with metal clips. With the medical treatment of fasting, gastrointestinal decompression and proton pump inhibitors, the patients would recover. There were 38 patients in this study in whom the tumor size was less than 1.2 cm; these patients were treated with ELR. Moreover, 3 of these patients were complicated by perforation; the perforations were closed with metal clips, and no surgical assistance was required. Because the texture of the stromal tumor was hard, it could not be as easily aspirated as mucosa or vascular tissue, so the ligation for the stromal tumor required a long period of aspiration. According to our experience, when the stromal tumor was ligated, a red-colored sign occurred when the tumor was aspirated, and at least another one-minute maximum aspiration was necessary to ensure that the rubber band could fully hitch the tumor.

ESE is another effective measure for the treatment of tumors originating from the submucosa or muscularis propria. For tumors of 3.0 cm or less in size originating from the muscularis propria, the achievement ratio of ESE was high and the patient suffered less, whereas the complication rate was low. ESE was able to excavate the whole tumor successfully, and a pathologic diagnosis could be performed using the resected specimen, which was able to provide a systemic and safe therapeutic method for each patient. In this study, there were 18 patients with gastric stromal tumors originating from the muscularis propria who underwent ESE; all of the tumors were excavated wholly, and no perforations occurred.

For patients with gastric stromal tumors originating from the muscularis propria, the perforation rate of endoscopic therapy is high; therefore, the traditional treatment method is surgical operation or therapeutic laparoscopy, especially for stromal tumors larger than 2 cm^[17-20]. A total of 13 patients in this study underwent EFR, and perforation occurred in all of the patients. The sizes of the 13 tumors were all larger than 2 cm, all of the perforations were closed with metal clips, the abdominal cavity pressure decreased with the use of abdominal paracentesis, all of the patients recovered with medical treatment, and no patients needed surgical help. The key point in avoiding complications was the use of endoscopic metal clips for oversewing; according to our experience, for larger perforations, the perforation should be closed with metal clips from rim to center, known as oversewing, and even some normal mucosa should be sewed up to decrease the perforation. The key point for successful EFR is to mend the perforation successfully, avoiding surgical mending and postoperative peritonitis^[21,22]. The most widely used method to mend perforation is metal clip sutura^[23,24]. For small perforations, one or several metal clips could oversee the per-

foration completely. For larger perforations, as the span of the metal clip is limited, the gas in the stomach must be aspirated properly to reduce the perforation, and several metal clips should be used to close the perforation. If the perforation is too large to be oversewn with metal clips directly, the retina mending method is recommended^[25,26]. The gas in the stomach should be aspirated with continuous negative pressure until the adipose tissue outside the gastric wall covers the perforation, at which point the perforation is closed with metal clips. The other essential point for EFR is to avoid too much gastric juice entering the abdominal cavity; to prevent postoperative infection, strict hemostasis for the wound surface should be performed, but repeat rinse hemostasis should be avoided during the procedure^[27]. The gas and liquid in the stomach should be aspirated fully before cutting the serous membrane. Postoperative gastrointestinal decompression, proton pump inhibitors and antibiotics were effective measures to prevent abdominal cavity infection, and no complications of peritonitis or peritoneal abscesses occurred in this study.

Pneumoperitoneum caused by perforation after EFR can influence the field of vision in the stomach, making endoscopic operation difficult; thus, during EFR, repeated abdominal touch was necessary. If the pressure in the abdominal cavity increased, prompt evacuation was needed; the puncture site was in the right lower quadrant, and the needle was a commonly used 20 mL injection needle. After puncture, the abdomen was compressed to exhaust the air, the needle was detained until the perforation was closed and the pneumoperitoneum had clearly improved, and the needle was removed when it was confirmed that there was no further gas exhausting from the abdominal cavity.

