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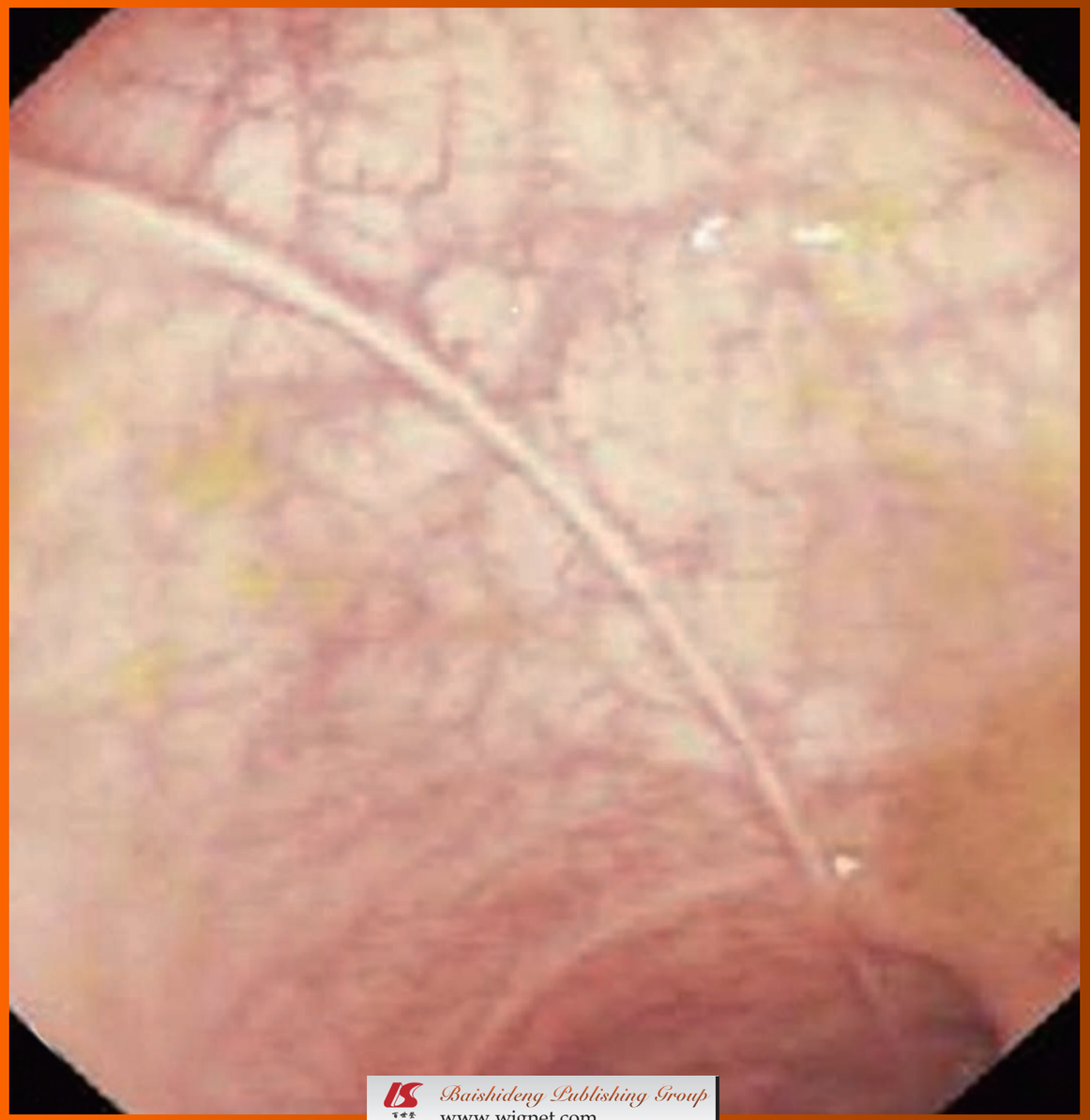

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EDITORIAL

Selection criteria for cytoreductive surgery and hyperthermic intraperitoneal chemotherapy in gastric cancer

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Abstract

Peritoneal carcinomatosis in gastric cancer is associated with a dismal prognosis. Systemic chemotherapy is not effective because of the existence of a blood-peritoneal barrier. Cytoreductive surgery and intraperitoneal chemotherapy can improve survival and quality of life in selected patients. Patient selection for this multimodal approach is one of the most critical issues, and calls for interdisciplinary evaluation by radiologists, medical and surgical oncologists, and anaesthetists. This article sets forth criteria for selection of gastric cancer patients suffering from peritoneal carcinomatosis.

Key words: Peritoneal carcinomatosis; Gastric cancer; Hyperthermic intraperitoneal chemotherapy; Cytoreductive surgery; Selection criteria

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INTRODUCTION

Gastric cancer is one of the most frequent causes of cancer-related mortality worldwide[1,2]. Surgical resection after neoadjuvant chemotherapy in primary locally extended cases remains the mainstay for treating patients suffering from this disease. Surgery is limited by various factors: impaired general status, severe concomitant diseases, and distant metastases. One form is peritoneal dissemination of cancer cells within the abdominal cavity. Peritoneal carcinomatosis (PC) is detected in more than 30% of patients with advanced gastric cancer, and almost 60% of deaths are caused by peritoneal dissemination. In contrast to lymphatic and hematogenous metastasis, peritoneal carcinomatosis can be considered a local disease limited to the peritoneal cavity. Based on this rationale, cytoreductive surgery and intraabdominal chemotherapy have become a relevant treatment option for patients.

Various intraabdominal chemotherapy protocols have been established, varying from hyperthermic intraperitoneal chemotherapy (HIPEC), to early postoperative intraperitoneal chemotherapy, normothermic intraperitoneal chemotherapy, and delayed postoperative intraperitoneal chemotherapy[3]. They differ in the heat of the administered agent, the chemotherapy dosage, and time of administration of the chemotherapy. HIPEC seems to have the most beneficial impact on overall survival[3]. Retrospective analyses of patients treated with cytoreductive surgery plus HIPEC show a clear survival benefit when complete cytoreduction was possible. The randomized trial by Fujimoto *et al*[4] in 141 gastric cancer patients, who were curatively resected, showed a significantly reduced peritoneal recurrence rate and improved long-term survival when HIPEC was part of the treatment as

compared to the "surgery alone" group. This observation was confirmed by Kim *et al*[5], who showed a significantly lower peritoneal recurrence rate and an improvement in the five-year survival rate in the multimodally treated patients as compared to the "surgery alone" group.

Systemic chemotherapy is not as effective as surgery plus HIPEC because of the blood-peritoneal barrier[6].

For metachronous peritoneal carcinomatosis from gastric cancer, there is no evidence to show which patient should be treated with the presented multimodal strategy. The dilemma arises when patients are very young and systemic chemotherapy is ineffective. In such cases, an individual approach with maximal tumor debulking may be an option and is justified in highly selected patients. However, in metachronous peritoneal carcinomatosis, the tumor often extensively involves the abdominal cavity with infiltration of the retroperitoneum, liver hilus, etc., which makes surgery impossible.

The aim of this article is to summarize the recent knowledge on patient selection for cytoreductive surgery and perioperative intraperitoneal chemotherapy for primary gastric cancer with peritoneal carcinomatosis or positive cytology.

GENERAL STATUS

Patients with limited peritoneal carcinomatosis from gastric cancer do not suffer from symptoms such as dysphagia or dysmotility, and PC is frequently found even in low T and negative N stages. Therefore, if the general status is acceptable, the option of radical surgical treatment should always be considered.

A detailed preoperative anesthesiological check-up is of importance and all patients should undergo a preoperative lung and cardiac function test. Concomitant diseases, that may influence surgical and anesthesiological risks, should be identified. As for most general elective operations, a low ASA score is mandatory. Age is still a matter of concern, because biological age does not always correlate with numerical age. However, most groups dealing with cytoreductive surgery and HIPEC restrict patient selection to an age below 65. Most importantly, informed consent with discussion of all alternative therapies must be obtained from the patients, and patients should be offered psychooncological support.

PREOPERATIVE DIAGNOSTICS

A high-end computed tomography is actually the standard and should be performed also to exclude extraabdominal spread and liver metastases. Recently, we demonstrated that positron emission tomography computed tomography (PET-CT) correlates well with intraoperative tumor load in peritoneal carcinomatosis[7]. However, gastric cancer with PC is frequently of mucinous character; therefore, PET-CT is not helpful in selecting candidates for radical resection. Nodules smaller than 5-8 mm cannot be consistently detected[8,9]. In particular, nodules on the small bowel and its mesentery are difficult to diagnose, but relevant for indication.

To date, there is no imaging method that can sufficiently predict intraoperative tumor load. Therefore, explorative laparoscopy is an invasive alternative for candidates in whom radiological work-up was not sufficient to determine operability.

Laparoscopy permits determination of the peritoneal carcinomatosis index (PCI) and cytology in locally advanced cases. Laparoscopy is highly accurate for the diagnosis of peritoneal carcinomatosis, with good correlation to the open surgical exploration found by Yonemura *et al*[9].

Therefore, every patient should undergo explorative laparoscopy before neoadjuvant therapy or primary gastrectomy. As the first step of treatment, some groups even administer HIPEC *via* laparoscopy in patients with synchronous PC or positive cytology[10,11].

PCI

PCI describes the tumor load in the abdomen and varies from 0 to 39, depending on the compartments involved[12].

In contrast to colorectal cancer, where the PCI should be lower than 20 so that patients potentially profit in terms of overall survival[13], in gastric cancer, the PCI should be much lower, because the biological behaviour of the tumor is more aggressive.

In a recent work by Yonemura *et al*[14], complete cytoreduction was achieved in 91% of the patients when the PCI was lower than 6, but in only 42% of the patients with a PCI $\leqslant$ 7. Overall survival was also better in the PCI $\leqslant$ 6 group. In gastric cancer with peritoneal carcinomatosis, lymph nodes should be removed only if they are infiltrated; however, prophylactic D2 lymphadenectomy is unnecessary.

Patients with liver metastasis, involving para-aortic lymph nodes and extraabdominal metastases, are not candidates for cytoreductive surgery and HIPEC. The treatment of metachronous metastases remains controversial, and cancer masses tend to infiltrate the retroperitoneum and liver hilus with vascular structures, which makes surgery impossible.

COMPLETENESS OF CYTOREDUCTION SCORE

The completeness of cytoreduction score CC score describes the completeness of cytoreduction after operation. Ideally, all tumor nodules can be removed macroscopically (CC0). Otherwise, a CC1, CC2 or CC3 score describes non-resectable tumor nodules that vary in size and influence on prognosis. Non-resectability is caused either by diffuse peritoneal carcinomatosis with a high PCI, where surgical resection is oncologically not justified, or by diffuse infiltration of the small bowel or the mesenterial axis, and infiltration of the retroperitoneum.

NEW PROTOCOLS

Neoadjuvant intraperitoneal systemic chemotherapy pro-

tocol (NIPS) is a newly developed neoadjuvant intraperitoneal treatment modality developed by Yonemura *et al*[15]. A good predictor of the possibility of achieving a CC0 status is preoperative cytology. Yonemura *et al*[16] performed NIPS and achieved CC0 status in 27 of 52 patients with negative cytology, but only in four of 27 with positive cytology. Peritoneal wash cytology may, therefore, be a good predictor of the potential for CC0 status.

SUMMARY AND FUTURE PERSPECTIVES

Gastric cancer with peritoneal carcinomatosis is a biologically aggressive tumor, and surgery is still the gold standard of treatment if abdominal spread is limited and PCI is low, ideally < 10. HIPEC may have a potential impact on remaining free cancer cells, although it has not been proven in randomized trials. In metachronous peritoneal carcinomatosis, the surgical approach is often limited by the extensive intraabdominal tumor load and by the aggressive biological behaviour of the tumor itself. NIPS is a promising therapy, and may improve resectability and survival. Intense research is currently being done in experimental peritoneal carcinomatosis, which will eventually modulate current indications.

Concerning promising biomarkers, Phosphoglycerate-kinase 1 (PGK1), an adenosine-triphosphate (ATP)-generating enzyme of the glycolytic pathway, which also affects DNA replication and repair, seems to be an interesting enzyme that is significantly involved in the pathogenesis of gastric cancer and PC[17-19]. Concerning tumorigenesis, it is assumed that genes involved in the glycolytic pathway are responsible for providing solid tumor cells with ATP. A newly discovered link between metabolic changes, including PGK1, and differentiation, has intriguing connections to an old hypothesis advocated by Otto Warburg for tumor metabolism. Further, recent *in vitro* and *in vivo* studies showed that PGK1 overexpression is associated with an elevated tumor invasion and metastatic rate in gastric cancer[17-19]. Those results demonstrate that PGK1 might be a crucial enzyme enabling cancer cells to metastasize, and, therefore, may serve as a target molecule for therapy in gastric cancer in the near future.

CONCLUSION

Nowadays, a radical combined treatment should be considered for a motivated patient with good performance status and low-grade peritoneal carcinomatosis. In addition, the patient should be sent to a peritoneal surface malignancy center.

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TOPIC HIGHLIGHT

Anastasios Koulaouzidis, MD, MRCP, *Series Editor*

Distinct colonoscopy findings of microscopic colitis: Not so microscopic after all?

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Abstract

Microscopic colitis (MC) is considered an "umbrella term", comprising two subtypes, i.e., collagenous colitis (CC) and lymphocytic colitis (LC). They are classically associated with normal or unremarkable colonoscopy. In the last few years, reports have been published revealing findings that are thought to be characteristic or pathognomonic of MC, especially CC. A systematic electronic and manual search of PubMed and EMBASE (to December 2010), for publications on distinct endoscopic findings in MC, resulted in 42 relevant reports for inclusion in this review. Eighty eight patients with collagenous colitis were presented. Only one publication describing a distinct endoscopic pattern in LC was found. Typical findings in CC are alteration of the vascular mucosal pattern, mucosal nodularity, a sequence of change from mucosal defects to mucosal cicatricial lesions, and perhaps (although of doubtful relevance) mucosal pseudomembranes. A causal connection of mucosal defects with the use of lansoprazole seems to exist. Adoption of the proposed lesion description herein is recommended in order to improve homogeneity of future reports.

Key words: Collagenous colitis; Microscopic colitis; Endoscopy; Mucosa; Lesion

Peer reviewer: Dr. Stefan Riss, Department of Surgery, Medical University of Vienna, Währingergürtel 18-20, 1090 Vienna, Austria

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INTRODUCTION

Microscopic colitis (MC), regarded as a rare entity in the early 80s (and certainly overlooked), has now emerged as an increasingly common cause of chronic, non-bloody/watery diarrhea[1].

MC is an "umbrella term", comprising two entities/subtypes, i.e., collagenous colitis (CC) and lymphocytic colitis (LC)[2]. The two entities are characterized by a variable, yet apparently benign, clinical course of protracted, non-bloody diarrhea and classically normal or unremarkable colonic mucosa on endoscopy[3]. In 1984, Gledhill[4] established that thickening of the colonic acellular basement membrane by > 15 μm is invariably associated with diarrhea.

The histological abnormalities in MC are discontinuous, subtle and often unequally located in the colon, making it necessary to take multiple biopsies from various

colonic regions for identification of the pathognomonic microscopy, i.e., thickened sub-epithelial collagen band and increased intraepithelial lymphocytes[5] (Figure 1).

However, there are occasions where endoscopy reveals findings that are thought to be characteristic or pathognomonic of MC, and especially CC. Although the estimated prevalence of MC is up to 10% in patients with chronic diarrhea[5], there are few reports of macroscopic findings in MC. This review attempts to describe the known characteristic endoscopy findings in MC and to categorize them in different types.

PATHOPHYSIOLOGICAL BACKGROUND

CC was first described in 1976, independently in Sweden by Lindström[6] and in Canada by Freeman[7], while LC was first described by Lazenby *et al*[8] in 1989. An increase in their incidence has been recently reported, but this is most likely an artifact secondary to increased awareness and prompt diagnosis[9]. In the absence of persistent endoscopic findings, diagnosis is based mainly on specific histological criteria[9].

It is not clear whether CC and LC are separate entities or part of the spectrum of a single disease[2]. With regard to pathogenesis, several hypotheses have been suggested, including inflammation secondary to medication, smoking, immune dysfunction, autoimmunity, and/or infection.

Studies of collagen typing in patients with CC have produced conflicting results. Electron microscopy findings have suggested that the collagen in CC appears similar to that found in granulation tissue, supporting the hypothesis that its presence would suggest a reparative response to injury[10]. In fact, it is plausible to assume that overproduced, multiple, and different collagen types may deposit in the sub-epithelial layer of the colon and manifest clinically as CC[11]. Günther *et al*[12] showed that increased connective tissue growth factor expression might be the final mediator of local fibrosis in CC.

Non-steroidal anti-inflammatory drugs (NSAIDs) have been implicated as causative factors, through their ability to inhibit prostaglandin synthesis from the colonic mucosa. More recently, several reports have been published incriminating proton pump inhibitors (PPIs), especially lansoprazole, in the induction of CC. Most of the findings to support this came from the temporal relationship of resolution of symptoms with cessation of NSAID or PPI therapy. PPI-induced conformational changes in the cytoskeleton of epithelial cells may result in alterations in the function of the tight junction, leading to increased paracellular permeability. Keszthelyi *et al*[13] postulated that this could allow the luminal contents to easily penetrate the lamina propria causing an immune and/or inflammatory reaction. On this basis, and in light of some recent reports[14], which incriminate lansoprazole as the main cause of linear mucosal defects in CC, it may be plausible to suggest that CC is a syndrome with various causes and perhaps graded histopathology.

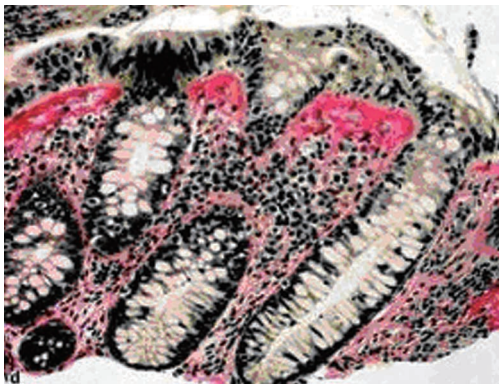

Figure 1 Van Gieson Stain, sub-epithelial collagen table.

The purpose of this review is to present the published experience on distinct endoscopic findings in MC and suggest a unifying lexicon for the reported lesions.

SEARCH STRATEGY

We conducted a PubMed and EMBASE computer search (to December 2010) in order to identify articles on microscopic colitis and endoscopic findings. Our search strategy for PubMed was ["Colitis, Microscopic" (MeSH) or "Colitis, Collagenous" (MeSH) or "Colitis, Lymphocytic" (MeSH)] and ["Endoscopy" (MeSH) or "colonoscopy" (MeSH) or "intestinal mucosa" (MeSH)]. We confined our search to articles in humans but we did not apply any language restriction. In order to search EMBASE we used the following key words: "collagenous colitis", "microscopic colitis" or "lymphocytic colitis", "endoscopy" or "colonoscopy". A further search of electronic journals was undertaken.

Duplicate articles identified in PubMed or EMBASE were manually deleted. The first selection, based on the title and/or abstract was carried out by one of the authors (AK). From the outset, we agreed not to include for further review reports or studies on endoscopic technology, e.g., confocal laser endomicroscopy, which is not yet widely available or restricted to a small number of tertiary institutions. The full paper of each potentially relevant report was then obtained. Thereafter, the two authors independently assessed publications for inclusion in the review. In addition, the reference lists of relevant reports and review papers were cross-searched, in order to identify papers that our initial computer search may have missed.

The following data were extracted from each included publication: year of publication and first author, country of origin, number of cases reported, gender and age of the cases, described endoscopic findings, histopathological diagnosis, post-endoscopy/clinical complications and any important clinical associations (Table 1).