It is difficult to distinguish gastric stromal tumors from leiomyomas and schwannomas based on cell shape. The clinical diagnosis of this tumor type mainly depends on histopathology, immunohistochemistry (CD34- and CD117-positive) and protocols in molecular biology^[28,29]. CD34 is the antigen marker of myeloid stem cells; it resides in the marrow hematopoietic stem cell tissue. CD117 is the product of the c-kit oncogene; it is the receptor of tyrosine kinase growth factor. The *Dog-1* gene is located at chromosome 11q¹³; it has 26 exons and is 114 Kb in length; the protein has 8 trans-membrane functional areas; it may be a chloride channel regulated by calcium ions, and it is currently recognized as the most sensitive and specific marker for gastrointestinal stromal tumors (GIST)^[30,31]. Typical leiomyomas and schwannomas do not express CD34; SMA-positive expression is the typical characteristic of leiomyoma, and S-100-positive expression is the typical characteristic of schwannomas^[32]. The positive expression rates of CD34, CD117, Dog-1, S-100 and SMA in the 69 patients were 81.2% (56/69), 82.6% (57/69), 82.6% (57/69), 0% and 17.4% (12/69), respectively, which demonstrated that, among the 69 patients, there were 12 leiomyomas, which express SMA, and the other 57 patients had gastric stro-

mal tumors confirmed with immunohistochemistry. Currently, the main treatment method for GISTs is surgical operation; the majority of benign GISTs in this study had a good prognosis after resection, and there was no recurrence in a short time. GISTs are not sensitive to radiotherapy or chemotherapy. For malignant GISTs that could not be completely resected or with recurrence, patients could take imatinib for therapy. Imatinib is an inhibitor of tyrosine kinase; it has significant therapeutic effects on malignant GISTs that express CD117^[33-37].

With the maturation of EFR, more and more gastric stromal tumors originating from the muscularis propria would avoid surgical operations, sharing the benefits provided by therapeutic endoscopy techniques.

COMMENTS

Background

The traditional method for the treatment of gastric stromal tumor originating from the muscularis propria was laparoscopic or surgical operation; in recent years, with the development of endoscopic techniques and instruments, endoscopic therapy for gastric stromal tumors originating from the muscularis propria has been made possible.

Research frontiers

Gastric stromal tumor is the most common mesenchymal tissue-originated tumor in the digestive tract; based on the tissue of origin, it can be defined as originating from the muscularis mucosae or the muscularis propria. As the location of the tumor originating from the muscularis mucosae was superficial, endoscopic resection or ligation was commonly performed. In contrast, the location of a tumor originating from the muscularis propria was deep, especially when the tumor grew outside gastric wall, perforation often occurred during endoscopic therapy, and the tumor could not be resected easily and thoroughly. Therefore, this tissue of origin was often considered to be a contraindication for endoscopic therapy, and the traditional treatment for such tumors was surgical or laparoscopic operation. In recent years, on the basis of sufficient clinical practice, endoscopic treatment for gastric stromal tumors originating from the muscularis propria has become possible.

Innovations and breakthroughs

Depending on the size of gastric stromal tumors originating from the muscularis propria, three types of endoscopic therapy could be used to treat the tumor. These techniques were endoscopic ligation and resection, endoscopic submucosal excavation and endoscopic full-thickness resection. The wound surface and the perforation of the gastric wall were closed with metal clips. These results showed that some gastric stromal tumor originated from muscularis propria can be treated successfully with endoscopic techniques, which could replace some surgical operations.

Applications

The study results suggest that some gastric stromal tumors originating from the muscularis propria could be treated successfully with endoscopic techniques, which could replace some surgical operations.

Terminology

Gastrointestinal stromal tumor (GIST): This term indicates a particular type of lobus intermedius-originating tumor in the gastrointestinal tract; usually CD117 was positively expressed. Histologically, this tumor type is often composed of spindle cells and epithelioid cells. With respect to immunophenotype, it expresses the c-kit protein, which is driven by mutated c-kit or platelet-derived growth factor receptors. It is recognized that GIST originate from the intestinal cells of Cajal in gastrointestinal tract, which are in the shape of a network structure distributed in the muscular layer of gastrointestinal tract; this network is the pacemaker of slow-wave activity in the gastrointestinal tract and participates in the mechanism of exertional disease and tumor formation in the gastrointestinal tract.

Peer review

This is a good paper on endoscopic resection of gastric stromal tumors and leiomyomas. The figures are excellent.

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Recurrent ischemic strokes in a young celiac woman with *MTHFR* gene mutation

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resonance imaging showed a subacute right occipital ischemic lesion, which was extended to the dorsal region of the right thalamus and the ipsilateral thalamo-capsular junction. Antitransglutaminase and deamidated gliadin peptide antibodies were no longer present, while antinuclear antibodies, antineuronal antibodies and immune circulating complexes were only slightly elevated. Since the patient was taking folic acid, her homocysteine levels were almost normal and apparently not sufficient alone to explain the clinical event. A conventional cerebral angiography showed no signs of vasculitis. Finally, rare causes of occipital stroke in young patients, such as Fabry's disease and mitochondrial myopathy, encephalomyopathy, lactic acidosis and stroke-like symptoms, were also excluded by appropriate tests. Thus, the most probable cause for the recurrent strokes in this young woman remained CD, although the mechanisms involved are still unknown. The two main hypotheses concern malabsorption (with consequent deficiency of vitamins known to exert neurotrophic and neuroprotective effects) and immune-mediated mechanisms. CD should be kept in mind in the differential diagnosis of ischemic stroke in young patients.

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Abstract

Celiac disease (CD) is frequently associated with neurological disorders, but very few reports concern the association with ischemic stroke. A 26-year-old woman affected by CD with secondary amenorrhea, carrier of a homozygous 5,10-methylenetetrahydrofolate reductase mutation with hyperhomocysteinemia, was affected by two occipital ischemic strokes within a period of 5 mo. At the time of the second stroke, while she was being treated with folic acid, acetylsalicylic acid and a gluten-free diet, she had left hemianopsia, left hemiparesis, and gait imbalance. Brain magnetic

Key words: Celiac disease; Female; Methylenetetrahydrofolate reductase; Stroke; Vasculitis; Young

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strokes in a young celiac woman with *MTHFR* gene mutation. *World J Gastroenterol* 2012; 18(26): 3472-3476 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i26/3472.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i26.3472>

INTRODUCTION

Celiac disease (CD) is an immune-mediated enteropathy, resulting from a permanent intolerance to the gliadin component in dietary gluten. Its clinical presentation may include signs and symptoms of malabsorption, frequently associated with extra-intestinal manifestations. Several neurological disorders like peripheral neuropathy, cerebellar ataxia and dementia were found to be associated with the disease; epilepsy, bilateral occipital calcifications and rarely stroke^[1-7] have also been reported in CD. This article illustrates the case of a young woman with asymptomatic CD, and carrier of the homozygous mutation of the gene encoding 5,10-methylenetetrahydrofolate reductase (*MTHFR*), who was affected by two occipital strokes within a period of 5 mo.

CASE REPORT

A 26-year-old woman with history of migraine without aura presented acute left hemiparesthesias (prevailing at lips and upper limb), mild weakness in her left arm, gait imbalance, unsteadiness and asthenia, while she was working hard in an archaeological site during a hot day. Since symptoms persisted, on the following day she was transported to the emergency department of our hospital and then admitted to the Stroke Unit.

The patient had a documented positivity of antitransglutaminase and antigliadin antibodies, which she had tested 7 years before because her sister was affected by CD. The patient had no gastrointestinal symptoms, but presented a secondary amenorrhea of at least two years duration. Duodenal biopsy showed a subtotal villous atrophy with an increased number of intraepithelial lymphocytes, so confirming the CD diagnosis. Her treatment consisted of a gluten-free diet, plus an estrogen-progestin therapy that she received for a period of 6 mo. The hormone therapy had been stopped 5 mo earlier when she was hospitalized elsewhere because of a left occipital ischemic stroke (transient right lateral homonymous hemianopsia). On that occasion, laboratory and genetic testing revealed the presence of a homozygous mutation of the *MTHFR* gene with hyperhomocysteinemia (31.5 mmol/L, normal value < 15 mmol/L). The patient was prescribed folic acid and acetylsalicylic acid.

On admission to our hospital, the patient appeared alert, oriented, cooperative and afebrile. Her neck was supple. She had left lateral homonymous hemianopsia, left hemiparesthesias and mild weakness in her left leg.

Investigations

A brain CT scan showed, in addition to the previous

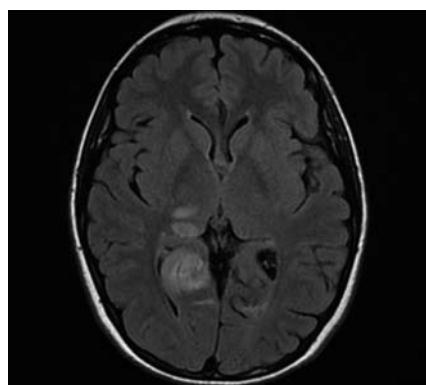


Figure 1 Magnetic resonance imaging showing a subacute right occipital lesion, extended to the dorsal region of the right thalamus and the ipsilateral thalamo-capsular junction.