SEARCH FINDINGS

Our initial computational search returned 89 articles in

Table 1 Overview of reports of endoscopic findings/appearance of collagenous colitis

Year	Ref.	No. of cases, gender, age	Endoscopic findings	Lesion location collagen table thickness	Clinical associations	Complications
1990	Giardiello *et al*[15]	1, M, 60	Pseudomembranes	S colon 50-70 μm	Watery diarrhea, received NSAIDs/antibiotics	None
1993	Richieri *et al*[16]	1, F, 43	Linear mucosal tears/lacerations	R colon	Watery diarrhea, abdominal pain	None
			Absent vascular mucosal pattern	30-40 μm	Successful therapy with steroids, some bloody stools	
			6 mo later; many linear cicatricial lesions			
1993	Smiley *et al*[17]	1, F, 53	Carpet-like patch with nodularity (5 cm)	R colon	Watery diarrhea	None
			Similar to sessile villous adenoma	20-40 μm	Therapy with bulking agents	
1995	Katanuma *et al*[18]	1, F, 72	Diminished vascular pattern	Pancolonic	RA on sulindac, diarrhea and wt loss	None
			Edematous/red mucosa	n/s	Treated by discontinuation of NSAID and salazosulphopyridine	
1997	Katsinelos *et al*[19]	1, M, 65	Multiple red mucosal spots	R colon	Watery diarrhea,	None
			Diminished vascular pattern	n/s	Successful therapy with steroids	
1997	Yabe *et al*[20]	1, F, 47	Multiple red mucosal spots	Pancolonic	6 F/U colonoscopies	None
			Diminished vascular pattern	n/s	showed no improvement	
1998	Sato *et al*[21]	1, F, 78	Crowded/tortuous vascular pattern	R + T colon	Watery diarrhea	None
			I/C spray: coarse/nodular surface	n/s		
1999	Bermejo *et al*[22]	1, F, n/s	Pseudomembranes and aphthae	n/s	Watery diarrhea, received	None
				n/s	NSAIDs/antibiotics	
2001	Freeman *et al*[23]	1, F, 37	Deep, elliptical mucosal defect/ulcer	S colon	Watery diarrhea, acute abdomen	Perforation
				n/s	Diagnostic laparotomy + IV antibiotics	
2001	Yagi *et al*[24]	1, F, 77	Mucous-covered lesions in R colon	R + T colon	Watery diarrhea, 4	None
			Ulcer in descending colon	30-60 μm	colonoscopy linear lesions in rectum, ASA-associated	
			2nd look: rectal pseudomembranes			
2002	Cruz-Correa *et al*[25]	2, F, (73/61)	Deep lacerations/tears	R and T colon	All had hypothyroidism	None
		1, M, 62	0.5-4 cm long (to muscularis mucosa)	n/s	Therapy with tetracycline/5-ASA	
2003	Kakar *et al*[26]	8, F, (a. r: 37-91)	Linear ulcers or lacerations (5)	R colon (5)	Aspirin and NSAID-associated CC	None
		1, M, 27	Diminished vascular pattern (2)	S colon (3)	Treated with discontinuation, bismuth	
			Aphthae (2), pseudomembranes (1)	n/s	Mesalamine or azathioprine/6-MP	
2003	Sato *et al*[27]	1, F, 78	1st colonoscopy: 3 mm nodule	Pancolonic	Watery diarrhea, wt loss	None
			2nd look: crowded/tortuous vascular pattern	R: 40-70 μm	ASA-associated	
			I/C spray: coarse and nodular, uneven surface	L: 20 μm		
2003	Byrne *et al*[28]	1, F, 27	Erythematous mucosa	S colon	Watery diarrhea,	None
			Multiple pseudomembranes	n/s	common variable immunodeficiency (CVID)	
2003	Yuan *et al*[29]	6, F, (a. r: 54-81)	Linear ulcers (1), R colon ulcers (2), inflamed rectum (1)	T colon n/s	Pseudomembranes in CC, only endoscopic cases included	None
2004	Buchman *et al*[30]	1, F, 58	Hemorrhagic mucosal spots and	R colon	Prednisolone, antibiotics,	None
		1, F, 46	erythema, granularity/ pseudomembranes		TPN, PPI, hypoalbuminemia	
2004	Sherman *et al*[31]	3, F, (a. r: 66-73)	Mucosal tears and fractures	R + T colon	Watery diarrhea, wt loss,	Perforation
		1, M, 69	Granularity of mucosa at places	40-50 μm	hypoalbuminemia	in 3/4 cases
2006	Wickbom *et al*[32]	3, F, (a. r: 73-86)	Mucosal tears and fractures (4-5 cm long)	R + T colon	All on aspirin	None
			Mucosal scars on repeat colonoscopy	14-40 μm	ACE/lansoprazole-induced (1 case)	
2006	Koulaouzidis *et al*[33]	1, F, 83	Mucosal tears	Cecum n/s	Iron deficiency anemia	None
2007	Poupardin-Moulin *et al*[34]	1, F, 80	Longitudinal mucosal fractures	R + T colon n/s	No significant clinical associations, diagnosis missed	None

2007	Smith *et al*[35]	1, F, 43	Long, linear mucosal fractures	R colon n/s	Treated with sulfasalazine	Perforation Hemicolectomy
2007	McDonnell *et al*[36]	3, n/s, n/s	Bright linear marks/parallel corkscrew lesions: "cat scratch" colon	R colon n/s	n/s	none
2008	Allende *et al*[37]	9, F, (a. r: 44-80)	Mucosal fractures to muscularis propria (7)	R colon (6)	2/12 underwent barium enema	Perforation all cases
		1, M, 71	Ragged mucosal defect (1)	T colon (3)		2 during colonoscopy
			Wall induration (1), constriction (1)	L colon (1)		
2008	Umeno *et al*[38]	7, n/s, (a. r: 37-92)	Longitudinal mucosal defects (ulcers/tears)	L colon	Only in the lansoprazole treated group	None
			Longitudinal scar in one case	12.5-50 μm		
2008	Hashimoto *et al*[39]	1, F, 66	Whirling/circling mucosal vessel network	Pancolonic	SLE, treated with mesalamine	None
			Linear (20 cm) ulcer/scar in the descending	n/s	2nd look: normal vessels, smaller scar	
2009	Watanabe *et al*[40]	1, F, 68	Multiple, longitudinal thin ulcers	L colon 30 μm	Lansoprazole, discontinued and healed	None
2009	Yusuke *et al*[41]	1, F, 78	Ragged and linear, long mucosal tear	S colon	Abrupt abdominal pain, PR blood	None
			Hypertrophic scar	n/s	Lansoprazole, discontinued	
2009	Cuoco *et al*[42]	1, F, 68	Deep linear ulcer-type defects	R + L colon	Watery diarrhea, abdominal pain	None
			7 cm long in ascending	n/s	4 L PEG for cleansing	
2009	Dunzendorfer *et al*[43]	1, F, 60	3 cm hypertrophic mucosal scar	S colon n/s	Long history of constipation Wt loss, combination therapy	None
2009	Chiba *et al*[44]	1, F, 70	Distinct diffuse mucosal cloudiness	Pancolonic	On lansoprazole and loxoprofen,	Reoccurred on a further Lansoprazole course
			Indistinct vascular pattern (UC-like pattern)	n/s	treated with sulfasalazine	
2009	Sekioka *et al*[45]	1, F, 82	2 longitudinal mucosal fractures	T colon	Lansoprazole-associated (6 mo)	Peritonitis, pre-endoscopy
			2nd look: A ridge-type cicatricial lesion	n/s	Treated by discontinuation	
2010	Couto *et al*[46]	1, F, 48	Hemorrhagic mucosal tears Longitudinal white ridges/lines	T + L colon n/s	OA on nimesulide and lansoprazole, abdominal pain, wt loss (10%)	None Colonoscopy halted at T colon
2010	Sawada *et al*[47]	1, M, 77	Disappearance of vascular network,	L colon	Lansoprazole-associated (6 years)	None
			Red (numerous) mucosal spots	25 μm	Wt loss, treated by discontinuation collagen table reduced on 2nd look	
2010	Koulaouzidis *et al*[48]	1, M, 83	Fine cicatricial line	L colon	n/s	None
		1, F, 45	Hypertrophic mucosal scar	n/s		
2010	van Velden *et al*[49]	1, F, 63	Linear tears Diminished vascular pattern and edema 2nd look colonoscopy: multiple linear scars	R + S colon 20 μm	Instrumentation-induced and insufflation-induced mucosal tears	Perforation Treated conservatively
2010	Nomura *et al*[50]	1, F, 67	Linear mucosal defect x 2, Linear scar in sigmoid, I/C spray	L colon n/s	Lansoprazole-associated Improved on discontinuation	None Painful left abdomen
2010	Miyagawa *et al*[51]	1, M, 81	Longitudinal mucosal defect	L colon n/s	Lansoprazole and hemodialysis	None
2010	Milestone *et al*[52]	3, F; 1, M (a. r: 57-75)	Long (5-20 cm) linear ulcers, non-hemorrhagic with evidence of healing	S colon n/s	Treated with budesonide and/or bismuth subsalicylate	None
2010	Kawamura *et al*[53]	3, n/s, n/s	Longitudinal mucosal ulcers	L + S colon n/s	Lansoprazole induced	None
2010	Fasoulas *et al*[54]	1, F , 68	"Cat scratch" colon	R colon n/s	n/s	None
2010	Cimmino *et al*[55]	4, F, (a. r: 24-77)	Mosaic pattern (honeycomb image), I/C spray: for delineation of pattern	Rectum+ S colon n/s	Case control study Mosaic pattern had high LR+/spec	None

n/s: Not stated; M: Male; F: Female; a. r: Age range; I/C spray: Indigo carmine spray; R colon: Right colon; T colon: Transverse colon; L colon: Left colon; S colon: Sigmoid colon; LR: Likelihood ratio; spec: Specificity; UC: Ulcerative colitis; TPN: Total parenteral nutrition; OA: Osteoarthritis; ASA: Acetyl salicylic acid; wt: Weight; PPI: Proton pump inhibitor; 6-MP: 6-mercaptopurine; PEG: Polyethylene glycol; ACE: Angiotensin converting enzyme; NSAIDS: Nonsteroidal antiinflammatory drugs; CC: Collagenous colitis; SLE: Systemic lupus erythematosus; RA: Rheumatoid arthritis.

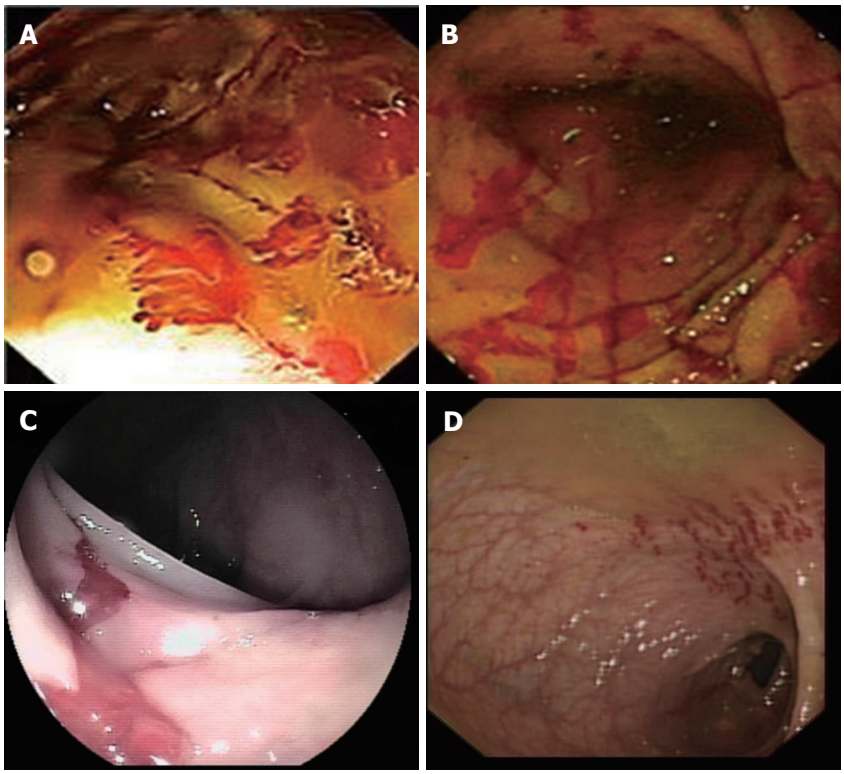

Figure 2 Colon lacerations/mucosal breaks in collagenous colitis. A, B and C: Colon lacerations/mucosal breaks; D: Cat-scratch colon.

PubMed and 499 in EMBASE. Nineteen and 50 articles, from PubMed and EMBASE respectively, were included for further review. After obtaining the full papers, 35 papers were selected. Another seven publications were identified from references lists and included in the final analysis.

The terms mucosal break, defect, tear, fracture or laceration were used indiscriminately. For the purpose of this review and in order to standardize the terminology, we agreed to use the term "mucosal defect" as a collective one, under which there are two subtypes of lesions: (1) mucosal lacerations/tears which are the longitudinal (superficial or deep) and mainly fresh/hemorrhagic in appearance mucosal breaks (Figure 2); and (2) mucosal fractures describing the deeper (with occasional exposure of the muscularis mucosa) and white-based or more chronic looking mucosal defects (Figure 3).

Although to an extent arbitrary, we believe that this terminology will aid the introduction of a universal lexicon for future reports of similar lesions. It is obvious that in accordance with the above, the "cat scratch colon" belongs to the first category, i.e., mucosal lacerations or tears.

Eighty eight cases [65 females, 10 males, 13 not stated (n/s); median age: 67 years] were reported in 41 publications. Of these, 14 publications were from Japan[18,20,24,27,37-41,44,45,47,50,51], 12 from the United States[15,17,25,26,28-31,35-37,43], three from the United Kingdom[33,48,52], two each from France[16,34], Sweden[21,32], and Greece[19,54], and one each from Argentina[55], Canada[23], Italy[42], the Netherlands[49], Portugal[46], and Spain[22]. Where reported, the submucosal collagen table thickness ranged from 14-70 μm. The only publication reporting endoscopic findings in LC described the presence of a subtle mucosal change in an 85-year-old female[56].

Gardiello *et al*[15] were the first to report distinct endoscopic findings in CC (i.e., pseudomembranes), but in fact it was Richieri *et al*[16] who first described the presence of multiple linear mucosal lacerations with sharp edges in the right colon of a 43-year-old female, with subepithelial collagen table thickness of 30-40 μm. Eventually, on repeat colonoscopy 6 mo later the lesions had healed, resulting in fine cicatricial lines on an otherwise unremarkable colonic mucosa. Therefore, Richieri *et al*[16] had effectively pointed to a pattern seen in some of the reports that followed, i.e., the continuum of laceration to cicatricial healing of the mucosa.

Since this report, 53 cases (34 females/6 males/13 n/s; median age: 69 years) of linear, long or shorter and finer (cat-scratch type) mucosal tears, fractures and ulcers have been reported[25,26,29,31-42,45,46,49-54]. Sixteen patients with mucosal defects were on lansoprazole, and in the majority, discontinuation of the medication resulted in symptomatic, endoscopic and histopathological improvement.

On the other hand, only 11 (10 females/1 male) cases of mucosal cicatricial lesions have been reported to date, identified either during the index colonoscopy that revealed the mucosal defects, or at follow-up colonoscopic examinations[16,32,38,41,43,45,46,48-50]. The lesions ranged from hypertrophic (celoid-type mucosal scars)[32,38,41,43,45,46,48-50] to fine, cicatricial lines[16,48] (Figure 4).

We did not manage to establish an association of any

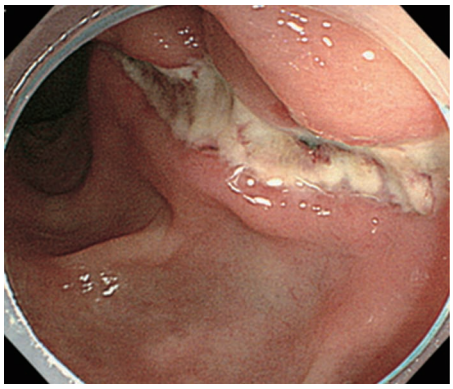

Figure 3 Colon mucosal fractures in collagenous colitis.

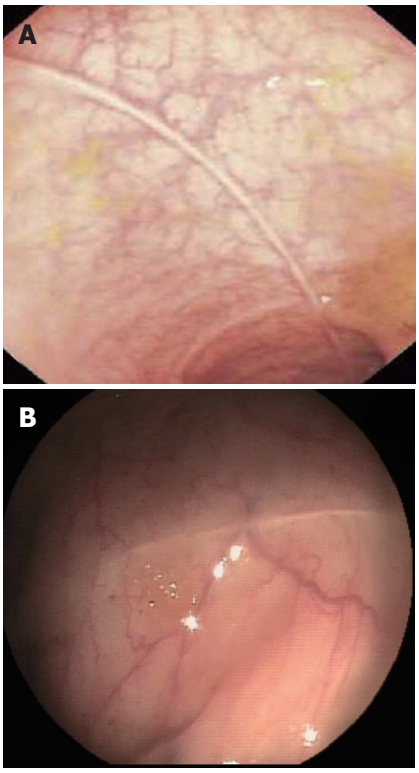

Figure 4 Cicatricial mucosal lesions in collagenous colitis (A and B).

of these lesions either with the collagen table thickness or with symptom severity in the review cohort.

The right colon (for the purpose of this review defined as the area from the cecum to the hepatic flexure), irrespective of the type of findings, was affected in 32 cases, the transverse colon in 16 and the left (descending, sigmoid and rectum) colon in 32. Five reports presented cases with pancolonic mucosal involvement[18,20,27,39,44].

Although the sign of a mosaic pattern or mucosa nodularity ("honeycomb mucosa") was noted first by Smiley *et al*[17] in 1993 in the ascending colon of a 53-year-old woman, a retrospective case-control study was only published in 2010[55]. In the appropriate clinical context of watery diarrhea, the "honeycomb pattern" had an odds ratio of 19.4 with a specificity of > 99% for diagnosis of CC. The authors though pointed out that, due to both the retrospective nature of the study and the high possibility of under-reporting, this may be an overestimation.

Dye spray (indigocarmine), for improved delineation of the identified lesions, was utilized in four reports[21,27,50,55], and seems helpful in the context of subtle mucosal changes and/or disturbed vascular architecture. However, this should be balanced against the greater resource implications and procedure time.

With regard to complications, there were 17 recorded perforations/peritonitis in the review cohort[23,31,35,37,45,49]. As expected, these were all associated with cases where mucosal defects (tears or fractures) were evident on colonoscopy[52,57].

WHAT IS CURRENTLY KNOWN

We found four broad categories of distinct endoscopic findings in CC: (1) pseudomembranes[15,22,24,26,28,30]; (2) mucosal vascular pattern alteration which includes an indistinct appearance of the blood vessels and a variable degree of pruning of the mucosal vasculature, or a crowded, dilated and tortuous capillary network[16,18-21,26,27,39,44,47]; (3) mucosal abnormalities such as red spots and some mucosal nodularity or textural alteration, evident with or without chromoendoscopy[17,19-21,27,30,31,55]; and (4) a continuum of mucosal breaks/defects, i.e., mucosal lacerations/tears, including the so-called "cat scratch colon" pattern, or fractures usually along the long axis of the colonic wall[16,25,26,29,31-42,45,46,49-54]to the fine linear cicatricial lines or thick scar-like ridges of the mucosal surface (effects of the mucosal healing process of mucosal defects)[16,32,38,41,43,45,46,48-50]. There was only one publication describing a characteristic endoscopic pattern in LC[56].

Hemorrhagic mucosal breaks have an appearance that could be liberally described as "colon craquelé"[16]. The term mucosal fracture was introduced by Sherman *et al*[31] in 2004 and it is admittedly a successful descriptive one. Thickened and abnormal sub-epithelial collagen table leads, at some areas, to loss of attachment with the epithelial component, and this in turn causes stretching of the mucosa over the deeper wall layers, and eventually tearing of the detached mucosal surface (in a "zip" fashion, hence the longitudinal lesions). The sharply demarcated margin of these mucosal defects, as if the mucosa has been slashed with a sharp knife, helps to differentiate them from ischemic colitis[50].

Mucosal defects are more likely to be found in the right colon as a result of a colonic insult, i.e., instrumentation or air insufflation due to the abundant presence of a thicker and denser (hence dysfunctional) collagen type Ⅲ table, in association with increased colon diameter on that side[25,37,58]. The right colon thinner wall and its expansion to a greater diameter during fecal storage and transit, produce greater relative wall tension (Laplace's law, i.e., tension on the wall of a cylinder is proportional to the radius). Therefore, a competent ileocecal valve and a deformed sigmoid are sufficient to cause colonic air

entrapment in a closed space[59], and eventually "cracking" of the brittle colonic mucosa[11,25,31,32,48,58]. Although the colon can not be seen as a simple cylinder[37], we suggest that these breaks can occur spontaneously, and postulate that increased intra-colonic pressure during peristalsis and defecation leads to mucosal stretching and defects that will heal with time leaving behind various types of cicatricial lesions[33,48].

McDonnell *et al*[36] coined the term "cat scratch colon" to describe the red linear marks in the cecum or ascending colon seen in 21 of 8277 patients undergoing colonoscopy. They reported a 14% prevalence of CC of in their cohort. They also postulated that these marks were due to barotrauma from insufflation[36,59-61]. However, it is unclear whether biopsies were taken in all patients undergoing the test for diarrhea, other than in those that had the "cat scratch" appearance. Furthermore, endoscopic findings are non-specific for CC and have been described in the normal colon (attributed to barotrauma from excessive insufflation during colonoscopy), in diversion colitis, and even in chronic cholestasis[54,61,62].

The true prevalence of mucosal tears is unknown due to the rarity of reported cases, but it is estimated to be around 1%. Under the assumption that not all of the relevant cases have been reported, the true prevalence may be much higher. However, based on the type of publications included in this review, i.e., case reports or series, it is not possible to estimate prevalence. In addition, practices vary worldwide and up until recently flexible sigmoidoscopy was considered sufficient to diagnose MC (it is believed that left-sided biopsies probably miss less that 5% of MC cases, due to its patchy nature), and as lesion awareness rises, the incidence of macroscopic findings will increase[63]. On the other hand, the increased frequency of reports published during the last decade show that there is an increased awareness of the distinct endoscopic appearances in MC, and perhaps endoscopist enthusiasm may result in over-diagnosis (as mucosal tears/scratches have been described in the normal colon, diversion colitis and in lansoprazole colitis[36,64,65]) of an entity whose main hallmark remains histological confirmation.

It is also now known that mucosal defects in CC represent a marker of increased risk of colonic perforation[52,54]. A recent review found 21 cases of perforation in CC. The majority of these were either colonoscopy-associated (15 cases) or barium enema-associated (four cases), while the rest seem to have occurred spontaneously[57].

There are several reports of remission, including disappearance of the collagen layer on follow-up. This would indicate that an environmental factor such as medication may be responsible in susceptible individuals. NSAIDs or PPIs have been implicated. It has also been suggested that collagen plate thickness is greater with lansoprazole[38]. The pathophysiologic mechanism by which lansoprazole induces microscopic colitis and mucosal defects is not well understood. Although a clear temporal correlation exists, it should be remembered that, due to the fluctuating nature of CC[66], it might simply represent a coincidence, as PPIs are one of the most commonly prescribed drug categories worldwide.

It has been postulated that this may be due to higher concentrations of drugs such as NSAIDs in the right colon[26]. However, it is possible that more right sided biopsies are taken because of endoscopic abnormalities, more likely to be observed in the right colon, as mentioned above. More case control studies and multivariate analysis may provide the answer[14].

In conclusion, the endoscopic appearances of CC are becoming more familiar amongst the endoscopic community. We recommend adoption of the proposed lesion description herein in order to improve homogeneity of future reports.

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REVIEW

Optimizing 6-mercaptopurine and azathioprine therapy in the management of inflammatory bowel disease

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Abstract

The thiopurine drugs, 6-mercaptopurine (6-MP) and azathioprine, are efficacious in the arsenal of inflammatory bowel disease (IBD) therapy. Previous reports indicate that 6-thioguanine nucleotide (6-TGN) levels correlate with therapeutic efficacy, whereas high 6-methylmercaptopurine (6-MMP) levels are associated with hepatotoxicity and myelotoxicity. Due to their complex metabolism, there is wide individual variation in patient response therein, both in achieving therapeutic drug levels as well as in developing adverse reactions. Several strategies to optimize 6-TGN while minimizing 6-MMP levels have been adopted to administer the thiopurine class of drugs to patients who otherwise would not tolerate these drugs due to side-effects. In this report, we will review different approaches to administer the thiopurine medications, including the administration of 6-mercaptopurine in those unsuccessfully treated with azathioprine; co-administration of thiopurine with allopurinol; co-administration of thiopurine with anti-tumor necrosis factor α; 6-TGN administration; desensitization trials; and split dosing of 6-MP.

Key words: Azathioprine; Drug levels; Inflammatory bowel disease; 6-Mercaptopurine; Thiopurine

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INTRODUCTION

Inflammatory bowel disease (IBD) encompassing Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory disorder caused by dysregulated immune responses in a genetically predisposed individual. Given the role that the immune system plays in IBD, the hallmark of therapy is immune modulation. The thiopurine drugs, 6-mercaptopurine (6-MP) and its prodrug azathioprine (AZA), remain the mainstay of immunomodulator therapy for IBD and are indicated in steroid-dependent and -refractory patients, as prophylaxis in CD[1-3]. Chebli found that AZA maintained steroid-free clinical remission for three years in UC patients, previously steroid-dependent[2]. Of note, however, AZA has not been shown to be effective in treating active UC flare[4]. Rather, thiopurines have also been noted to induce and maintain remission in UC and CD patients, more effectively than 5-aminosalicylic acid[1,5-10]. However efficacious, their use

is often limited, as an estimated 30% to 50% of patients discontinue these drugs due to either side-effects or lack of clinical efficacy[11-13]. The lack of response to these immunomodulators has been attributed to differences in individual variations in drug metabolism[14,15]. The 6-thioguanine nucleotide (6-TGN) metabolite of 6-MP and AZA appears to be the predominant active metabolite responsible for therapeutic efficacy, whereas 6-methylmercaptopurine (6-MMP) levels correlate with the risk of hepatotoxicity and possibly myelotoxicity[15,16]. Theoretically, if the thiopurine metabolite profile can be shifted to 6-TGN, a greater percentage of IBD patients would benefit from immunomodulator therapy. A meta-analysis has confirmed that higher 6-TGN levels are associated with remission among IBD patients[17]. In this review, we will discuss the thiopurine metabolic pathway, monitor the drug metabolite levels, and evaluate the different approaches that have been developed to enhance clinical efficacy and minimize the side-effects of AZA and 6-MP.

THIOPURINE METABOLIC PATHWAY

To achieve the active cytotoxic form, AZA is metabolized *via* a series of biochemical pathways summarized in Figure 1. Initially, approximately 90% is non-enzymatically cleaved to 6-MP in the liver[18,19]. There are three competitive metabolic pathways in 6-MP metabolism. It can be inactivated to 6-thiouric acid (6-TU) *via* xanthine oxidase (XO), activated to 6-MMP *via* thiopurine methyltransferase (TMPT), or to the therapeutic 6-TGN *via* enzymes hypoxanthine phosphoribosyl transferase (HPRT), inosine monophosphate dehydrogenase (IMPDH), and guanosine monophosphate synthetase (GMPS)[11,20,21]. A complete understanding of its mode of action is unknown[19]; however, based on its structural similarity to the purine guanine, 6-TGN is a purine antagonist that inserts within the DNA of leukocytes[22]. Intracellular build up of 6-TGN is thought to be the cytoactive form that inhibits DNA synthesis and downstream T cell proliferation for its immunosuppressive activity[20,23,24]. Using a genome-wide expression profiling approach, 6-TGN was found to inhibit several immune and inflammation-related genes including tumor necrosis factor-related apoptosis-inducing ligand, tumor necrosis factor receptor superfamily member 7, and α4-integrin in activated but not resting T lymphocytes[25]. Thus, 6-TGN may additionally exert its immunosuppressive effect by down-regulating the expression of pro-inflammatory and gut-homing factors. Another report found that the immunosuppressive role of thiopurine medications may in part be due to its metabolite 6-thioguanine triphosphate (6-TGTP) suppression of the Rac1 protein, which participates in T cell maturation and proliferation, thus inducing T lymphocyte apoptosis[19,26].

MONITORING THIOPURINE METABOLITE LEVELS

Because AZA is 55% of 6-MP by molecular weight and

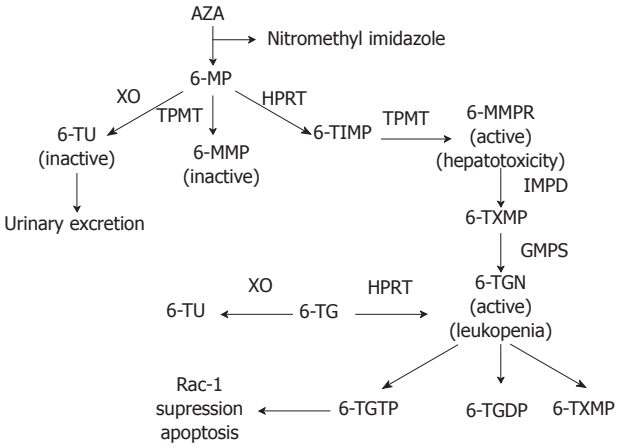

Figure 1 Thiopurine metabolic pathway. Metabolic pathway for AZA and 6MP is shown in the diagram. AZA: Azathioprine; 6-MP: 6-mercaptopurine; 6-TU: Thiouric acid; 6-MMP: 6-methylmercaptopurine; TIMT: Thiopurine methyltransferase; 6-MMPR: Methyl-mercaptopurine ribonucleotide; TXMP: 6-thioxanthosine monophosphate; 6-TGN: Thioguanine nucleotide; 6-TG: Thioguanine; 6-TGDP: 6-thioguanine diphosphate; 6-TGTP: 6-thioguanine triphosphate; XO: Xanthine oxidase; TPMT: Thiopurine methyltransferase; HPRT: Hypoxanthine phosphoribosyl transferase.

88% of AZA is converted to 6-MP, historically, thiopurines are dosed by the patient's weight; the maintenance dose of AZA is 2-2.5 mg/kg per day and 6-MP is dosed at half that of AZA, or 1-1.5 mg/kg per day in IBD patients[1,22,7-29]. Individual variation in drug metabolism account for the differences in therapeutic efficacy and development of adverse reactions[30,31]. Fortunately, advances in thiopurine metabolite monitoring can help predict which patients are more at risk of developing side effects, allowing for adjustments in drug dosages[11,15].

TPMT is a key enzyme whose activity determines the level of 6-MMP as well as 6-TGN metabolite levels[15,20,24,30]. TPMT methylates 6-MP to 6-MMP and 6-TIMP to 6-methylmercaptopurine ribonucleotide (MMPR) (Figure 1)[15]. Elevated levels of 6-MMP (> 5700 pmol/ 8×10^8 erythrocytes) are associated with hepatotoxicity, whereas 6-TGN is the metabolite responsible for the therapeutic activity of thiopurines. Monitoring the thiopurine metabolite levels can help to optimize immunomodulator therapy and minimize adverse events. A retrospective study showed that patients who did not respond to AZA or 6-MP either had a high 6-MMP concentration or 6-MMP/6-TGN ratio[12]. Furthermore, a subset of IBD patients preferentially metabolize thiopurines to the hepatotoxic 6-MMPR which explains why some patients develop toxic metabolite accumulation, side effects and ultimately cannot be maintained on thiopurine therapy[30]. Even though 6-TGN is associated with therapeutic immunosuppressive activity, an excess amount of this metabolite poses an increased risk for myelosuppression[32]. The therapeutic efficacy of 6-MP or AZA are correlated with 6-TGN levels between 235-450 pmol/8×10^8 erythrocytes[15,22,33,34]. Because several studies have shown that weight-based dosing is poorly correlated with 6-TGN levels[16,35,36], monitoring thiopurine metabolite levels can help optimize immunomodulatory therapy while mini-

mizing adverse effects[15,30,34].

Differences in patient response to thiopurines may in part be due to patient-specific metabolism and genetic variation[30]. TPMT activity is inversely related to clinical response to AZA[11]. Different genetic polymorphisms code for the level of TPMT activity[37]. 0.3% of the Caucasian population are homozygous for low enzyme activity; 11% are heterozygous and 89% are homozygous for high enzyme activity[38]. Allelic frequency patterns vary among different ethnic groups. In Caucasian populations, intermediate or low TPMT activity is most frequently associated with TPMT*2, TPMT*3A or TPMT*3C alleles[38], while in African-Americans, TPMT*3C is the most prevalent variant allele[39]. Wild-type or heterozygous TPMT deficient patients have high TPMT activity > 14 units/mL RBC, which were associated with higher levels of 6-MMP and lower levels of 6-TGN, and thus a decreased likelihood of achieving complete remission (termed 6-MP resistance) and increased risk for hepatotoxicity[11,13,30]. Dose escalation of thiopurine level may optimize 6-TGN levels, but must be done under caution given that TPMT also catalyzes the formation of the toxic metabolite 6-MMPR[40]. A meta-analysis found that TPMT polymorphisms are related to adverse drug reactions and myelotoxicity, but not hepatotoxicity or pancreatitis[37]. It is thought that the higher level of TPMT activity may cause higher 6-MP catabolism resulting in higher 6-MMP and decreased 6-TGN levels[11]. Low TMPT and thus high 6-TGN is associated with a higher risk for leukopenia[13,15].

Studies have found that checking TMPT activity may be cost-effective as compared to standard therapeutic dose administration[33,41]. Traditionally, AZA or 6-MP was started at a low dose and progressively titrated up because of safety concerns (bone marrow suppression, hepatotoxicity, etc.). Using this strategy, time to initial response is delayed and can take up to 6 mo to reach therapeutic response[6,33,42,43]. Compared to traditional thiopurine dosing, monitoring TMPT can allow faster achievement of initial response (22.4 wk *vs* 18.9 wk) and lower costs at 1 year ($7142 *vs* $3861)[33]. Thus, patients found to have normal TPMT could have dose escalation sooner therefore avoiding delay in achieving response[33]. The cost-effectiveness of measuring TPMT activity was independently shown in a separate study[41]. Furthermore, awareness of TMPT activity can help to avoid potential deleterious consequences of thiopurine therapy. For example, in patients with low TPMT activity, a lower initiation dose or avoidance of either 6-MP or AZA is recommended due to risks of leukopenia[16,44]. Albeit, TMPT activity monitoring is not universally available to all practitioners; in these cases, thiopurine may be started at a low-dose (50 mg daily) and titrated up with weekly monitoring of CBC and liver function tests during the first 2 mo, and once every 3 mo thereafter[20].

APPROACHES TO OPTIMIZING THIOPURINE METABOLITES

Use of 6-MP in patients who are intolerant of AZA

In addition to bone marrow suppression and hepatotoxicity, early hypersensitivity reactions including fever and gastrointestinal side effects including diarrhea, nausea, and emesis can occur in as many as 10% of patients[45]. These adverse reactions often cause IBD patients to discontinue thiopurine therapy. Several studies have shown that among patients intolerant of AZA, 6-MP may be a safe and effective alternative[29,45,46]. One study showed that 20 of 29 (69%) IBD patients with a history of AZA hypersensitivity tolerated 6-MP[46]. An AZA to 6-MP change appears to be more effective in UC compared to CD patients as by the end of the first year, none of the CD patients were maintained on 6-MP[46]. In addition to hypersensitivity reactions, up to 60% of IBD patients with AZA intolerance due to nausea, emesis, and flu-like illness tolerated switching to 6-MP[45]. In contrast, patients who discontinued AZA due to hepatotoxicity or pancreatitis were less likely to tolerate 6-MP[45]. Another study found that 48% of patients previously intolerant to AZA due to myalgia and arthralgia were able to tolerate 6-MP[16]. Another report showed that 11 of 15 (11 CD, 4 UC) patients (73.3%) who discontinued AZA due to epigastric pain, nausea and vomiting tolerated 6-MP and reached therapeutic goals[47]. A retrospective study showed that 19 out of 140 patients discontinued AZA therapy (4 patients for clinical inefficacy, 13 due to side-effects, 2 due to leucopenia)[48]. Of these 19 patients, 11 (58%) tolerated the switch to 6-MP[48]. Consistent with the above findings, another report showed that 6 of 11 patients who initially could not tolerate AZA, were able to tolerate 6-MP and achieve response[18]. The reasons behind the observation that 6-MP bypasses the adverse reactions caused by AZA are unclear, but may in part be due to the nitro-imidazole structure that is released as AZA is cleaved to 6-MP[49].

Based upon the above studies, we propose that 6-MP should be considered in IBD patients who require continuing immunosuppressive therapy but are intolerant of AZA. We caution that there has been variable success among those who are switched to 6-MP (Table 1), and unfortunately many of the same reactions to AZA develop with 6-MP over time.

Use of AZA in 6-MP intolerant patients

The converse treatment strategy of administering AZA to patients who did not tolerate initial 6-MP therapy has not proved as effective[48,50,51]. In a trial of AZA after 6-MP adverse reactions, similar side-effect profiles were seen[18,48,50,51]. This is likely due to the fact that AZA is converted in the liver to 6-MP (Figure 1), thereby, yielding similar adverse reactions (Table 1). Based upon the lack of clinical efficacy, we do not recommend using AZA in patients who were previously intolerant of 6-MP.

Desensitization

Some investigators propose desensitization in the subset of patients who experience hypersensitivity reactions to AZA or 6-MP within the first month of treatment. Korelitz *et al*[51,52] retrospectively reviewed 591 charts of IBD

Table 1 Summary of strategies to optimize thiopurine metabolite levels

Method	Effectiveness (%)	Side effect
AZA to 6-MP	48-73[45-48]	Nausea, vomiting, hepatotoxicity, neutropenia, pancreatitis
6-MP to AZA	Not effective[48,50,51]	Nausea, vomiting, hepatotoxicity, neutropenia, pancreatitis
Desensitization	25[51]	Hypersensitivity reaction
Combination infliximab/ thiopurine	25[72,74]	Lymphoma, infection
6-TG	46-82[50,58]	NRH, veno-occlusive disease and possible tumor
Allopurinol supplementation	25-75[68,69]	Skin rash, renal impairment, leukopenia
Split-dosing	60[75]	Reduces 6-MP, AZA associated adverse effects

AZA: Azathioprine; 6-MP: 6-mercaptopurine; NRH: Nodular regenerative hyperplasia; 6-TG: 6-thioguanine.

patients treated with 6-MP. Four of 16 patients who had early hypersensitivity reactions were successfully desensitized to 6-MP or AZA and achieved long-term clinical remission. One patient tolerated the direct switch from 6-MP to AZA. Of the remaining 11 patients, 5 needed surgery, 2 were changed to methotrexate (MTX), and 4 had chronic symptoms. In this study, desensitization began at one-quarter tablet per day, with an increase in the dose every 3 d for several weeks until a full dose was reached[51,52]. A similar case report describes a CD patient who developed skin rash after 4 wk of treatment with AZA[53]. Upon drug withdrawal, the rash resolved. A skin test was positive for AZA allergy, suggesting an IgE mediated hypersensitivity; and the patient was desensitized with AZA, with the patient's CD successfully in remission[53]. Another case report describes a CD patient who developed a macular, erythematous truncal rash and fever after treatment with 6-MP[54]. This patient was able to tolerate 6-MP after desensitization[54]. The process of desensitization for patients with hypersensitivity reactions to AZA or 6-MP may be an empiric strategy for maintenance of immunomodulator therapy. However, we caution that more studies are needed to confirm the efficacy of this strategy.

6-thioguanine

As a possible alternative to those who cannot be maintained on 6-MP or AZA, treatment using 6-TG has been proposed. This drug has been used in children with acute lymphoblastic leukemia[55]. Compared to AZA, 6-TG is directly converted to 6-TGN by HPRT (Figure 1)[56,57]. Since 6-TG is a poor substrate for TPMT, hepatotoxic 6-MMPR production would be low[50,55]. Therefore, 6-TG bypasses several of the steps in thiopurine metabolism that are responsible for producing toxic metabolite build-up and its association with the aforementioned potential adverse effects[13,50]. One study found that of the 49 CD patients who were either resistant or intolerant to AZA or 6-MP, 46% of patients at 6 mo and 79% of patients at 12 mo were in remission and none of the patients developed pancreatitis or bone marrow toxicity[58]. Although up to 82% of patients tolerated 6-TG[50], this drug has been associated with several possible toxicities, notably nodular regenerative hyperplasia (NRH). One study found that the prevalence of NRH to be 16 out of 26 (62%) biopsies taken from patients treated with 6-TG[59]. Several studies further found that the incidence of NRH associated with 6-TG use varied from 4%-27% among thiopurine naive patients (Table 1)[60-62]. Other 6-TG associated adverse effects include secondary liver tumors, veno-occlusive disease, and other vascular liver pathologies[50,58,63-65]. Formal dose-ranging studies for 6-TG are lacking and only limited data are available on the therapeutic efficacy and dosing regimes[13]. As a general rule, dosage should not exceed 25 mg daily, as higher dosing has been associated with an increased risk of developing NRH[13,66].