left occipital infarction, a right occipital low-density area (maximum diameter 40 mm) suggesting an acute ischemic stroke involving the territory of the right posterior cerebral artery. There was no contrast enhancement of these lesions. Brain magnetic resonance imaging (MRI) confirmed the subacute right occipital lesion, which was extended to the dorsal region of the right thalamus and the ipsilateral thalamo-capsular junction (Figure 1). A conventional angiography of the aortic arch, supraortic branches and intracranial arteries showed a substantially normal picture, with poor visualization of the P3 segment and distal branches of the right posterior cerebral artery. In particular, no signs of vasculitis were found. Chest X-ray, abdominal ultrasound, electrocardiogram, electrocardiogram Holter monitoring, transthoracic echocardiogram, supraortic ultrasound and transcranial echo-Doppler with microbubbles to test the presence of patent foramen ovale, were all normal. Moreover, a bicipital muscle biopsy and an MRI spectroscopy of the gastrocnemius muscles before, during and after exercise were performed to test the hypothesis of mitochondrial disease and, in particular, of mitochondrial myopathy, encephalomyopathy, lactic acidosis and stroke-like symptoms (MELAS). Biochemical and molecular studies were performed to assess Fabry's disease. The results of all these investigations were negative.

Laboratory analyses, including complete blood count, C-reactive protein, erythrocyte sedimentation rate, renal function, cobalamin and folate levels, were normal. Serological tests for Human Immunodeficiency Virus, markers of hepatitis B and C, antigranulocyte antibodies (p-ANCA and c-ANCA), cryoglobulins, antiphospholipid antibodies and lupus anticoagulants phenomenon were also normal. The serum markers of CD, antitransglutaminase and deamidated gliadin peptide antibodies were no longer present. However, a control duodenal biopsy was not performed and antinuclear antibodies, anti-neuron antibodies and immune circulating complexes were slightly increased. Factor V Leiden and prothrombin mutation were absent. Similarly, other markers of a possible hypercoagulable status (antithrombin, protein C and

protein S) were normal. A mild hyperhomocysteinemia (16.1 mmol/L) still persisted, despite treatment with folic acid. Other laboratory tests documented secondary hypogonadism and hypothyroidism (increased FSH, LH, TSH and reduced FT4).

During hospitalization, there was a gradual improvement of neurological symptoms. The previous treatment with acetylsalicylic acid (100 mg/d) was replaced with clopidogrel (75 mg/d), and L-thyroxine (50 mcg/d) was started. Folic acid (5 mg/d), in addition to a gluten-free diet, was confirmed. At the time of discharge, the left lateral homonymous hemianopsia was still present. Five months after admission, the neurological picture was unchanged, but the patient was able to return to her job. There were no new acute cerebrovascular events.

DISCUSSION

Ischemic strokes occurring in patients younger than 30 years are a relatively rare event. The most important causes of cerebral ischemia in the young are cardioembolism, dissection of extracranial arteries, migraine, drugs and hypercoagulable states. Furthermore, premature atherosclerosis can also play an important etiological role. Uncommon causes include vasculitis, metabolic disorders and inherited diseases. However, the undetermined etiology is the most frequent reported cause of ischemic stroke in young patients^[8]. In this case report, we have considered in the differential diagnosis two rare causes of occipital stroke in young adults: MELAS syndrome and Fabry's Disease. MELAS^[9] is a progressive disease characterized by acute neurological events resembling cerebral ischemia, associated with mitochondrial myopathy and hyperlactataemia. Ten different mutations of mitochondrial DNA have been identified, but most cases (about 80%) are due to the A3243G mutation encoding the transfer RNA (tRNA) of leucine. CT and MRI often show lesions located in the occipital lobes, which are sometimes associated with brain atrophy and intracranial calcifications. Increased levels of lactate are often present in the blood, and almost constant in the cerebrospinal fluid. Muscle biopsy is abnormal in 85% of patients, showing atypical mitochondrial proliferation with presence of "ragged red fibers" at the Gomori's stain. The diagnosis is based on mitochondrial DNA analysis. Fabry's disease^[10] is a progressive multisystem inherited disease of glycosphingolipid metabolism. It is caused by mutations in the gene of α galactosidase A (chromosome Xq22). Its classic form, which mainly affects hemizygous males, is characterized by angiokeratomas, renal insufficiency with proteinuria, cardiomyopathy, and cochlear, vestibular and cerebrovascular symptoms. The final diagnosis for hemizygous males is based on the demonstration of α -galactosidase A deficiency, while in heterozygous females enzyme analysis is often inconclusive and genetic analysis is needed. In the present case, the diagnostic investigations performed for both MELAS and Fabry's disease were negative.