6-TG can be considered a rescue drug in IBD patients intolerant of or refractory to AZA or 6-MP. However, given the potential complications including NRH, and the small number of long-term safety monitoring and limited formal dose-range studies, we do not recommend 6-TG therapy at this time.

Allopurinol supplementation

In some patients, with AZA or 6-MP dose escalation, rather than achieving therapeutic levels of TGN, 6-MMP levels increase and resultant hepatotoxicity ensues[67]. These patients are known as preferential 6-MMP metabolizers[30]. Among IBD patients with high TPMT activity who favor 6-MMP production with reduced 6-TGN levels, adding the XO inhibitor allopurinol can favor the production of 6-TGN over 6-MMP[68,69]. The addition of allopurinol to 6-MP or AZA resulted in improved disease activity as measured by the partial Harvey Bradshaw index in CD and Mayo scores in UC patients; decreased prednisone maintenance dose, and improved liver function laboratory values[68,69]. However, at the 18 mo follow-up, 25% of these patients were escalated to anti-tumor necrosis factor (TNF) α therapy and 2 required surgery.

The exact mechanism of shifting thiopurine metabolites from 6-MMP to 6-TGN by the XO inhibitor is unknown, but may involve reduced production of the inactive thiouric acid (TU) metabolite in favor of the cytoactive metabolites 6-MMP and 6-TGN[67,70,71]. However, if XO inhibition favors MMP and TGN production, then the toxic 6-MMPR would also be expected to increase (Figure 1). Interestingly, allopurinol does not increase the level of 6-MMPR or its associated hepatotoxicity, but mainly shifts the metabolite to 6-TGN.

Potential side effects of allopurinol include skin rash and renal impairment[68]. In addition, the addition of allopurinol to thiopurine therapy may lead to supra-

therapeutic levels of 6-TGN, leading to leukopenia[40,68]. Therefore, it is recommended that if allopurinol is used in combination with 6-MP or AZA, the dose of thiopurine medications should be reduced by at least 50% with close laboratory monitoring for leukopenia with weekly CBC monitoring during the first month, followed by every other week for the next month.

Combination therapy: Thiopurine and anti-TNF

Anti-TNF therapies are generally used for patients who are refractory to first-line medications[72,73]. Colombel *et al*[72] conducted a randomized, double blind study of moderate-to-severe CD patients, comparing infliximab and AZA alone *vs* in combination and found that the primary endpoint, steroid-free remission at 26 wk, was achieved in a greater number among those treated in combination *vs* monotherapy. Mucosal healing, a secondary endpoint was also greater among patients who received combination therapy. These patients were immunosuppressant- and biologic therapy-naïve patients. Caution must be used, however, as studies have found an increased risk of non-Hodgkin's lymphoma in patients treated with anti-TNF with a history of thiopurine use[74].

Split dose administration of thiopurines

As discussed above, patients who are preferential 6-MMP metabolizers exhibit high 6-MMP levels with subtherapeutic 6-TGN levels when thiopurines are dosed in the traditional weight-based, once-a-day fashion. 6-MP/AZA dose escalation in this subset of patients in an attempt to push the 6-TGN level into the "therapeutic range"-often results in dose-dependent leukopenia, transaminitis and/or flu-like symptoms (headache, nausea, myalgia, fatigue, general malaise). Overproduction of 6-MMP and side-effects resolve with dose reduction, but the lower dose often fails to adequately suppress IBD disease activity, resulting in suboptimal symptom control.

Anecdotally, we observed that simply splitting the daily dose of thiopurine (e.g., 50 mg *BID* rather than 100 mg once daily) can reduce the 6-MMP metabolites while maintaining 6-TGN levels. To confirm our observation, we performed a retrospective chart review of patients with baseline 6-MMP levels greater than 7000 pmol/8 × 10^8 red blood cells (RBC) who underwent split dosing (n = 20). Dividing the daily thiopurine dose led to a significant reduction in 6-MMP levels (11 879 *vs* 5955 pmol/8 × 10^8 RBC; $P < 0.0001$) without adversely affecting clinical disease activity (HBI) or 6-TGN levels (250 *vs* 227 pmol/8 × 10^8, P = NS)[75]. Side-effects associated with 6-MMP, such as abnormal liver function test (LFT), leukopenia and flu-like symptoms, improved in seven of eight patients[75]. After a mean follow-up of 42 mo, 12 of 20 patients were able to be maintained on a split dose of 6-MP with control of their IBD activity[75]. To our knowledge, this is the first study to demonstrate the effectiveness of dose splitting on preferential metabolism. This approach has several advantages over other strategies. Dose splitting does not sacrifice potential efficacy associated with dose reduction, and may even allow for further upward titration of thiopurine to efficacy if needed. It avoids the introduction of possible additional medication side effects as can be seen with co-administration of allopurinol and the potential cost burden of designer biologic inventions. This maneuver is relatively simple for both patients and practitioners alike.

Independent studies are needed to confirm that split-dose administration of thiopurine is an effective approach to manage 6-MMP preferential metabolizers. However, in an IBD patient who might otherwise not tolerate immunomodulator therapy and require ongoing steroid exposure and/or escalation of therapy to biologics, splitting the daily dose of 6MP or AZA may be attempted.

POTENTIAL RISK OF LYMPHOPROLIFERATIVE DISEASE

Thiopurines have been linked with chromosomal abnormalities and an increased risk of lymphoma among rheumatoid arthritis patients[76]. Whether the same risk exists in IBD patients is controversial. A meta-analysis of six studies showed a four-fold increased risk of developing lymphoma among IBD patients treated with immunomodulators[77]. Similarly, in the CESAME prospective cohort study by Beaugerie, a five-fold increased risk of lymphoproliferative diseases was shown among IBD patients on thiopurines[78]. Whether this risk stems from underlying disease or iatrogenic medication is unclear. Studies have shown that the risk is greater in IBD patients who are male and between 15 to 40 years old[79,80]. One study focused on hepatosplenic T-Cell lymphoma and noted cases among men younger than 35 years who had been treated with either anti-TNF and thipopurines or thiopurine monotherapy[79]. In contrast, a study by Vos *et al*[81] found no increased risk of lymphoma among a nationwide study of 17 834 IBD patients. Interestingly, however, they did find an association between Epstein-Barr Virus-positive lymphoma and thiopurine use. EBV-positive lymphoma implies the effect of immunosuppression as a factor[77,80,81]. Nevertheless, the consensus has been that the benefits of thiopurines outweigh the risk[77,82]. Lewis *et al*[82] conducted a decision analysis and found that a 9.8-fold increase in lymphoma is needed to favor an alternative therapy over AZA. Further studies are needed, as it has yet to be determined how this risk changes with discontinuation of thiopurines[77].

CONCLUSION

Given the complex metabolism of thiopurines and the individual variability among patients in response to this medication, various dosing strategies have been adopted. Several approaches have been promising among patients who develop toxicities to the initial strategy. However, no single strategy has proven completely effective in all patients. Further studies will inevitably provide more in-

formation to assist in optimizing administration of these vital IBD medications. This is significant given that there are a limited number of medications available in the IBD arsenal. In all dosing strategies, however, close monitoring of metabolites as well as determination of pharmacogenetics are pivotal for patient safety and medication efficacy.

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REVIEW

Barrett's esophagus with high-grade dysplasia: Focus on current treatment options

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Abstract

High-grade dysplasia (HGD) in Barrett's esophagus (BE) is the critical step before invasive esophageal adenocarcinoma. Although its natural history remains unclear, an aggressive therapeutic approach is usually indicated. Esophagectomy represents the only treatment able to reliably eradicate the neoplastic epithelium. In healthy patients with reasonable life expectancy, vagal-sparing esophagectomy, with associated low mortality and low early and late postoperative morbidity, is considered the treatment of choice for BE with HGD. Patients unfit for surgery should be managed in a less aggressive manner, using endoscopic ablation or endoscopic mucosal resection of the entire BE segment, followed by lifelong surveillance. Patients eligible for surgery who present with a long BE segment, multifocal dysplastic lesions, severe reflux symptoms, a large fixed hiatal hernia or dysphagia comprise a challenging group with regard to the appropriate treatment, either surgical or endoscopic.

INTRODUCTION

In the era of minimally invasive therapies, numerous treatment options for Barrett's esophagus (BE) with high-grade dysplasia (HGD) are available. Nevertheless, since therapy is individualized, the standard of care remains debatable for a large number of patients without clear-cut guidelines. The aim of this review is to briefly present and compare current therapeutic modalities with an emphasis on endoscopic approach, outline factors that can aid in the choice of the appropriate treatment (medical, endoscopic or surgical) and underline the lack of a properly designed study so far that compares the outcomes of these therapies.

BE is the result of chronic gastroesophageal reflux disease (GERD) and represents the end stage of the natural course of this disease. It has been estimated that 20% of the population in the United States suffers from gastroesophageal reflux[1] and that about 10% of

these patients are diagnosed with BE[2]. Commonly, BE is discovered during endoscopy for the evaluation of GERD symptoms. The severity of GERD symptoms is not considered an indicator of BE presence, whereas the chronicity of GERD symptoms may be related to the possibility of BE transformation[3]. It is documented that longstanding exposure of esophageal mucosa to gastric acidity results in cellular damage of the stratified squamous epithelium and creates an abnormal environment, which stimulates repair in the form of intestinal epithelial metaplasia[4,5]. Moreover, BE is related to a serious mechanical insufficiency of the lower esophageal sphincter, a functional derangement of the esophageal body, as well as an insufficient esophageal clearance[6-8].

In BE it is possible to encounter three histologic types of columnar epithelium: (1) the specialized intestinal metaplasia type, in which the epithelium exhibits a villous surface and intestinal-type crypts lined by columnar cells that secrete mucous and goblet cells containing mucin; (2) the gastric fundus epithelial type; and (3) the junctional type. Among these three histological types, only the intestinal type represents an important premalignant state.

In BE, the stratified squamous epithelium, which physiologically lines the esophageal mucosa, is replaced by a pathological, specialized columnar epithelium which is neither of cardiac nor of stomach type, but exhibits features of the intestinal type of epithelium[4]. This pathological type of epithelium usually demonstrates DNA alterations that predispose to malignancy[2,9,10]. The alterations in BE are histologically classified into three categories, depending on whether or not they exhibit dysplasia: (1) BE without dysplasia; (2) BE with low-grade dysplasia; and (3) BE with HGD[11-13]. In BE with HGD, dysplasia is confined to the mucosa without crossing the basement membrane. If dysplasia extends beyond the basement membrane into the lamina propria through the in-coming lymphatic network, it is defined as intramucosal (superficial) adenocarcinoma, whereas if it invades the muscularis mucosa layer it becomes invasive adenocarcinoma. Thus, BE with HGD is considered a precursor of invasive adenocarcinoma. Six to twenty percent of patients with BE and HGD are at greatest risk of developing adenocarcinoma within a short period of time, ranging from 17 to 35 mo at follow-up[14]. Esophagectomy specimens from patients with BE and HGD revealed invasive adenocarcinoma in 30%-40% of cases[15]. A recent meta-analysis demonstrated that patients with BE and HGD developed esophageal adenocarcinoma with an average incidence of 6 every 100 patients per year, during the first 1.5 to 7 years of endoscopic surveillance[16]. Furthermore, the majority of esophageal adenocarcinoma is thought to have evolved from cells that have undergone Barrett's metaplasia[17].

BE is also classified into two categories according to the extent of intestinal metaplasia above the gastroesophageal junction: (1) long segment BE, if the extent of the intestinal epithelium is greater than 3 cm; and (2) short segment BE, if it is less than 3 cm[18]. Among patients who undergo endoscopy for symptoms of GERD, the incidence of long segment BE is 3%-5%, whereas short segment BE occurs in 10%-15%[4]. Whether long and short segment BE share the same pathogenetic alterations or the same predisposition to malignancy still remains unclear; however, both conditions are currently treated in the same manner[19].

ENDOSCOPIC SURVEILLANCE IN PATIENTS WITH BE

Concerning the therapeutic management of BE, endoscopic follow-up of the patient at regular intervals, so-called endoscopic surveillance, plays a pivotal role. There is great difference of opinion when dealing with the problems of therapeutic management of BE. The value of endoscopic surveillance in patients with BE represents one of the many controversies that exist amongst gastroenterologists.

As aforementioned, BE represents a strong risk factor for developing adenocarcinoma, which is a particularly lethal malignancy[19]. In order to diminish the risk of cancer development, the American College of Gastroenterology introduced the application of a surveillance protocol that is, in summary, as follows[20]: (1) patients who are diagnosed with BE at two consecutive endoscopies should undergo endoscopy every 3 years; (2) if Barrett's dysplasia is diagnosed, it should be confirmed by a second specialized pathologist; (3) patients who are definitely diagnosed with low-grade dysplasia after examination of sufficient biopsy specimen should undergo endoscopic surveillance every year; (4) patients diagnosed with HGD should undergo a new endoscopy with a second adequate biopsy specimen, to check the possible presence of invasive cancer; (5) if the results are positive, the biopsy specimen should be examined by a second specialized pathologist; and (6) if multiple HGD foci are confirmed, then the patient should undergo either surgical therapy (esophagectomy) or endoscopic surveillance every 3 mo.

This protocol, concerning high-risk patients, is considered strict in various aspects. Many authors believe that surveillance is not justified in a cost-effectiveness analysis[21-23]. Others compare endoscopic surveillance of patients with BE with the endoscopic follow-up of patients with ulcerative colitis for early detection of malignancy or mammography for early diagnosis of breast cancer and conclude that the former is lacking, in terms of cost-effectiveness, compared to the other two[24,25].

Furthermore, many studies have shown that the survival of patients with BE is not different from that of the general population[26]. This observation, as paradoxical as it may appear, can be explained by the low absolute number of adenocarcinoma cases in patients with BE[19]. Current data demonstrate that patients with BE develop adenocarcinoma at a low rate of 0.5% which is, nevertheless, 30-40 times higher than that of the general population[27,28]. The subgroup of patients with BE and HGD

develop esophageal adenocarcinoma at a higher rate of 6.58 per 100 patient-years, as shown in a recent meta-analysis[16]. Moreover, survival studies in patients with BE primarily include elderly individuals, for whom the risk of death from other lethal co-morbid conditions is much higher than the annual 0.5% risk of death from esophageal adenocarcinoma[29]. Apart from the above, a long-term prospective study involving young patients with BE demonstrating a decreased life span in these patients has not yet been published[19].

Concerning the endoscopic surveillance of patients with BE, this is not a risk-free procedure. It is estimated that the risk of development of adenocarcinoma is one in every 200 or 300 patients with BE, whereas the risk of a major complication from an endoscopic procedure is one in 1000 esophagogastroscopies[28-30]. The program of the American College of Gastroenterology also differs from that of the British Society of Gastroenterology[31] and the NHS Technology Review[32] in the value of endoscopic surveillance as a screening tool. Despite the previously mentioned contradictory views, many authors indicate a benefit of endoscopic surveillance in cost-effectiveness analyses for the early diagnosis of cancer[33-36].

CURRENT MANAGEMENT OF BE WITH HGD

Controversy is also perpetuating between surgeons and gastroenterologists. BE with HGD carries a high risk of developing esophageal adenocarcinoma, at a rate of 6%-20%, within a short period of time (17-35 mo)[14,16]. Therefore, in the presence of such risk, the traditional standard therapy was en bloc esophagectomy with regional lymph node dissection. This approach has been supported by the fact that invasive adenocarcinoma was previously diagnosed in patients with HGD at a rate of 30%-40%[15], although more recent data have revealed a significantly lower incidence (12%)[27]. Nevertheless, en bloc esophagectomy carries a high mortality (4%-19%)[37], high postoperative morbidity (20%-47%)[38] and unacceptable late postoperative quality of life[39].

During the last few years, while surgeons try to improve their surgical technique and the results of esophageal resection (esophagectomy without lymph node dissection and/or without thoracotomy, esophagectomy with vagal preservation or laparoscopic esophagectomy), endoscopists have been developing minimally invasive therapeutic methods for the management of BE with HGD. It should be noted that the problem of GERD persists with these endoscopic methods and endoscopic surveillance is necessary for all endoscopic treatment options.

Management of gastroesophageal reflux disease

The therapeutic goal in patients with BE is similar to that of patients with GERD, i.e., relief of symptoms and reversal of the epithelial damage caused by increasing gastric reflux. In cases of BE with HGD, the question is whether medical or surgical management of GERD can have beneficial effects on the dysplastic lesions. Therefore, the following questions come into play:

Can either surgical or medical antireflux therapy achieve regression of the epithelium in BE?

Evidence clearly indicates that medical therapy of GERD does not lead to acceptable results, with regard to the regression of dysplastic epithelial lesions[40]. Surgical therapy may have better results than conservative therapy in terms of regression, but is far from being considered adequate. In a series of five publications that included 151 patients submitted to surgical management of gastroesophageal reflux (fundoplication), surgical therapy achieved full regression of these lesions in 6 patients only, whereas in 31 patients only a decrease in the length of BE lesions was observed and 6 patients developed invasive adenocarcinoma. Furthermore, other published data corroborate that antireflux surgery does not decrease the rate of adenocarcinoma in patients with gastroesophageal reflux[41-43]. Data from the subgroup of patients with BE are also conflicting and pose an unsettled issue[44].

Does antireflux surgery prevent the metaplastic evolution of the mucosa in BE?

Evidence suggests that surgery is superior to conservative therapy as it can abolish, at high rates, the progression of metaplastic mucosal lesions in BE[45,46] and therefore protect from dysplasia and malignancy. On the other hand, systematic review indicates that antireflux surgery in patients with BE is associated with regression of BE lesions and/or dysplasia, but evidence supporting the assertion that surgery decreases the rate of adenocarcinoma comes from non-controlled studies[47]. In a study from the Mayo Clinic in 118 patients who underwent antireflux surgery and follow-up for 18.5 years, it was stated that only 3 patients developed adenocarcinoma within the first three years postoperatively[48]. This outcome suggested that the lesion probably existed during the operation[49,50]. Encouraging data come from patients with low-grade dysplastic mucosa and antireflux surgery who, at endoscopic surveillance, showed conversion from a dysplastic to a non-dysplastic mucosa at a rate of about 70%[51]. Concerning the endoscopic antireflux interventions (Stretta procedure, Bard EndoCinch, Wilson-Cook Endoscopic Suturing Device, NDO Plicator, Enteryx, Gatekeeper Reflux Repair System and Plexiglas), these are currently under evaluation and evidence is lacking to support their role in the therapy of BE with HGD[52].

Should antireflux therapy accompany other treatment modalities when confronting metaplasia or dysplasia of BE epithelium?

The combination of medical or surgical antireflux therapy with endoscopic mucosal ablation has yielded promising results[53,54]. These early observations concluded that the resected mucosa undergoes re-epithelialization by normal squamous epithelium and is preserved with the aid of antireflux therapy; usually proton pump inhibitors. Further research is nevertheless needed in this field.

Endoscopic treatment of BE with HGD

It is documented that BE with HGD or intramucosal adenocarcinoma constitute diseases amenable to cure in most cases. Data from high volume centers of esophageal surgery have indicated rare lymph node metastasis, ranging in incidence between 2%-6%[36,49,55,56]. Newer, less invasive treatment modalities such as endoscopic therapies or less aggressive surgical operations are currently being evaluated in an effort to achieve the least postoperative morbidity and the best quality of life.

Current endoscopic methods include two major therapeutic categories: (1) endoscopic ablation of Barrett's mucosa that can be achieved by thermal, photodynamic and/or radiofrequency energy; and (2) endoscopic mucosal resection.

Thermal therapy

In methods implementing thermal energy, the endoscopic elimination or destruction of the diseased superficial esophageal mucosa is achieved by the administration of heat with one of the following specialized devices: (1) electrocoagulation; (2) argon plasma coagulation (APC); (3) heat probe; and (4) Nd: neodymium-doped yttrium aluminium garnet laser. Another version of thermal therapy consists of cryospray ablation, but experience with this method is limited[57].

The more widely used first two methods of thermal therapy, probably due to greater availability in endoscopy units, provoke a superficial mucosal injury with a low rate of serious complications. APC has been evaluated at twelve independent centers in 444 patients with BE, making this technique by far the most commonly applied method[55]. However, the significant variation in the regression of intestinal metaplasia and the formation of new squamous epithelium, together with the complications of this method, resulted in dismissal of APC as the method of choice[55]. In published series, full regression of BE has ranged from 36%[58] up to 98%[59] in an average time frame of 36 and 12 mo, respectively.

Two studies have focused on the effect of APC on intestinal metaplasia in association with the amount of administered energy. In one, no recurrence of BE was noted, while in the other, recurrent disease occurred in 30% of cases. It is noted that in the patients of the first study (n = 70) a higher energy device (90 W) was utilized and higher doses of omeprazole (40 mg three times a day) were administered. In 69 patients (98.6%) complete BE eradication with associated squamous regeneration was achieved after a median of two APC sessions (range 1-5). During a median follow-up of 12 mo (range 2-51 mo) with continuous acid suppression, no case of dysplasia relapse was noted. Of these patients, only 3 developed stenosis (4.3%), for whom dilatation was advocated for therapy[59]. In the second study, where low energy was administered in 27 patients, 70% showed regeneration of squamous epithelium with no persistent intestinal metaplasia and in 30%, areas of intestinal metaplasia were present under the new squamous epithelium, after a median follow-up of 9 mo (range 6-18 mo). Overall, two cases of perforation were reported, one of which was fatal[60]. In a third study of 33 patients treated with APC energy between 65 W and 70 W and 60 mg omeprazole daily, complete restoration of the normal squamous epithelium was noted in all cases after 1.96 sessions (range 1-4). Esophageal stenosis occurred in 3 patients, for whom dilatation was deemed necessary, 5 patients developed mediastinal syndrome (high fever and pleural effusion) and one patient pneumomediastinum. After a follow-up period of 10.6 mo, only one recurrence of BE was observed[61]. Accordingly, the amount of energy administered with APC seems to be directly related to the recurrence rate of BE, favoring the use of high energy devices for a median follow-up of 9 to 12 mo, although data for long-term effectiveness are still lacking. It should be stated that the emergence of APC-related complications depends not only on the amount of energy, but also on other parameters such as mucosal contact at different pressures and repetitive therapy in the same area[55].

Photodynamic therapy

Photodynamic therapy requires previous administration of a photosensitizer and selection of a specific wavelength of light that stimulates a specific target area or the whole of BE. As a result, singlet oxygen is formed that causes damage to the esophageal mucosa. 5-aminolevulineic acid (5-ALA) is an oral photosensitizing agent that incites severe superficial injury in the patients with HGD and superficial cancer. In the United States, intravenous porfimer sodium, which causes deeper injury, is used. Overholt *et al*[62] applied a technique of introducing a cylindrical inflatable balloon through which light was administered in 101 patients with HGD. After a follow-up of at least 4 years, the analysis of the therapeutic effect showed that in 54% of cases there were no residual BE lesions. Successful eradication of low- or high-grade dysplasia or cancer reached 93%, 78% and 48%, respectively. It is thus suggested that HGD and cancer exhibit the greatest resistance to therapy. The total rate of stenosis reached 30%, reflecting the effect of this therapy in deeper esophageal layers.

Great value to this type of therapy is attributed by a large multicentric, semi-blinded, randomized study by Overholt *et al*[62] in 208 patients with HGD. Patients were randomly divided, in a 2:1 ratio, into a study group treated with photodynamic therapy and omeprazole and a control group receiving only omeprazole. A statistically significant difference ($P < 0.0001$) regarding the complete eradication of HGD was noted in favor of photodynamic therapy (106/158, 77%), compared to the control group (27/70, 39%). The therapeutic response persisted even after 5 years of follow-up. It should be noted that endoscopic ablation was combined with a long-term follow-up and was, thus, more costly. Nevertheless, this approach has proved to be a better treatment option in terms of cost-effectiveness, compared to the standard follow-up and radical surgery for the treatment

of dysplasia, although clinical trials directly comparing these strategies are warranted[63]. Additionally, esophagectomy provided 11.82 quality adjusted life years (QUALYs) compared to photodynamic therapy with 12.31 QUALYs and long-term follow-up[63]. Furthermore, anecdotal time-life analysis of several cases has revealed that many patients with HGD and even early cancer could be controlled with ablative techniques and careful follow-up for 5-10 years[39].

Radiofrequency energy ablation

This method is a novel therapeutic approach employing (1) energy emitted from a controlled radiofrequency (RF) source [Halo360 or Halo90 RFA (where A stands for ablation), BARRX Medical Inc, Sunnydale, CA]; (2) a sizer balloon catheter, that is introduced into the esophagus and measures esophageal width; and (3) an EFA balloon catheter. The controller of the RFA source is preset to deliver energy of 12 J/cm^2 which causes complete destruction beyond the lamina propria[64]. The RFA balloon is 3 cm long and consists of 60 narrowly spaced electrode rings in a bipolar fashion. After the esophageal diameter is measured by the sizer balloon, the RFA balloon catheter is introduced in the esophagus and placed in its position. The balloon is then inflated and the RFA source releases energy circumferentially on the esophageal surface for 300 ms. The whole procedure is performed under general anesthesia[65].

The use of radiofrequency for the ablation of the dysplastic epithelium in BE is more effective, posing less risk for damage beyond the desired limits, while also controlling the depth of the damage[66]. In contrast to photodynamic therapy, radiofrequency mucosal ablation is not associated either with the development of esophageal strictures or with recurrent disease resulting from buried Barrett's glands. According to current opinion, the development of strictures after photodynamic or thermal therapy has been attributed to the circumferential destruction of the mucosa. Despite the fact that during RFA therapy destruction is also circumferential, no strictures are observed, as a result of better control of the depth of ablation attained by this method[65,66]. In order to safely evaluate this method and its long-term effects, studies with larger series, longer duration of follow-up and endoscopic surveillance are expected, so as to document the recurrent dysplasia-free interval.

Recently, endoscopic radiofrequency ablation was evaluated in a study as the definitive treatment of 25 patients with ultralong-segment (≥ 8 cm) BE, using balloon- and/or plate-based devices (BARRX Medical Inc., Sunnydale, CA). Complications for all 25 patients included hemorrhage in one, stricture in two, and nausea and vomiting in two cases. The time from the initial procedure was such that 15 patients underwent at least one post-ablation biopsy. One patient was elected to undergo esophagectomy based on biopsies. Of these patients, 78.5% (11/14) had a complete response. The number of ablations in this group was 2-3 (median 2.5). The authors concluded that the method is safe and feasible in patients with ultralong-segment BE and can be applied to the entire length of intestinal metaplasia during one session[67]. Radiofrequency ablation has also been recommended as a single-modality therapy for flat type mucosa, or as a supplementary therapy after endoscopic resection of visible lesions. The treatment protocol consists of initial circumferential ablation, using a balloon-based electrode, followed by focal ablation of residual Barrett's epithelium. The authors believe that radiofrequency is less frequently associated with stenosis and buried glandular mucosa, in contrast to other ablation techniques. This method has been shown to be safe and effective in the treatment of patients with BE and early cancer[68].

Endoscopic mucosal resection

Endoscopic mucosal resection (EMR) with a curative intent, beyond the scope of mucosal resection for biopsy, is being investigated more than any other endoscopic method for the treatment of HGD in BE. Since the first publication in 2000[69], several other similar reports have emerged in the United States as well as in Europe[56,70-76].

The landmark study by Ell *et al*[69] included 35 low-risk patients with superficial cancer and well or moderately differentiated BE less than 2 cm in diameter who underwent EMR. With an average of 1.3 interventions and an average follow-up time of 1 year, complete regression was observed at a rate of 97% and local recurrence or metachronous cancer at a rate of 17%, with only one case of hemorrhage that was controlled endoscopically. In another study, a group of 70 patients with HGD or early cancer similarly underwent EMR, with an average follow-up interval of 34 mo, and demonstrated regression of lesions in 98% of cases, with a complication rate of 9.5%. Metachronous or recurrent disease occurred in 30% of cases[77].

EMR has demonstrated satisfactory rates of complete regression; up to 82.5% in 550 patients with HGD or Barrett carcinoma, at an average follow-up interval of 12 mo. The best results were documented in patients with HGD and small (< 20 mm), well or moderately differentiated Barrett carcinomas, at a rate of 97%[78]. Recently, in a retrospective, single center study from the University of Chicago, 49 patients, 33 with high-grade dysplasia and 16 with early carcinoma, underwent complete Barrett's eradication with the aid of EMR. The rate of stenosis was significant, but it resolved easily with endoscopic dilatation. The authors noticed the presence of Barrett's epithelium underneath the squamous resection margin (Z line) in 13 of 47 patients (28%) at initial mucosectomy. Based on their findings and surveillance biopsies, they concluded that ablative therapy should extend to 1 cm proximal to the endoscopically determined squamocolumnar junction. They also concluded that EMR, with close endoscopic surveillance, is an effective treatment modality for BE with HGD and intramucosal carcinoma[79]. Another recent study, originating from two Australian academic hospitals, involved 75 patients; 89% with Barrett's HGD

and 11% with early esophageal cancer, who were treated by EMR over a 7-year period. The treatment resulted in complete Barrett's excision in 94% of cases with short segment BE. During the mean follow-up of 31 mo (range, 3-89) there was no recurrence although 11% developed metachronous lesions. Five patients underwent esophagectomy because the endoscopic resection specimen demonstrated submucosal invasion. The complications were one aspiration and six strictures, which were managed with endoscopic dilatation. This study concluded that EMR alters histological grade or local T stage in 48% of patients and dramatically reduces esophagectomy rate, thus providing a safe and effective therapy[80].

The development of EMR allows full eradication of the neoplastic mucosal lesions and simultaneous accurate staging. Nevertheless, the greatest value of this method is focused on the ability to detect a metachronous lesion, in 50% of cases, in the residual portion of BE. From a therapeutic approach, EMR is promising but has been associated with persistent HGD, persistent Barrett's epithelium and serious recurrence rates of dysplasia or neoplasia in the residual Barrett's epithelium, thus necessitating endoscopic surveillance after resection. It is, therefore, obligatory to completely extirpate intestinal metaplasia at its whole extent, a target that can be accomplished with the combination of EMR with other therapeutic modalities. Combinations of EMR and photodynamic therapy with porfimer sodium, 5-ALA, or meta-tetrahydroxyphenylchlorine[73,76,77] have been applied in selected patients and have yielded successful results with regard to the eradication of dysplastic lesions[55].

Another novel therapeutic approach with the intent to eliminate local recurrence involves the use of circumferential mucosal resection with complete excision of the visible Barrett's epithelium[81]. High success rates in eradication of Barrett's epithelium with a low rate of complications have been demonstrated[82,83]. These findings suggest that this method could be beneficial for all patients with BE and HGD or intramucosal cancer. Larghi *et al*[84] have used the technique of cylindrical mucosal excision in 26 patients with BE and HGD as a way to achieve complete excision of Barrett's mucosa. The technique utilized either endoscopic cap suction or endoscopic snare mucosectomy or a combination of both methods. The method of endoscopic cap suction was applied as previously described[80,84], with the aid of commercially available kits (K001 and K002, Olympus America Inc.). The method of endoscopic snare mucosectomy was performed in the way described by Soehendra *et al*[85], in which a single-channel therapeutic endoscope (type GIF-IT, Olympus America Inc.) and a single-channel mucosectomy snare (type D3422161 M-C, Endo-Flex GmbH, Voerde, Germany) were used. From the follow-up of 23 patients over an average period of 28 mo, complete eradication of lesions occurred in 21 patients (87.5%), whereas in one patient Barrett's epithelium developed underneath the neo-squamous epithelium three mo after excision, and in another an HGD nodule was detected and excised at twelve mo during follow-up. Finally, many authors appreciate this method owing to its high therapeutic yield, but also stress the need for additional larger cohort studies, with longer duration of follow-up and endoscopic surveillance, in order to deduce definitive conclusions. It is also suggested that new equipment will aid in the en bloc resection and possibly prove more effective in completely excising the mucosa along with eliminating the possibility of residual Barrett's epithelium. Improvement in the skills needed to perform such techniques with optimal results is expected to accompany technological advances[84,86,87].

CHOICE OF THERAPEUTIC APPROACH

The classical therapy of BE with HGD has been based on the well renowned, *en bloc* esophagectomy with thoracotomy, vagotomy and lymph node dissection; an operation that, as already mentioned, carries high perioperative mortality, morbidity and a poor quality of life. Considering these disadvantages, many patients are considered unfit for such an operation, whereas others fail to accept it as an option. Additionally, studies from high volume centers in esophageal surgery have demonstrated rare lymph node metastasis, in the region of about 5%, rendering lymph node dissection unnecessary[36,88,89]. The above data have led surgeons as well as gastroenterologists to in-depth research regarding less invasive endoscopic procedures and operations with decreased mortality, morbidity and an acceptable quality of life, such as laparoscopic vagal-sparing esophagectomy. However, the decision for the appropriate therapeutic approach is often difficult and currently there is considerable controversy over which method is better, i.e., surgery or endotherapy (techniques involving endoscopy). Nevertheless, it must be noticed that a number of other parameters may affect the choice of the therapeutic method.

The histopathological diagnosis of HGD, or even the distinction between low- and high-grade dysplasia, remains alarmingly subjective. The kappa values for intra-observer and inter-observer variability are 0.64 and 0.45[90] and the accordance for the diagnosis of dysplasia attains a rate of 94% and 88%, respectively. Furthermore, agreement between specialized and non-specialized pathologists as to the definition and the histopathological characteristics of HGD exists in only 50% of cases[88]. When the need to distinguish HGD from intramucosal carcinoma arises, agreement is even poorer. It is also common knowledge that the natural course of dysplasia differs from patient to patient. Thus, some researchers announce cancer development in 60% of patients at 8 mo, while others report a cumulative cancer rate of 9% at 5 years and only 16% over a 15.9-year period, as documented by endoscopic surveillance[91].

The presence of esophageal cancer in BE represents yet another diagnostic problem. With meticulous examination of esophagectomy specimens in an effort to detect invasion through the submucosal layer, the kappa values for intra-observer and inter-observer variability

are 0.56 and 0.42, respectively[92]. There is also significant discrepancy between the prevalence of carcinoma in esophagectomy specimens of patients who are operated for HGD (0% up to 75%)[93-95] and that of invasive adenocarcinoma in patients with HGD who are under endoscopic surveillance (16% up to 60%)[88,93,95]. Further disagreement exists as to the presence of occult cancer in patients with BE and HGD. Cameron *et al*[94] histologically mapped esophagectomy specimens from patients operated for early adenocarcinoma and depicted areas with occult cancer that are extremely small and can easily evade attention.

Obviously, current data are not sufficient to dictate clear-cut therapeutic indications for this specific patient population. The question in doubt is whether to choose endoscopic therapy, particularly EMR, over esophagectomy. Nevertheless, this does not apply to patients who reject surgical intervention or are considered unfit to undergo a major operation. The therapeutic indication for these patients is limited to the choice of an appropriate endoscopic method. Additionally, it must be pointed out that endoscopic therapy should probably be precluded for a group of patients with BE and HGD who are young (about 55 years old), otherwise healthy without significant co-morbidities, with a high risk of developing invasive adenocarcinoma[95]. Therefore, the selection of the appropriate treatment is questionable for older patients who are eligible for esophagectomy.

Proponents of endoscopic treatment, even in the absence of comparative studies between surgical units and endoscopic departments, advocate that endoscopic therapy carries lower morbidity and mortality than esophagectomy. They also raise the argument that the FDA has already approved porfimer sodium for the photodynamic eradication of premalignant lesions in patients with BE who do not undergo esophageal resection. It seems that even technology works in favor of the endoscopic therapy argument. Recently, in the field of optical spectroscopy, a technique that allows detection of molecular degeneration and minute dysplastic alterations in real time was developed. This technique is expected to allow simultaneous detection and destruction in a single endoscopic session[79,80].

On the other hand, surgeons argue that the patient is subject to the risk of being lost during the follow-up with endoscopic surveillance and may reappear later with inoperable disease. Moreover, the techniques of endoscopic destruction of the lesion may not provide adequate samples for histological examination. At EMR, residual foci of dysplastic cells remain deeper in the regenerated squamous epithelium; however, HGD is often multifocal and early reports of endoscopic excision have documented an unacceptably high rate of positive excision margins. The majority of studies evaluating endoscopic treatment of BE with HGD were neither randomized nor controlled, included small numbers of patients and the duration of follow-up was relatively short, thus unreliable for extraction of safe conclusions. From a surgeon's point of view, before choosing a therapeutic approach, the severity of GERD as well as the gravity of symptoms should be taken into account. Thus, avoiding esophagectomy and implementing an endoscopic therapy should be considered for patients with few symptoms, normal esophageal function and short segment BE, with associated low risk of intramucosal cancer. Accordingly, esophagectomy is reserved for patients with BE and HGD or intramucosal cancer who present with severe symptoms of GERD or dysphagia, long segment BE, a large hiatal hernia and poor function of esophageal body[96].

Currently, the optimal therapy of BE with HGD is, at best, controversial, despite the vast number of emerging new techniques in the fields of both surgery and endoscopy. No properly designed prospective randomized controlled trial, comparing the various therapeutic modalities, has yet been conducted, rendering the undertaking of such a study mandatory in order to elucidate the ideal therapy[97].

CONCLUSION

The modern era surgeon is confronted with multiple dilemmas concerning the best therapeutic management of patients with BE and HGD, which represents an area of dispute between esophagogastric surgeons and gastroenterologists. The ideal therapy for BE with HGD is further perplexed by the unclear natural history of the disease, the discordance of histopathologic diagnosis and its relation to malignancy, i.e., coexistent disease or subsequent development of esophageal adenocarcinoma. When considering the best therapeutic approach for these patients, multifocality, extent and pretreatment staging of the disease, as well as patient's preference and performance status, should all be taken into account. Therefore, the ideal therapy should be individualized. Many advocate esophagectomy as the gold standard therapy for BE with HGD. Nevertheless, new and emerging minimally invasive, endoscopic and ablative techniques have more recently yielded significant results and gained popularity. Randomized controlled trials are still required to properly define their optimal role in the armamentarium against BE with HGD and current research is expected to lead to the incorporation of these techniques in standard clinical practice.

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ORIGINAL ARTICLE

Hepatic steatosis prevents heme oxygenase-1 induction by isoflurane in the rat liver

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Abstract

AIM: To characterize the inductive effects of isoflurane (ISO) on hepatic heme oxygenase-1 (HO-1) in an animal model of hepatic steatosis.

METHODS: Lean (LEAN) and obese (FAT) Zucker rats were randomized into 4 groups: 1: LEAN + pentobarbital sodium (PEN); 2: LEAN + ISO; 3: FAT + PEN; 4: FAT + ISO. The animals were mechanically ventilated for 6 h. *In vitro* analyses of liver tissue included determination of HO-1 mRNA and protein expression as well as measurement of HO enzyme activity and immunohistochemical analyses.

RESULTS: Compared to PEN treatment, ISO administration profoundly induced hepatic HO-1 mRNA and protein expression and significantly increased HO enzyme activity in lean Zucker rats. In contrast, no difference in *HO-1* gene expression was observed after ISO or PEN anesthesia in obese Zucker rats.

CONCLUSION: The present study demonstrates that ISO is an inducer of hepatic *HO-1* gene expression in non-steatotic organs but failed to upregulate HO-1 in steatotic livers.