Our patient presented mild hyperhomocysteinemia, which is recognized as an independent risk factor for cardiovascular diseases^[11], acting through several possible prothrombotic mechanisms including the toxic action on vascular endothelium resulting in endothelial dysfunction, enhancement of both platelet adhesion and aggregation, endothelial factor V activation, protein C inhibition, and reduction of antithrombin III levels. Hyperhomocysteinemia may result from reduced elimination due to renal insufficiency, from inadequate intake or malabsorption of folic acid and B vitamins, or from a mutation of genes coding for one of the three enzymes involved in homocysteine metabolism: cystathionine β synthetase (CBS), MTHFR and methionine synthetase^[12]. The MTHFR gene mutation was carried by our patient. This is a fairly common condition in the Caucasian population, with a prevalence of 8%-15% for the homozygous genotype and 65% for the heterozygous genotype. It does not seem to be more prevalent among patients with CD^[13,14]. The MTHFR gene is located in the short arm of chromosome 1. Its mutation may be a cardiovascular risk factor only in subjects with a low intake of folic acid. At the time of the second stroke the patient was already taking folic acid. Thus, her homocysteine levels were only slightly elevated and apparently not sufficient alone to explain the clinical event.

Since no other cerebrovascular risk factors were found, we concluded that CD could be involved in the pathogenesis of recurrent strokes in this patient. CD is an immune-mediated disorder of the small bowel, resulting from a permanent gluten intolerance. Its prevalence in the general population is about 1% and its presentation may be at any age: childhood, adolescence and also adulthood. The clinical picture of CD varies greatly, ranging from asymptomatic forms, to the "classic symptomatic" form with mainly intestinal symptoms, to "atypical" forms dominated by extra-intestinal symptoms^[15]. The systemic involvement of CD may include skin, thyroid, pancreas, heart, joints, muscles, bones, reproductive system, liver, central and peripheral nervous system^[16]. Approximately 10% of CD patients show neurological symptoms, including idiopathic cerebellar ataxia, peripheral neuropathy, various forms of epilepsy, myoclonus, migraine and dementia^[17-20].

There are two main possible hypotheses concerning the pathogenesis of neurological manifestations in CD. Firstly, malabsorption with consequent deficiency of vitamins known to exert neurotrophic and neuroprotective effects (especially folic acid, vitamin B12, vitamin E) has long been thought to contribute to the neurodegenerative changes observed in patients with CD-related neurological manifestations. However, even plenty of vitamin supplementation does not improve the clinical course in these patients. Secondly, an emerging hypothesis is the possible participation of immune-mediated mechanisms. This hypothesis is strengthened by the evidence of lymphocytic infiltration of the central and peripheral nervous system^[21-22] and the presence of serum antineuronal

antibodies^[23] in CD patients with neurological complications. In this regard, neurological manifestations might result from a combination of sub-threshold neurotoxic effects caused by gluten-related immune markers (e.g., antigliadin antibodies, deamidated gliadin peptide antibodies and antibodies to one or more tissue transglutaminase isoenzymes) in combination with antibodies directed against nervous system epitopes, including anti-Purkinje cell and antiganglioside antibodies^[24]. However, the role of these antibodies in the pathogenesis of neurological dysfunction is not yet understood, even if there are elements supporting a mitochondrial-dependent neuronal apoptosis^[25].

Although the association between CD and ischemic stroke has been described by some authors, a causal link between these two conditions has never been demonstrated^[1-7]. Rush *et al*^[1] described the case of a 51-year-old white man affected by CD who had an ischemic stroke with tonic-clonic seizures, resulting from a vasculitis of the central nervous system that was documented by brain biopsy. Also, Ozge *et al*^[2] hypothesized that a vasculitis of the central nervous system could be a possible explanation for the association between CD and stroke. Moreover, Goodwin *et al*^[4] described the case of a child with CD and recurrent episodes of transient hemiplegia, suggesting an autoimmune vascular disease as the underlying mechanism. In fact, tissue transglutaminase, which is the major autoantigen of CD^[26], is also present in the central and peripheral nervous system, and it is thought to maintain vascular endothelial integrity. Pratesi *et al*^[27] found that anti-endomysium IgA antibodies, which were shown to bind to transglutaminase^[28], reacted with human brain vessel structures. This suggests an autoimmune mechanism for the association between CD and ischemic stroke. This hypothesis has also been proposed by Dogan *et al*^[7], who presented a case of stroke and dilated cardiomyopathy associated with CD.