Key words: Isoflurane; Heme oxygenase; Hepatic steatosis; Heme oxygenase-1; Volatile anesthetics

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INTRODUCTION

The development of hepatic ischemia/reperfusion (I/R) injury is a fundamental problem in major hepatic surgery including liver transplantation, causing a higher rate of morbidity and mortality[1,2]. Surgical interventions such as warm hepatic inflow occlusion (Pringle maneuver) or cold ischemia in the transplant setting followed by reperfusion are important and often unavoidable techniques used to reduce blood loss or preserve organs for subsequent transplantation. I/R injury frequently results in apoptosis and necrosis of hepatocytes, which could consequently lead to organ failure or graft dysfunction[3,4]. In recent years liver surgery has become safer due to improvements in surgical techniques, anesthetic procedures

and postoperative care. However, due to the epidemic increase in obesity, the prevalence of hepatic steatosis has significantly increased during the last few decades. The Dallas Heart Study reported a prevalence of hepatic steatosis in their population of about 38% indicating the high relevance for today's health care system[5]. It has been repeatedly shown that steatotic livers are especially vulnerable to I/R injury. Liver surgery in patients with severe hepatic steatosis is associated with higher morbidity and mortality rates due to the underlying pathogenic features affecting important mechanisms during I/R, liver regeneration and recovery[6-8]. Various strategies have been proposed to improve the postoperative outcome of these patients including pharmacological approaches aiming at upregulation of cytoprotective genes.

Heme oxygenase-1 (HO-1) and its catalytic products have been identified as major players in cell protection in different organs[9-12]. Whereas HO-1 represents the inducible form of the HO family, HO-2 is expressed constitutively. HO catabolizes the first and rate-limiting step in heme degradation producing carbon monoxide (CO), free iron and biliverdin, which is converted into bilirubin by biliverdin reductase[13]. A multitude of HO-1 inducers are well known, but most of them are toxic which limit their therapeutic application in humans[14,15]. We have previously shown that volatile anesthetics are potent non-toxic inducers of *HO-1* gene expression in the rat liver[16]. Isoflurane (ISO) pretreatment induces hepatic HO-1 mRNA and protein followed by an increase in HO activity, thereby reducing portal resistance[17]. Experimental and clinical evidence support the hypothesis that administration of volatile anesthetics could be a promising approach to limit I/R injury and to improve the outcome of patients undergoing liver surgery[18,19]. To date, no data are available regarding the effects of anesthetics on hepatic HO-1 induction in steatotic livers. Therefore, the present study was designed to characterize the effects of ISO administration on hepatic *HO-1* gene expression in an established animal model of hepatic steatosis using genetically modified Zucker rats.

MATERIALS AND METHODS

Reagents

Isoflurane was obtained from Abbott (Wiesbaden, Germany) and pentobarbital sodium from Alvetra (Neumuenster, Germany). Pancuronium was purchased from Organon (BH Oss, Netherlands). All other reagents used were purchased from Sigma Aldrich (Deisenhofen, Germany), if not specified otherwise.

Animals

All animal experiments were approved by the local animal care and use committee and were in accordance with the Guide for the Care and Use of Laboratory Animals. Homozygous obese (FAT) male Zucker rats and heterozygous lean (LEAN) male Zucker rats aged 12 wk were obtained from Charles River (Sulzfeld, Germany). Animals were fasted for 6 h before the beginning of the experiments but were allowed free access to water.

Experimental protocol

The animals were assigned to 4 groups: group 1, LEAN + PEN (pentobarbital sodium, 40 mg/kg per hour i.v.); group 2, LEAN + ISO; group 3, FAT + PEN; group 4, FAT + ISO. Animals treated with PEN received one initial intraperitoneal injection of pentobarbital sodium (40 mg/kg) followed by an intravenous infusion of 40 mg/kg per hour. For compensation of evaporative losses 10 mL/kg per hour of saline solution 0.9% were continuously infused. Rats in the ISO groups were anesthetized by inhalation of ISO (2.8-3.1 Vol%). After induction of anesthesia, a tail vein was cannulated and a tracheostomy was performed. Relaxation was achieved by injection of pancuronium (1 mg/kg i.v.) and all animals were mechanically ventilated (Rodent Ventilator UB 7025-10, Harvard Apparatus, March-Hugstetten, Germany). Doses of 0.5 mg/kg pancuronium were repeated every 3 h to maintain muscle paralysis. Cannulation of the left carotid artery with polyethylene (PE-50, Smith Medical, Ashford, United Kingdom) tubing was performed for arterial blood pressure monitoring and blood gas analysis. The right jugular vein was cannulated for fluid administration. Blood gas analyses were performed using an autoanalyzer ABL 800 Flex (Radiometer, Willich, Germany). At the end of the experiment (6 h after onset), the animals were killed and blood and liver tissue were removed for subsequent analyses.

Enzyme determination

Blood samples were collected at the end of each experiment and immediately centrifuged at 4 ℃. Rat α-glutathione s-transferase (α-GST) serum concentration was evaluated using an anti-rat α-GST enzyme immunoassay (Argutus Medical, Dublin, Ireland). The procedures were performed according to the manufacturer's instructions.

RNA isolation

Total RNA was extracted from liver tissues using the TRIzol method (Invitrogen, Carlsbad, CA, Untied States) according to the manufacturer's recommendation. RNA amounts were normalized to a concentration of 50 ng/μL diluted with RNAse-free Water (Qiagen, Hilden, Germany).

Semi-quantitative real-time reverse transcriptase-polymerase chain reaction

Total RNA was reverse transcribed to single-stranded cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc, Foster City, CA 94404 Untied States) according to the manufacturer's protocol. Briefly 250 ng of purified total RNA were subsequently used in 50 μL reverse transcription reactions employing random hexamers. 50 ng of the resulting cDNA were used in semi-quantitative real-time polymerase chain reaction (PCR) analysis in a 50 μL final volume. Reactions were performed on an ABI Prism 7000 (Applied Biosystems, Foster City, CA, United States) in duplicate for each animal using TaqMan Mastermix reagents (part number

4309169, Applied Biosystems, Foster City, CA, United States) with a specific TaqMan® Probe against HO-1 cDNA (Assay ID: Rn00561387_mL) as described in the manufacturer's protocol. Parameters for quantitative PCR were as follows: 10 min at 95 ℃, followed by 40 cycles of amplification for 15 s at 95 ℃, and 1 min at 60 ℃. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as endogenous control in all real time analyses using a VIC®/MGB labeled Probe (part number: 4352338E, Applied Biosystems, Foster City, CA, United States). The obtained data were analyzed by the ΔΔCT method.

Determination of heme oxygenase enzyme activity

The heme oxygenase (HO) enzyme activity assay was performed as previously described[20]. Briefly, frozen liver tissue was homogenized and added to a reaction mixture containing NADPH, liver cytosol, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and hemin. The reaction was performed at 37 ℃ for 1 h in the dark and stopped by the addition of chloroform. The extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm.

Hematoxylin-eosin staining of liver sections

For histological analysis, liver sections were fixed with 4% buffered formalin (pH 6.9) and embedded in paraffin. Livers were sliced (5 μm) and stained with hematoxylin-eosin according to a standardized protocol.

Immunohistochemical staining

Liver tissue samples were formalin-fixed, paraffin embedded and cut in a microtome to 4 microns. The slides were deparaffinized with xylene, rehydrated and then rinsed with tap water. Antigen retrieval was performed by microwave irradiation in a sodium citrate buffer (pH 6) and slides were blocked with a ready-to-use peroxidase blocking reagent (Dako North America, Inc., CA 93013 United States) for 10 min at room temperature. After subsequent treatment with normal goat serum, slides were incubated with the primary antibody (dilution 1:50) as used in Western blotting for 1 h at room temperature. Following three washing steps with phosphate buffered saline (PBS) the slides were incubated with the HRP-conjugated secondary antibody (Goat A-rabbit-HRP, Dako, Denmark) diluted 1:200. The slides were again washed 3 times with PBS and then incubated with liquid diaminobenzidine and substrate (Dako North America, Inc., CA, United States) as the chromogen for 6 min and rinsed with deionized water. Finally the sections were counterstained with Mayer´s hematoxylin (Merck KG,, Darmstadt, Germany), dehydrated and mounted in an organic mounting media. For assessment of the severity of hepatic steatosis, hematoxylin and eosin-stained sections were evaluated without immunohistochemical treatment.

Western blotting analysis

Western blotting analysis was performed with total cell lysates as described previously[19]. Briefly, frozen liver tissue was homogenized on ice in activated RIPA buffer (Santa Cruz, CA, United States). Total protein concentration was determined in the supernatant using the Bradford assay (Bio-Rad Laboratories, Munich, Germany). Each lane of a 10% sodium dodecyl sulfate gel contained 100 μg of total protein. After separation and electroblotting, HO-1 was detected by a rabbit polyclonal anti-HO-1 antibody (1:1000 dilution, SPA 895; Stress Gen Biotechnologies, Victoria, British Colombia, Canada) using the enhanced chemiluminescence detection kit (Amersham Pharmacia) according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SE of the mean with n = 5 animals per group as indicated. Statistical differences within each group were determined using a one-way analysis of variance (ANOVA) for repeated measurements and between the different groups by one-way ANOVA followed by the post hoc Student-Newman-Keuls test for pairwise comparisons. When criteria for parametric tests were not met, Kruskal-Wallis ANOVA on ranks followed by Dunn´s test was used. These data are presented as median (box: 25th and 75th percentiles; error bars: 5th and 95th percentiles) for n = 5 animals per group. Data were considered significant when $P < 0.05$. Statistical analysis was performed using the Sigma Stat and Sigma Plot 11 software package (Jandel Scientific, San Rafael, CA, United States).

RESULTS

Analysis of vital parameters and weight

The animals in the LEAN + PEN group had a significantly higher mean arterial pressure at the respective time points during the experiments (Table 1). There were no differences in heart rate between the different groups. Body temperature dropped during induction of anesthesia but returned to normal values after at least 2 h in all groups (Table 1). Homozygous Zucker rats (FAT) had a higher body weight compared to the age-matched heterozygous controls (LEAN) (Table 2). There were no significant differences in blood gas parameters.

Effect of ISO treatment on hepatic HO-1 mRNA expression

Semi-quantitative HO-1 mRNA real-time analysis of isolated liver extracts is shown in Figure 1A. ISO treatment over 6 h led to a significant increase in HO-1 mRNA in lean rats (4.81 ± 0.82) compared to all other groups. ISO inhalation in FAT Zucker rats (2.57 ± 0.22) did not lead to a significant induction of HO-1 compared to the respective PEN group (2.83 ± 0.39). A significant difference in HO-1 mRNA levels was detected between liver extracts of animals from the FAT+PEN and FAT + ISO group compared to rats assigned to the LEAN + PEN group (2.03 ± 0.38).

Effect of ISO administration on hepatic HO-1 protein levels and HO enzyme activity

In line with RT-analyses, representative Western blotting showed higher HO-1 protein levels in liver extracts

Table 1 Time course of mean arterial pressure, heart rate and temperature

	Time (h)	LEAN+PEN	LEAN+ISO	FAT+PEN	FAT+ISO
MAP	0	127[a] ± 5	82 ± 11	106 ± 5	99 ± 5
(mmHg)	2	86[a] ± 3	72 ± 2	77 ± 4	73 ± 2
	4	85[a] ± 5	69 ± 2	74 ± 4	69 ± 2
	6	84[a] ± 7	69 ± 2	72 ± 3	70 ± 2
HR	0	305 ± 3	302 ± 4	314 ± 2	310 ± 2
(bpm)	2	302 ± 2	300 ± 0	319 ± 3	312 ± 0
	4	310 ± 2	305 ± 3	310 ± 2	310 ± 4
	6	307 ± 3	307 ± 3	310 ± 2	310 ± 2
Temp.	0	34.4[c] ± 0.3	34.7[c] ± 0.6	34.5[c] ± 0.2	34.7[c] ± 0.3
(℃)	2	36.6 ± 0.3	37.2 ± 0.3	36.6 ± 0.3	36.9 ± 0.1
	4	37.1 ± 0.2	37.5 ± 0.1	37.2 ± 0.1	37.0 ± 0.2
	6	37.1 ± 0.2	37.5 ± 0.0	37.2 ± 0.1	37.0 ± 0.1

Data are presented as mean ± SE of the mean for n = 5 animals per group. [a]P < 0.05 *vs* all other groups, [c]P < 0.05 *vs* all other time points. MAP: Mean arterial pressure; HR: Heart rate; Temp.: Temperature; LEAN: Lean Zucker rats; FAT: Obese Zucker rats; ISO: Isoflurane; PEN: Pentobarbital sodium.

Table 2 Body weight and baseline values of blood gas parameters

	LEAN+PEN	LEAN+ISO	FAT+PEN	FAT+ISO
Weight (g)	321 ± 19	294 ± 16	462[b] ± 14	426[b] ± 18
pH	7.46 ± 0.03	7.48 ± 0.03	7.48 ± 0.04	7.42 ± 0.01
pCO_2 (mmHg)	40 ± 3	37 ± 3	32 ± 4	33 ± 4
pO_2 (mmHg)	211 ± 52	279 ± 69	258 ± 33	308 ± 74

Data were obtained after induction of anesthesia. Data are presented as mean ± SE of the mean for n = 5 animals per group ([b]P < 0.001 *vs* Lean + Pentobarbital sodium and Lean + Isoflurane). pCO_2: Arterial carbon dioxide partial pressure; pO_2 : Arterial oxygen partial pressure; LEAN: Lean Zucker rats; FAT: Obese Zucker rats; ISO: Isoflurane; PEN: Pentobarbital sodium.

from animals in the LEAN + ISO group compared to the other groups (Figure 1B). Equal loading was verified by reprobing the membrane with a GAPDH antibody. In addition, HO enzyme activity was significantly higher in ISO treated lean Zucker rats (Figure 1C). Interestingly, we found no differences in HO activity between the FAT + PEN and FAT + ISO group.

Expression pattern of hepatic HO-1 protein after ISO treatment

HO-1 immunoreactive protein was restricted to spindle-shaped sinusoidal lining cells in PEN anesthetized control animals (Figure 2A). HO-1 protein was markedly upregulated in hepatocytes predominantly located in the perivenular area after ISO treatment in lean Zucker rats (Figure 2B). In sharp contrast, we did not detect any upregulation of HO-1 protein in hepatocytes of the perivenular area in obese Zucker rats after treatment with ISO (Figure 2C and D).

Hematoxylin-eosin staining

For confirmation of steatosis hepatis, hematoxylin-eosin staining was performed. Obese Zucker rats (FAT) showed

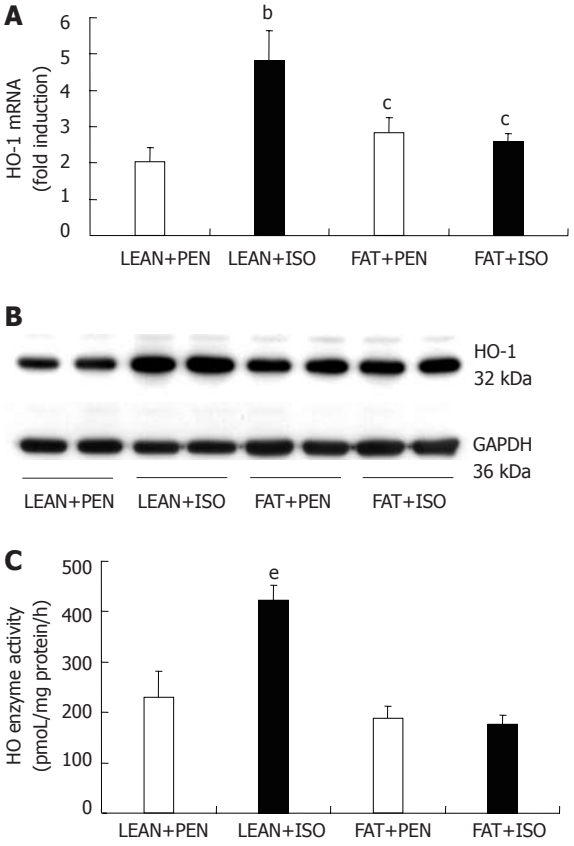

Figure 1 Hepatic *heme oxygenase-1* gene expression and heme oxygenase enzyme activity in lean and obese Zucker rats after treatment with pentobarbital or isoflurane. A: Reverse transcriptase-polymerase chain reaction was performed for determination of HO-1 mRNA expression. Data are presented as mean ± SE of the mean for n = 5 per group. [b]P < 0.01 *vs* all other groups; [c]P < 0.05 *vs* LEAN + PEN; B: Western blotting analysis for determination of HO-1 protein expression in two representative animals from each group; C: Measurement of HO enzyme activity. Data are presented as mean ± SE for n = 5 per group. [e]P < 0.001 *vs* all other groups. HO-1: Heme oxygenase-1; PEN: Pentobarbital; ISO: Isoflurane; LEAN: Lean Zucker rats; FAT: Obese Zucker rats; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

severe macrovesicular and microvesicular fatty infiltration in hepatocytes (Figure 3C and D). In contrast, we did not detect any accumulation of lipid droplets in LEAN animals (Figure 3A and B).

Effect of ISO treatment on serum levels of α-glutathione s-transferase

As shown in Figure 4, α-GST, one of the most specific serum enzymes identifying hepatocyte injury did not differ between the groups.

DISCUSSION

In the present report we demonstrate that ISO induced upregulation of *HO-1* gene expression, which was reproducibly shown in normal livers and serves as a major protective mechanism against hepatic ischemia and reperfusion injury, is abrogated in the presence of hepatic steatosis. ISO treatment of heterozygous lean Zucker rats representing a normal phenotype led to a profound induction of HO-1 mRNA and protein with a subsequent

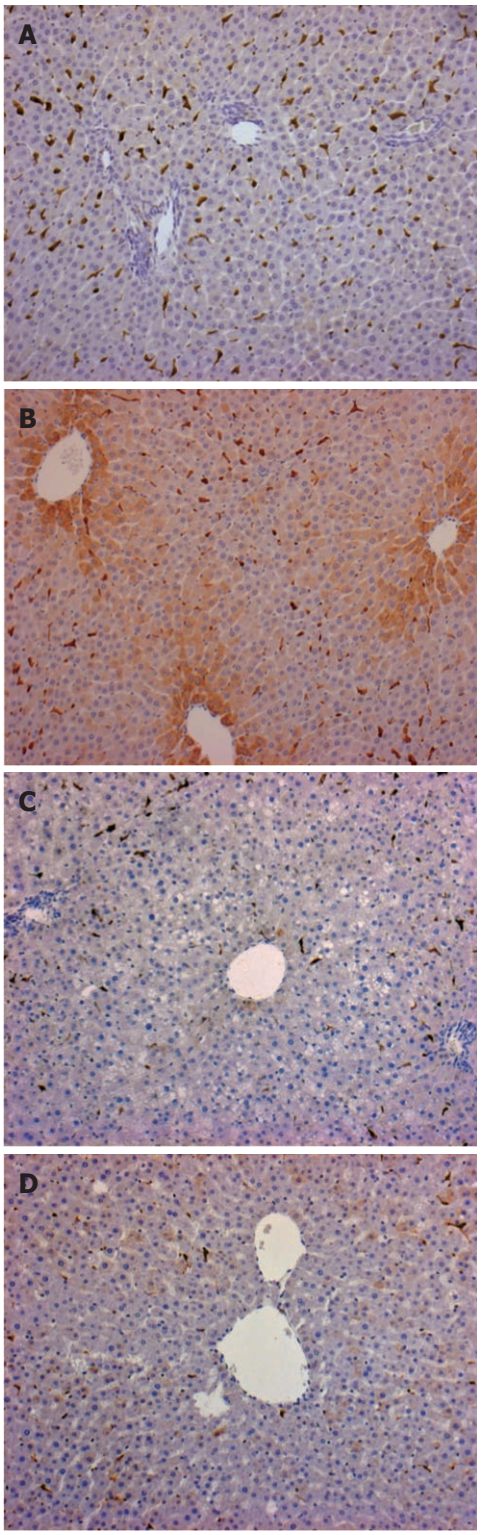

Figure 2 Heme oxygenase-1 protein expression pattern in liver sections from lean and obese Zucker rats after treatment with pentobarbital or isoflurane. A: Heme oxygenase-1 (HO-1) protein was restricted to spindle-shaped sinusoidal lining cells in pentobarbital treated animals; B: Isoflurane (ISO) administration led to markedly upregulated HO-1 protein expression in hepatocytes of the perivenular area; C and D: No detectable induction of HO-1 protein in hepatocytes of obese animals was detected after ISO treatment. PEN: Pentobarbital; LEAN: Lean Zucker rats; FAT: Obese Zucker rats.

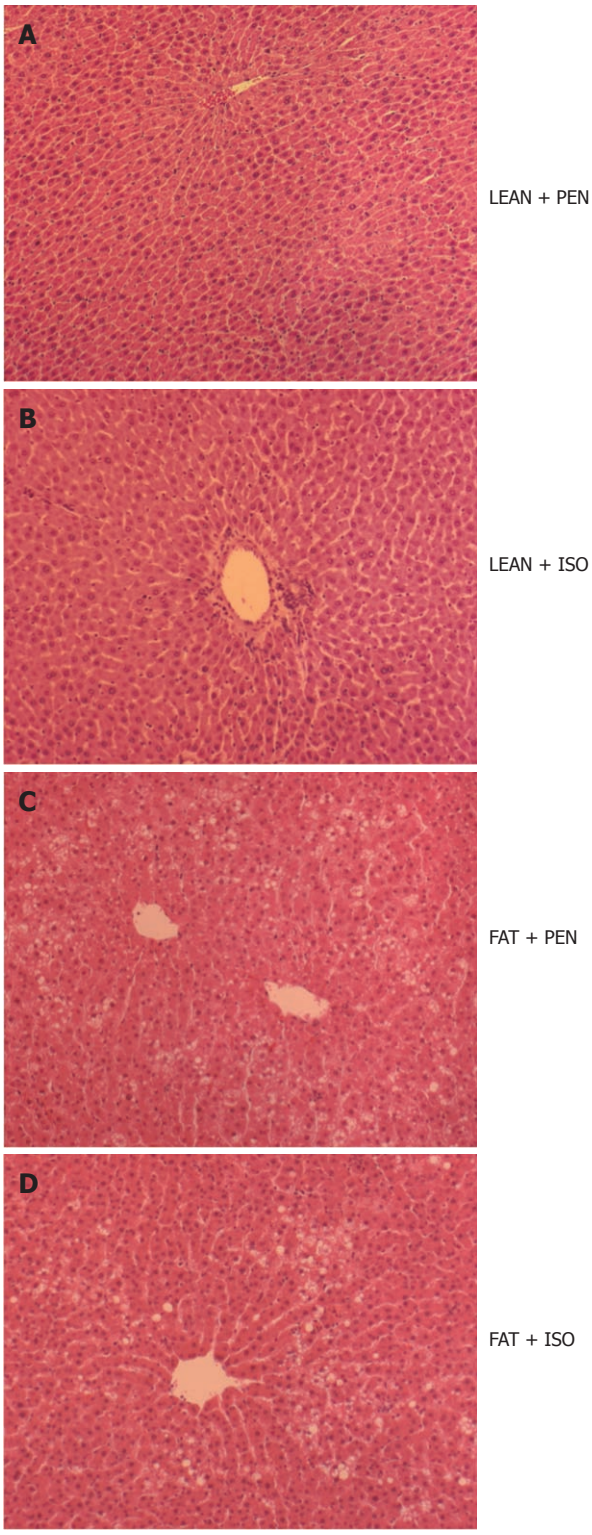

Figure 3 Hematoxylin and eosin staining of liver sections. To assess the degree of hepatic steatosis, hematoxylin-eosinstaining was performed. A, B: No lipid droplets were detected in lean Zucker rats; C, D: In obese Zucker rats numerous intracellular lipid vacuoles were observed within the liver tissue. PEN: Pentobarbital; ISO: Isoflurane; LEAN: Lean Zucker rats; FAT: Obese Zucker rats.

increase in HO enzyme activity in the liver. In contrast to these findings, which confirm earlier reports from our laboratory in Sprague Dawley rats, ISO administration to homozygous obese Zucker rats, an established animal model for steatosis hepatis, had no effect on *HO-1* gene

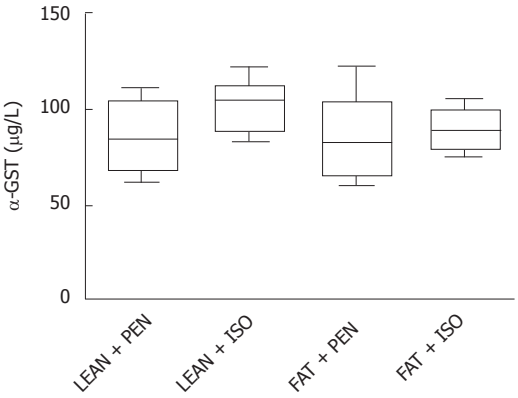

Figure 4 Determination of serum levels of α-glutathione s-transferase. There were no significant differences in α-glutathione s-transferase serum levels between the different groups. Data are presented as median (box: 25th and 75th percentiles; error bars: 5th and 95th percentiles) for *n* = 5 animals per group. PEN: Pentobarbital; ISO: Isoflurane; LEAN: Lean Zucker rats; FAT: Obese Zucker rats.

expression in the liver of these animals.

Volatile anesthetics are non-toxic inducers of hepatic *HO-1* gene expression[16,17,19,21]. We previously showed that pretreatment with ISO leads to an improvement in hepatic macro- and microvascular blood flow and reduces portal vascular resistance in the normal liver[17]. Furthermore, ISO induced upregulation of hepatic HO-1 is an important hepatoprotective mechanism against I/R injury. It specifically upregulates HO-1 protein in hepatocytes of the perivenular area, the primary localization of cellular injury in low flow states like I/R. HO-1 induction improves microcirculation in the early reperfusion period, decreases the oxidative burst and significantly reduces serum levels of liver enzymes and morphological signs of hepatic injury after I/R[19]. In addition to these experimental data, Beck-Schimmer and colleagues recently published the first randomized clinical study demonstrating the hepatoprotective effect of the volatile anesthetic, sevoflurane, in patients undergoing liver resection[18]. Interestingly, they found an even more pronounced beneficial effect of sevoflurane in patients with steatotic livers. No information is available regarding sevoflurane treatment on *HO-1* gene expression in their study. However, we hypothesize that the protective effect of sevoflurane in this trial is independent of HO-1 since the duration of pretreatment (30 min) and the concentration applied (1.5 of minimal alveolar concentration) is most likely not sufficient to upregulate hepatic *HO-1* gene expression. Therefore, volatile anesthetics might mediate liver protection by at least two different mechanisms, one dependent and one independent of HO-1. To exclude the possibility that HO-1 upregulation in homozygous Zucker rats is generally prevented by genetic modifications in these animals, we screened the literature in this regard. It has been repeatedly shown by different authors that the administration of a variety of compounds can profoundly induce HO-1 in obese Zucker rats[22,23]. Therefore, the inability to upregulate HO-1 by ISO seems to be substance specific rather than based on a general lack of inducibility in these animals. As indicated in Figure 1A and B, hepatic HO-1 induction in obese animals was slightly but significantly higher than in the livers of lean controls. However, this did not affect HO enzyme activity in our experiments (Figure 1C).

To exclude hepatotoxic effects of the anesthetics in our experiments, we performed serum α-GST measurements. α-GST levels, which serve as a very specific marker of hepatocyte injury, did not differ between the respective groups indicating the non-toxic action of ISO.

Obese Zucker rats develop hypertensive blood pressure values accompanied by improper autoregulation caused by an impairment of sympathetic baroreceptor reflexes[24-26]. Therefore, anesthetics (e.g., barbiturates) may have an even more pronounced effect on blood pressure in obese rather than lean Zucker rats. This could be an explanation for the higher blood pressure in the LEAN + PEN group compared to the other animals observed in the present study.

Patients with hepatic steatosis are at higher risk for postoperative complications after major hepatic surgery including liver transplantation, and adverse outcomes have been repeatedly documented[8,27-30]. Due to the increasing gap between the number of available organs and the number of patients awaiting an organ, the amount of so-called "marginal livers" considered for transplantation is increasing. Based on this dilemma, it is important to develop protective strategies particularly for the above-mentioned type of organs comprising severely steatotic livers to expand the pool of available liver grafts.

The present study demonstrates that ISO is a potent inducer of *HO-1* gene expression in non-steatotic livers but failed to upregulate HO-1 in steatotic organs. If validated in humans, this observation may have an impact on the anesthetic regimen in patients undergoing liver surgery.

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COMMENTS

Background

Experimental and clinical evidence support the hypothesis that administration of volatile anesthetics could be a promising approach to limit ischemia/reperfusion (I/R) injury and to improve the outcome of patients undergoing liver surgery. The volatile anesthetic isoflurane is a potent non-toxic inducer of heme oxygenase-1 *(HO-1)* gene expression in the normal liver. The authors previously showed that these livers are protected from I/R injury.

Research frontiers

There are no studies currently available characterizing the inductive effects of volatile anesthetics on steatotic livers which are especially vulnerable to I/R injury. Therefore, we examined the effects of isoflurane (ISO) on hepatic HO-1 induction in lean (non-steatotic livers) and obese (steatotic livers) Zucker rats.

Innovations and breakthroughs

The findings of the present study demonstrate that isoflurane is a potent inducer of *HO-1* gene expression in non-steatotic livers but failed to upregulate HO-1 in steatotic livers.

Applications

If verified in humans, this observation may have a crucial impact on the anesthetic regimen in patients undergoing liver surgery.

Terminology

HO-1 also called heat shock protein-32 and its catalytic products were recently identified as major players in cell protection in different organs. Hepatic I/R injury is a fundamental problem in major hepatic surgery including liver transplantation. The Zucker rat is a genetic research model for obesity and hypertension named after Lois M Zucker. Homozygous Zucker rats have high levels of lipids in their blood and an increased size and number of fat cells. Therefore, these animals serve as a model for steatosis hepatis.

Peer review

In this manuscript, the authors compare the effects of the volatile anesthetic isoflurane in the induction of HO-1 among lean and obese rats. They measure HO-1 expression at the mRNA and protein level, and also assess the activity of this enzyme. The overall data suggest that isoflurane efficiently induces HO-1 in lean, but not in obese animals. The study is well designed, appropriate controls are included and the experiments are of high technical quality. The conclusion is fully supported by the presented data.

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ORIGINAL ARTICLE

Decreased accumulation of ultrasound contrast in the liver of nonalcoholic steatohepatitis rat model

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Author contributions: Miyata Y and Miyahara T contributed equally to this work; Moriyasu F designed research; Miyata Y and Miyahara T performed research; Miyata Y and Miyahara T contributed new reagents/analytical tools; Miyata Y wrote the paper.

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Abstract

AIM: To investigate the diagnosis of nonalcoholic steatohepatitis (NASH) using contrast ultrasonography in the NASH rat model.

METHODS: The liver in methionine choline-deficient diet (MCDD) rats, a NASH model constructed by feeding an MCDD, was examined by contrast ultrasonography at weeks 2, 4, 8, 12 and 16, with late phase images of contrast ultrasonography (Kupffer imaging) in which contrast enhancement was achieved by incorporation of a contrast agent by Kupffer cells (KCs), and images were compared to those in rats taking a regular chow.

RESULTS: Decrease in contrast enhancement was observed first in MCDD rats at week 2. KCs were counted based on immunohistochemistry, but their numbers were not reduced and it was assumed that attenuation of contrast enhancement was attributable to reduced phagocytic activity of the KCs.

CONCLUSION: It is suggested that clinical application of contrast ultrasonography may be valuable for non-invasive diagnosis of NASH.

Key words: Nonalcoholic steatohepatitis; Leptin; Kupffer cell; Methionine choline-deficient diet; Contrast ultrasound

Peer reviewer: Søren Rafaelsen, MD, Consultant Radiologist, Associate Professor, Department of Radiology, Vejle Hospital, Vejle 7100, Denmark

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INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is a relatively new disease entity, proposed by Ludwig in 1980[1], which exhibits a clinical manifestation similar to that of alcoholic steatohepatitis in terms of liver histology, despite a lack of history of alcohol abuse causing hepatitis. The diagnosis of fatty liver without a history of drinking alcohol is generally termed nonalcoholic fatty liver disease (NAFLD), which is most simply fatty liver with an excellent prognosis, while NASH is a subtype of NAFLD[2].

It is important to differentiate NASH from NAFLD at an early stage to start relevant treatment. Although there have been a number of reports aimed at differentiating NASH patients[2], histological diagnosis based on liver biopsy is the only diagnostic method at present. However, liver biopsy is an invasive method, with a small risk of complications, and is too invasive for diagnosis of a benign disease, such as NASH.

It has been previously reported that liver-specific

Kupffer images of contrast ultrasound can be used for differential diagnosis between NASH and NAFLD[3]. In this study, using a rat model of NASH, we carried out contrast ultrasonography to compare contrast enhancement between the NASH model and control rats that were fed a regular chow. Levovist™ (Schering AG, Berlin, Germany) and Sonazoid™ (GE Healthcare, Oslo, Norway) were used as contrast ultrasonography agents. Microbubbles of Levovist™ and Sonazoid™ are phagocytosed and incorporated by Kupffer cells (KCs) in the late contrast phase (Kupffer imaging)[4]. There is a report that contrast enhancement by the contrast agent in the late contrast phase was decreased in patients diagnosed with NASH, compared to NAFLD patients[3,5]. The reason for decreased microbubble accumulation in the NASH liver is presumed to be because phagocytosis of microbubbles by KCs is decreased.

We constructed a NASH model by feeding a methionine choline-deficient diet (MCDD, Oriental Yeast Co., Tokyo, Japan) to rats[6-8]. Then, examining the liver using contrast ultrasonography, we compared the phagocytic activity by KCs in rats eating an MCDD and those given regular chow, using contrast enhancement in the late contrast phase.

MATERIALS AND METHODS

Animals and construction of a NASH model

Male Wistar rats weighing about 50 g at the time of purchase were used and kept in a room at 21 ℃ ± 2 ℃ with a 12-h cycle of light and darkness. The NASH model was constructed by feeding an MCDD to 15 rats, and the group was designated the MCDD group. Conversely, a diet of regular chow was fed to 5 rats and they were designated the control group. Rats were subjected to experiments at 2, 4, 8, 12 and 16 wk after beginning the diet, and three rats in the MCDD group and one in the control group were used for experiments at each time point. During the follow-up period, water and regular chow were given *ad libitum*. Body weight was measured in all rats in both groups at the designated time points. In addition, to observe the histological changes in liver tissue in MCDD rats at the designated time points, part of the resected liver was fixed in 10% formalin after the experiments, and paraffin slices were prepared and stained with hematoxylin and eosin for histological examination. All animals received humane care and the experimental protocols were approved by the Animal Ethical Committee of Tokyo Medical University.

Incorporation of the contrast ultrasonography agent

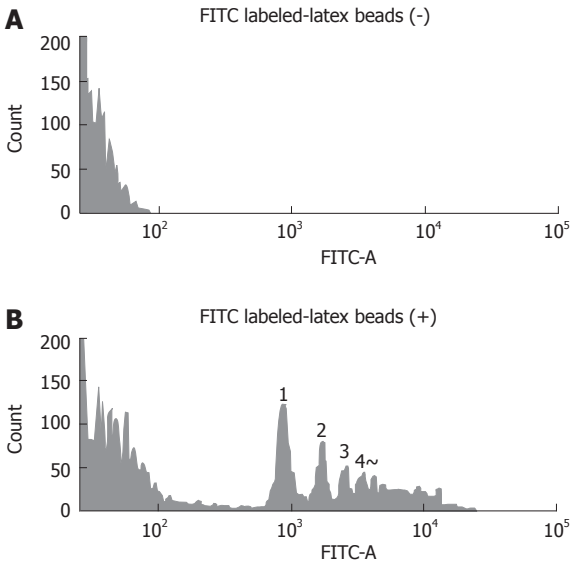
After rats were anesthetized at the designated time point by inhalation of diethyl ether (Wako Pure Chemical Industries, LTD., Tokyo, Japan), the abdomen and thighs were shaved and the fascia exposed by an excision of abdominal skin. At the same time, the thigh was incised to expose the femoral vein and a 24-gauge indwelling needle (TERUMO Corp., Tokyo, Japan) was inserted to secure the vessel. From this site, a total of 500 μL of Levovist™ solution (10 μL/g body weight) with physiological saline was infused as a bolus. In both groups, the liver was scanned through the exposed fascia with an echo probe 10 min later. Images were scanned at mechanical index (MI) of 1.5 and then any Levovist™ remaining in the liver was destroyed by sweep scanning over the whole liver. Next, as for Levovist™, a total of 500 μL of Sonazoid™ 10 times diluted solution (1 μL/g of Sonazoid™ solution/body weight) with physiological saline was infused intravenously as a bolus and, 10 min later, images were scanned and recorded at Advanced Dynamic Flow™ (ADF) ultrasound mode at MI of 1.5. An Aplio™ (Toshiba Medical Systems, Tokyo, Japan) was used in this study. The frequency used was 7.5 MHz and the type of the transducer was linear. ADF was harmonic power Doppler mode from Toshiba Medical Systems.

A region of interest (ROI) was determined arbitrarily in the constant area of ultrasound images of the liver parenchyma obtained at 10 min, and contrast enhancement was quantified in the MCDD and control groups by intensity analysis software Image J (http://rsb.info.nih.gov/ij/). Quantified values in the MCDD group were expressed as relative values compared to those in the control group at each designated time point. An ROI was set in the region covering a wide area of the liver parenchyma, excluding large vessels as far as possible. The average intensity in the ROI was measured and relative values were compared.

In vivo administration of FITC-latex beads and isolation of KCs

After experiments using contrast ultrasound, 50 μL of FITC-latex beads (2 μm in diameter, Polyscience, Warrington, PA, United States) were mixed with 500 μL of physiological saline and infused from the tail vein to examine the phagocytic activity of KCs in rats in the MCDD and control groups. One hour later, KCs were isolated according to the following methods: Rats were anesthetized by inhalation of diethyl ether and a midline abdominal incision was made. A 20-G indwelling needle was inserted into the portal vein and after perfusion with Hanks Balanced Salt Solution (HBSS, Sigma, St. Louis, MO, United States), the portal vein was perfused with 100 mL of Dulbecco's Modified Eagle's Medium (DMEM/F-12, Sigma) containing 200 mg pronase (Roche Diagnostics Corp., Indianapolis, IN, United States), followed by 100 mL of DMEM/F-12 containing 25 mg collagenase (Nitta Gelatin Inc., Osaka, Japan). When collagenase leaked out of the blood vessel because of rupture during perfusion, and it was judged that the liver was not perfused, the procedure was stopped. After completion of perfusion, the digested liver was extracted carefully and incubated in DMEM/F-12 supplemented with 0.035% pronase and 62.5 units/mL of DNase (Sigma) for 20 min at 37 ℃ on a shaker incubator.

Next, the suspension was filtered through gauze and undigested liver tissue was discarded. The liver cell sus-



KC phagocytic index = [FITC (+) cells/total KC (gated cells)] × 100%

Figure 1 Measurement of Kupffer cells phagocytic activity by flow cytometry. A: The negative control, into which fluoresceinisothiocyanate (FITC)-labeled latex beads were not injected; B: The positive control to which FITC-labeled latex beads were administered. Note the several peaks of the FITC from the beads in the positive control. The numbers on the peak shows the number of latex beads phagocytosed. KC: Kupffer cell.

pension containing KCs was centrifuged for 1 min at 50 *g*/min and the supernatant collected. Then, the supernatant was centrifuged again at 50 *g*/min and parenchymal liver cells were removed as far as possible. Subsequently, the supernatant was centrifuged at 500 *g*/min for 8 min and a pellet of non-parenchymal cells was obtained. The supernatant was discarded and the pellet was resuspended with a small amount of washing buffer. The non-parenchymal liver cell suspension containing KCs was centrifuged at 900 *g*/min for 15 min with 50% and 25% Percoll (Pharmacia, Uppsala, Sweden) for density-gradient centrifugation, and the first layer from the top was collected. Next, the suspension was rinsed with 40 mL of HBSS and percentages of KCs that had phagocytosed FITC-latex beads were measured by flow cytometry. The purity of the KCs was examined by incorporation of latex beads 2 μm in size and confirmed to be always 96% or greater. Viability was examined by the trypan blue dye exclusion test and unstained cells accounted for 90% or more.