As far as the present case is concerned, the cause of recurrent ischemic strokes remains uncertain. The negativity of indices of inflammation and autoantibodies would exclude systemic vasculitis. The hypothesis of a primary vasculitis of the central nervous system still remains. In fact, this condition cannot be ruled out even by the negativity of cerebral angiography^[29], and also cerebral biopsy is of limited value for the diagnosis due to its low sensitivity^[29]. However, in addition to the lack of small vessel involvement on neuroimaging, the benign course of the disease in our patient in the absence of immunosuppressive treatment makes this hypothesis unlikely.

In conclusion, although the involved mechanisms are still unclear, CD should be considered as a potential etiology of stroke of unknown cause, particularly in young patients, even in the absence of gastrointestinal manifestations.

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Endoscopic diagnosis of Barrett's esophagus

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Abstract

The Prague C and M Criteria have been developed for the objective endoscopic diagnosis of Barrett's esophagus (BE). BE arises between the squamocolumnar junction and the gastroesophageal junction at the proximal margin of the gastric folds. In this study, we reported that 43.0% of the subjects examined were diagnosed with BE based on the Prague C and M Criteria. Previous criticism by John Dent proposed that our data should be considered invalid because the prevalence of BE reported in our study was extraordinarily high and discordant with previous studies. Dent predicted that the position of the gastroesophageal junction in our study was judged to be lower than the actual position due to the effacement of the proximal ends of the gastric folds because of the routine use of a high degree of air distension during typical Japanese

endoscopic examinations. The endoscopic evaluation of the superior gastric folds is certainly influenced by the degree of air distension of the esophagus. However, in our study, the proximal limit of the gastric mucosal folds was prospectively imaged while the esophagus was minimally insufflated. Then, under a high level of air distension, the distal ends of the palisade-shaped longitudinal vessels were imaged because they are more easily observed when distended. In the majority of patients, the distal ends of the palisade-shaped longitudinal vessels correspond to the proximal limit of the gastric mucosal folds. Our endoscopic evaluation was appropriately performed according to the Prague C and M Criteria. We suspect that the high prevalence of BE in our study may be due to the inclusion of ultra-short-segment BE, which defines BE with an affected mucosal length under 5 mm, in our positive results.

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Key words: Barrett's esophagus; Gastroesophageal junction; Squamocolumnar junction; Digital endoscopic images; Endoscopy

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TO THE EDITOR

In our previously published retrospective study^[1], we reported that a total of 43.0% [short-segment Barrett's esophagus (SSBE, 42.6%); long-segment Barrett's esophagus (LSBE, 0.5%)] of the patients examined were diagnosed with Barrett's esophagus (BE) based on the Prague

C and M Criteria^[2]. The study population included 869 patients, of whom 463 were men and 406 were women. The median age of the patients was 66 years and the patient age ranged from 29 to 91 years. The study population consisted of patients who underwent upper gastrointestinal endoscopic examination as part of a routine health check at the Yokohama City University Hospital. During endoscopic examination and imaging of the esophageal mucosa, the gastroesophageal junction (GEJ) was prospectively photographed prior to air distension. All of the endoscopic images were digitalised and were independently and retrospectively reviewed by two endoscopists trained to diagnose BE based on the Prague C and M Criteria^[2]. Any inconsistencies in the assessment of the endoscopic images were resolved by a joint review of the questionable endoscopic images by the two endoscopists. The Prague C and M Criteria for the endoscopic diagnosis of BE is based on the presence of columnar-appearing mucosa between the squamocolumnar junction and the GEJ, which composes the proximal margin of the gastric folds.

The findings of our study are consistent with a previous Japanese study on the occurrence of BE diagnosed based on the Prague C and M Criteria^[3]. The reported frequency of BE could be affected by differences in the interpretation of what constitutes BE, especially with regard to whether histological confirmation of specialised intestinal metaplasia of the esophagus is required. Many physicians from Western countries believe that confirmation of intestinal metaplasia by an esophageal biopsy is necessary to correctly identify BE^[4] because this condition is considered a risk factor for esophageal adenocarcinoma^[5]. However, in our study, BE was diagnosed endoscopically based on the Prague C and M Criteria without histological confirmation; therefore, positive results were referred to as endoscopic BE.

In a recent review article, Dent^[6] opined that our study appeared to be fatally flawed to the extent that our data should be considered invalid. His rationale was that the reported prevalence of BE in our study was extraordinarily high and discordant with previous studies. Dent predicted that the position of the GEJ was judged to be lower than its actual position due to the effacement of the tops of the gastric folds by the routine use of high levels of air distension during our endoscopic explorations.

The endoscopic evaluation of the proximal limit of the gastric folds is certainly influenced by the degree of air distension of the oesophagus. Furthermore, the American Gastroenterological Association workshop in Chicago has concluded that the proximal limit of the gastric mucosal folds is best visualised when the esophagus is minimally distended, although further work

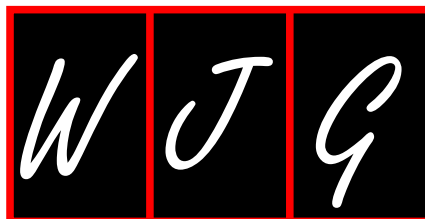
may be required to define minimal insufflation^[2]. When endoscopically examining and imaging the GEJ, our protocol includes the prospective imaging of the proximal limit of the gastric mucosal folds while the esophagus is minimally insufflated. Then, we image the extreme distal ends of the palisade-shaped longitudinal vessels, which are considered topographical landmarks for the GEJ, under high levels of esophageal air distension^[7]. The distal ends of the palisade-shaped longitudinal vessels are much more easily observed under high levels of oesophageal air distension than when the oesophagus is minimally insufflated. In most study populations, the most distal ends of the palisade-shaped longitudinal vessels correspond to the proximal limit of the gastric mucosal folds.

Therefore, our endoscopic evaluation of the position of the GEJ was appropriately performed according to the Prague C and M Criteria without histopathological examination. We suspect that the relatively high prevalence of BE reported in our study may be due to the inclusion of ultrashort-segment BE, which defines BE with an affected mucosal length under 5 mm, in our positive results.

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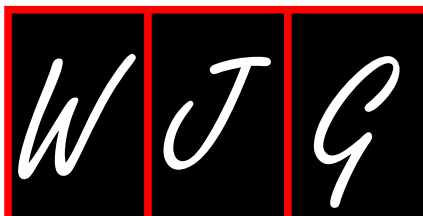
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MEETINGS

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori*
Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical
Oncology 2012 Gastrointestinal
Cancers Symposium
San Francisco, CA 3000,
United States

January 19-21, 2012
2012 Gastrointestinal Cancers
Symposium
San Francisco, CA 94103,
United States

January 20-21, 2012
American Gastroenterological
Association Clinical Congress of
Gastroenterology and Hepatology
Miami Beach, FL 33141,
United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing
Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts
Oesophagus: Everything you need
to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week
2012
Montreal, Canada

March 1-3, 2012
International Conference on
Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal
and Endoscopic Surgeons Annual
Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on
Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and
Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and
Hepatology
San Antonio, TX 78249,
United States

March 31-April 1, 2012
27th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on
Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary
Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress
2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166,
United States

April 20-22, 2012
Diffuse Small Bowel and Liver
Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual

Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian
Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-
American Society of Colon and
Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology
Nurses and Associates Annual
Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and
Rectal Surgeons Annual Meeting
San Antonio, TX 78249,
United States

June 18-21, 2012
Pancreatic Cancer: Progress and
Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International

Neurogastroenterology and Motility
Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory
Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society
2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of
Gastroenterology and Abdominal
Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies
in the Management of Viral Hepatitis
Prague, Czech

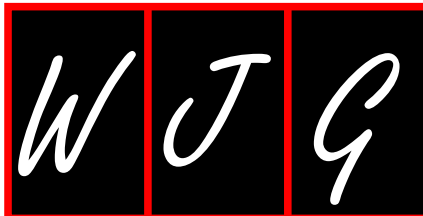
October 19-24, 2012
American College of
Gastroenterology 77th Annual
Scientific Meeting and Postgraduate
Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in
Diagnosis and Treatment of
Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101,
United States

November 9-13, 2012
American Association for the Study
of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel
Diseases
Hollywood, FL 33028, United States



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ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

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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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- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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

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

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 15 Morse SS. Factors in the emergence of infectious dis-

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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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