Changes in phagocytic activity of KCs

Phagocytosis by KCs isolated from each group was examined using a BD LSR-Ⅱ flow cytometer (Becton Dickenson, Franklin Lakes, NJ, United States). First, in a preliminary experiment, it was determined whether FITC-latex beads phagocytosed by KCs were measurable by the flow cytometer. The flow cytometry histogram of KCs in the control group is shown in Figure 1. The upper panel shows the flow cytometer histogram of KCs in the control rat without adding FITC-latex beads and the lower panel shows the flow cytometer histogram of KCs in the control rat given FITC-latex beads. The amount of FITC-latex beads added was the same as in the subsequent experiments. There were peaks representing the number of FITC-latex beads, but the number of KCs that phagocytosed five beads or more was not determined. Nevertheless, it was possible to detect phagocytosed FITC-latex beads. KCs isolated in the control group were used to determine the position of gating and the gates were set a little wider. After the flow of 10 000 counts of KCs, the percentage of cells positive for fluorescence of FITC-latex beads in all gated cells was calculated in the MCDD and control groups and taken as the phagocytic activity.

Changes in KCs

Because there was a possibility that microbubble accumulation in the liver was dependent on the number of KCs in the liver, the liver was removed from one rat in each group, embedded in O.C.T. compound (Sakura Finetechnical Co. LTD, Tokyo, Japan) and snap-frozen immediately in liquid nitrogen. Later, 5 μm frozen sections were cut with a cryostat. Sections were fixed with acetone and subjected to immunohistochemistry for KCs with anti-rat macrophage antibody (ED2, SEROTEC Ltd, Oxford, United Kingdom) to count positive cells under the same magnification in the MCDD and control groups. In addition, 5 μm frozen liver specimens were cut with the cryostat and immunofluorescent latex beads were observed.

Statistical analysis

Unpaired Student's *t* test was employed for all statistical analyses and a *P* value less than 0.01 was considered statistically significant.

RESULTS

Body weight changes and liver histology in the MCDD and control groups

Body weight initially was about 50 g and increased in the MCDD group to 270 g at week 9, but decreased slightly thereafter. Although body weight reduced, general conditions were stable. In the control group, body weight increased steadily to about 440 g at week 16. General conditions also were stable (data not shown). In terms of histological changes in the liver, large-droplet fat deposition was recognized in the entire liver lobules at week 2 and later. At week 8, necrotic inflammatory changes were observed around the central vein and inflammatory findings were evident. At week 16, fibrosis was observed extending from the central vein and the MCDD group was judged to be a NASH model. In contrast, no fatty liver, inflammation, or fibrosis was observed up to week 16 in the control group (Figure 2).

Changes in contrast ultrasonographic findings

Signal intensity of the liver obtained by contrast ultrasound with Levovist™ was compared between the two groups.

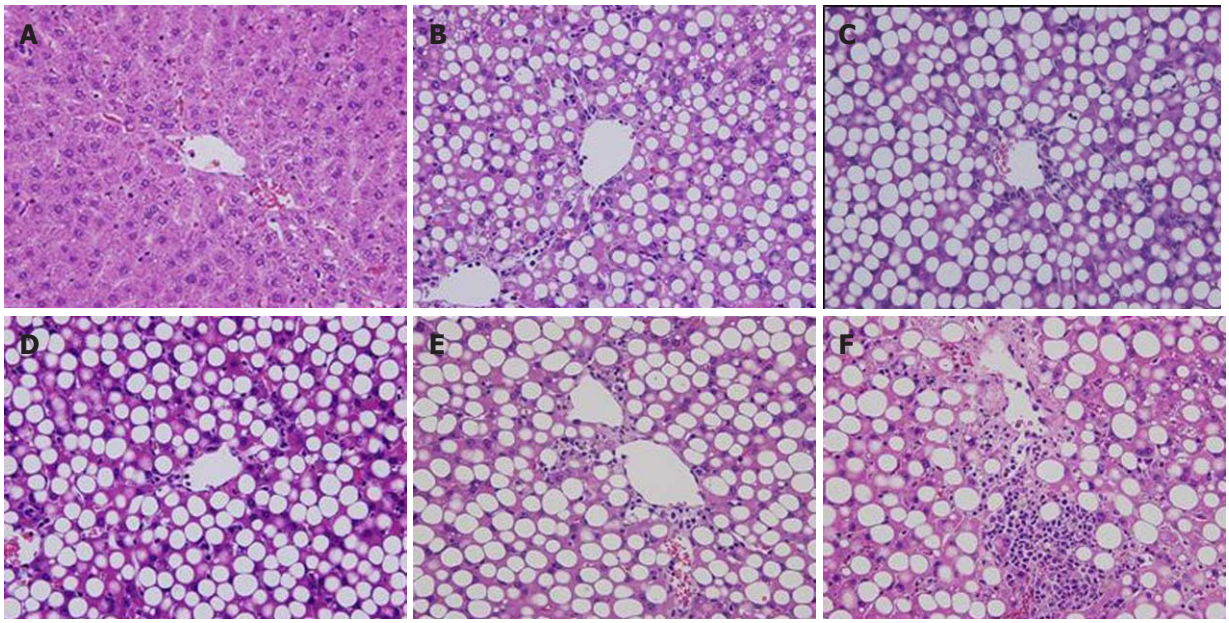


Figure 2 Histological changes of methionine choline-deficient diet-fed rat liver (hematoxylin eosin x 200). Extremely large vesicle fat deposits in almost the entire lobules were detected even as early as two weeks after the start of the methionine choline-deficient diet. By 8 wk, spotty necrosis was dispersed and by 16 wk, there was fibrosis extending from the central vein. A: Control; B: Two weeks methionine choline-deficient (MCD); C: Four weeks MCD; D: Eight weeks MCD; E: Twelve weeks MCD; F: Sixteen weeks MCD.

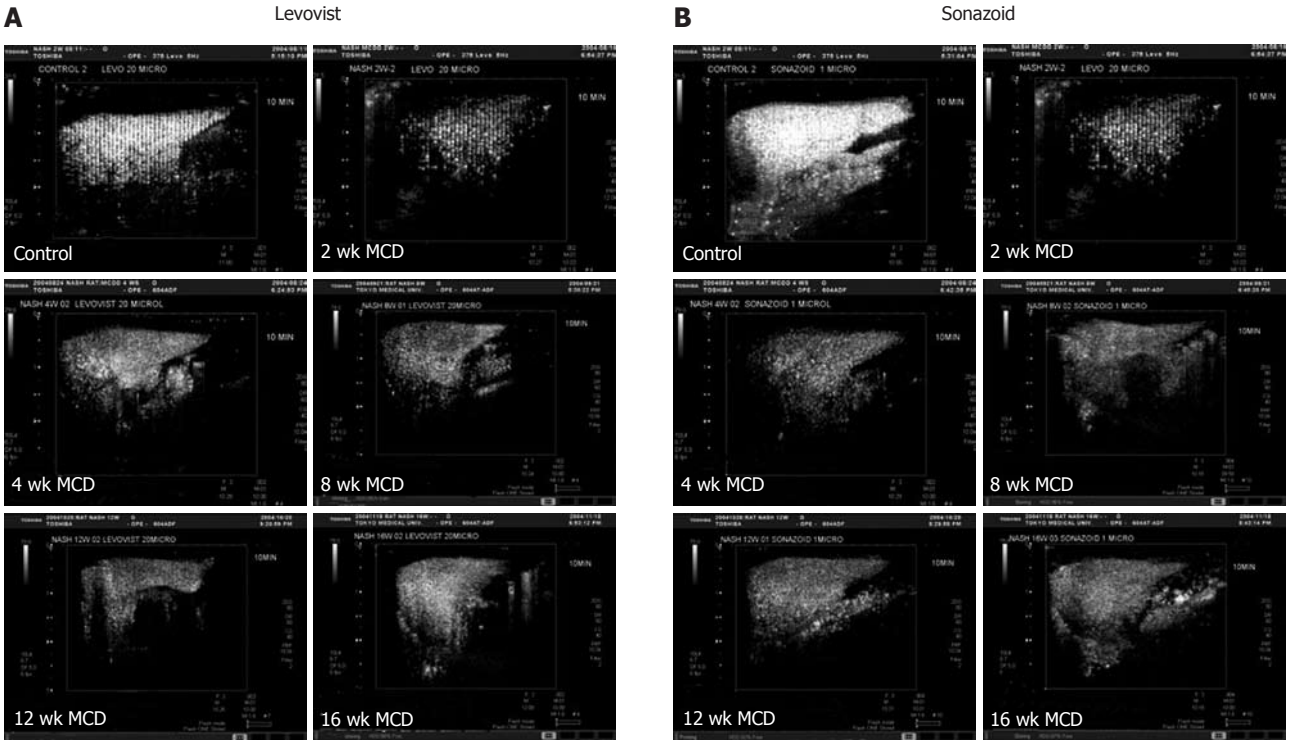


Figure 3 Ultrasound images of the liver 10 min after Levovist™ (A) and Sonazoid™ injection (B). Note signal intensity of the liver was lower in all methionine choline-deficient diet rats than in the controls. MCD: Methionine choline-deficient.

In the MCDD group, the intensity showed a significant decrease at weeks 2, 8 and 12, and a decrease, although not significant, at weeks 4 and 16 (Figures 3A and 4A). On the other hand, signal intensity of the liver obtained by contrast ultrasound with Sonazoid™ showed a significant decrease at all weekly time points in the MCDD group, compared to the control group (Figures 3B and 4B).

Phagocytic activity of KCs in each group

Phagocytic activities (control *vs* MCDD) of KCs examined with fluorescent latex beads were 44.3% ± 12.6% *vs* 18.5% ± 9.8% ($P < 0.01$) at week 2, 55.1% ± 0.1% *vs*

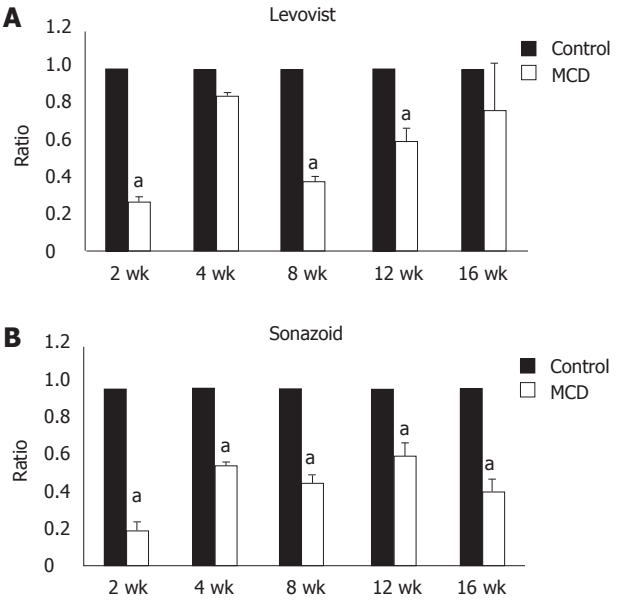


Figure 4 Changes in the signal intensity between control and methionine choline-deficient diet livers. A: Levovist™ contrast enhanced ultrasonography (CEUS). In the methionine choline-deficient diet (MCDD) liver, there was a significant decrease in Levovist™ signal intensity, compared to control livers at 2, 8 and 12 wk. While there was a lower tendency in signal intensity in MCDD liver than control liver at 4 and 16 wk, however, significance was not obtained; B: Sonazoid™ CEUS. In the MCCD liver, there was a significant decrease in Sonazoid™ signal intensity, compared to control livers at all time points. MCD: Methionine choline-deficient. $^{a}P < 0.05$ *vs* control.

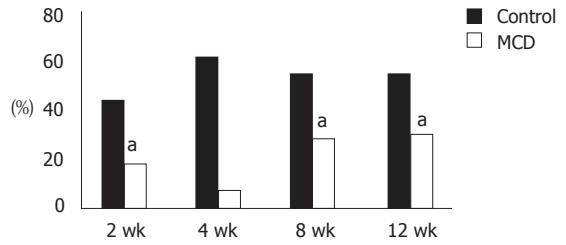


Figure 5 Decreased phagocytic activity in Kupffer cells from methionine choline-deficient dietrats compared to Kupffer cells from control rats. Control and methionine choline-deficient diet (MCDD) rats were injected with fluorescein-isothiocyanate (FITC)-labeled latex beads and Kupffer cells (KCs) were isolated 1 h later. KCs which had phagocytosed FITC-labeled latex beads were counted by flow cytometry and changes in the phagocytic activity were compared between control and MCDD KCs. Note that there was an almost 50% decrease in phagocytic activity in KCs from MCDD, compared to KCs from controls. $^{a}P < 0.05$ *vs* control.

18.5% ± 9.8% ($P < 0.01$) at week 8, and 57.4% ± 3.4% *vs* 30.3% ± 0.6% ($P < 0.01$) at week 12, and the activities in the MCDD group were about half those in the control group. At week 4, only one rat was examined but the activities were 61.9% in the control and 7.6% in the MCDD group, and again they tended to be lower in the MCDD group (Figure 5).

Counting of KCs and images of phagocytosed fluorescent latex beads

Because the decrease in the accumulation of the ultrasound contrast agent in the MCDD group potentially was attributable to a decrease in the number of KCs compared to the control group, KCs were counted histologically based on immunohistochemistry with anti-rat macrophage antibody. As a result, there was no significant difference between the MCDD and control groups in the number of KCs (Table 1). In addition, fluorescence microscopy revealed a decrease in the accumulation of fluorescent latex beads in the MCDD group compared to the control group (Figure 6).

Table 1 Numbers of Kupffer cells

Group ($n = 10$)	[1]Total ED-2(+) cells
Control	122 ± 14
MCDD	[2]110 ± 25

Numbers of Kupffer cells (KCs) in control and methionine choline-deficient diet (MCDD) livers were counted after immunostaining with ED-2 antibody, which recognizes residential macrophages or KCs in the liver. There was no significant difference in the numbers of KCs between controls and MCDD livers. [1]mean ± SD; [2]No significance.

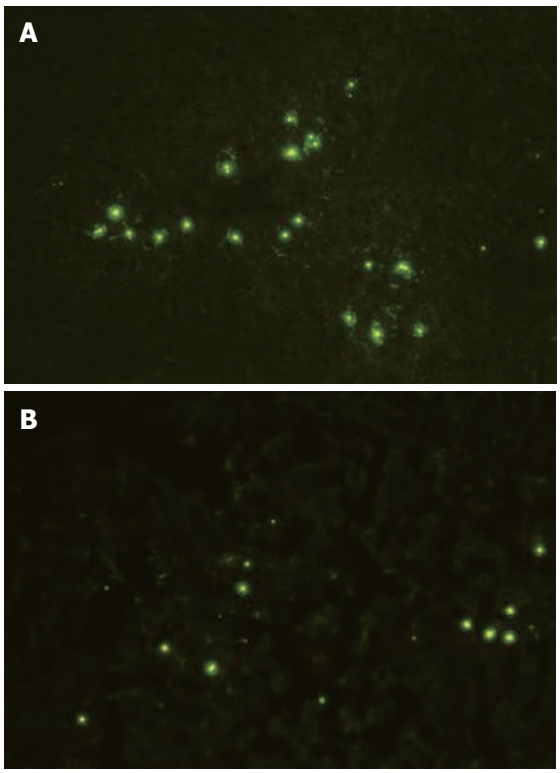


Figure 6 Fluoresceinisothiocyanate-labeled latex beads were injected through the tail veins of control and methionine choline-deficient diet rats. One hour later, small samples of the liver were snap frozen and sectioned using a cryostat. After immediate drying, unfixed tissues were observed by fluorescence microscopy. Note that there were fewer fluoresceinisothiocyanate-labeled latex beads in the livers from methionine choline-deficient diet (B) compared to the controls (A). Magnification: x 200.

Serum leptin concentrations

Serum leptin concentrations (control *vs* MCDD) were 120.6 ng/L ± 39.4 ng/L *vs* 31.5 ng/L ± 77.6 ng/L ($P < 0.01$) at week 2, 396.7 ng/L ± 95.0 ng/L *vs* 99.3 ng/L ±

14.5 ng/L ($P < 0.01$) at week 4, 618.6 ng/L ± 0 ng/L *vs* 79.2 ng/L ± 7.0 ng/L ($P < 0.01$) at week 8, and 397. 2 ng/L ± 221.3 ng/L *vs* 93.7 ng/L ± 21.5 ng/L ($P < 0.01$) at week 12, and were significantly lower in the MCDD group at all time points.

DISCUSSION

Fatty liver disease is a state in which triglycerides accumulate in hepatocytes. A manifestation of fat-related liver disease in non-drinkers is known as NAFLD, and consists of simple fatty liver and NASH accompanied by inflammatory changes. At present, it is difficult to differentiate NASH patients from a number of NAFLD patients by any diagnostic method other than liver biopsy. However, biopsy is invasive and potentially causes critical complications. Because it has been reported that 5% of NAFLD patients develop liver cirrhosis[9], it is important to diagnose NASH at an early stage. There have been reports of differentiating NASH patients from NAFLD patients without the invasiveness of liver biopsy[2,10]. It has been reported that contrast enhancement is decreased in the late contrast phase in NASH patients compared to healthy subjects[3,5]. In this study, contrast enhancement was compared by injection of the contrast ultrasonography agent in a NASH model receiving MCDD and in controls taking a regular chow.

MCDD rats have been used in research to investigate the pathogenesis of NASH[6,11,12]. We used MCDD rats as the most general NASH model developing steatohepatitis, which was induced quickly and easily by the administration of MCDD alone. In this study, the rats started to show fat deposition two weeks after the beginning of MCDD administration, steatohepatitis at week 8 and fibrosis at week 16.

Levovist™ is a first generation ultrasound contrast agent consisting of air as the inner gas within a lipid shell. SonoVue™, Optison™, Definity™, Imagent™ and Sonazoid™ are available commercially for liver imaging. Levovist™ and Sonazoid™ are easily phagocytosed by KCs[13] and have a liver-specific contrast phase. The ultrasound contrast agent Levovist™, a high MI contrast agent consisting of microbubbles, is destroyed when exposed to ultrasound with a high acoustic pressure. Most Doppler signals are retrieved when all microbubbles in the scan volume are destroyed with high MI ultrasound. On the other hand, Sonazoid™ is a new-generation ultrasound contrast agent, and develops resonance and vibration under the conditions of lower acoustic pressure than that for Levovist™, because of an elastic phospholipid shell around the inner gas, perfluoro-butane. It is a low MI contrast agent to visualize harmonic signals. Compared to Levovist™, Sonazoid™ has the advantage of enabling evaluation of contrast enhancement in a real-time manner.

In order to quantify the accumulated microbubbles in the liver parenchyma, we destroyed microbubbles by exposing high MI ultrasound to both contrast agents, Levovist™ and Sonazoid™. The period of about 2 min from the injection of the ultrasound contrast agent reflects the early vascular phase, while the time period from about 10 min later reflects the delayed parenchymal phase, or Kupffer imaging, representing the time for phagocytosis of microbubbles by KCs[14,15]. Therefore, in the delayed parenchymal phase, it is possible to obtain information similar to that provided by superparamagnetic iron oxide (SPIO)-enhanced MRI[16]. Clinically, about 25% of the ultrasound contrast agent injected from the peripheral vein is phagocytosed by KCs and visualized as the hepatic parenchyma-specific contrast[17]. It has been reported that dysfunction of KCs is involved in NAFLD[18].

In this study, images were scanned to measure the signal intensity 10 min after infusion of the ultrasound contrast agent, and accumulation of the medium by KCs was compared between the MCDD and control groups. As a result, contrast enhancement was decreased in MCDD rat livers, as in clinically diagnosed NASH patients. There are two potential mechanisms for the decrease of contrast enhancement in Kupffer imaging, which represents phagocytosis of microbubbles by KCs. These mechanisms are a reduction in phagocytosis by KCs and a decrease in the number of KCs despite normal phagocytic activity. To count the number of KCs, KCs were immunostained with anti-rat macrophage antibody, and it was found that there was no statistical difference in the number of KCs. Therefore, it was thought that decrease of contrast enhancement was attributable to a reduction in phagocytic activity of KCs, rather than a decrease in the number of KCs.

As for the pathogenesis of NASH, phagocytic activity of KCs and involvement of leptin have been suggested. Leptin is an adipocytokine produced by adipose tissue which interacts with a receptor in the hypothalamus. Leptin controls the amount of body fat by suppressing appetite and stimulating sympathetic nerve activity. NAFLD patients with obesity and insulin resistance exhibit leptin resistance and, thereby, serum leptin concentrations increase[18].

In *ob/ob* mice defective in the leptin gene[19], fatty liver is present in association with insulin resistance and obesity, and leptin administration improves fatty liver[20]. In addition, *db/db* mice harboring an abnormality in the leptin receptor do not respond to leptin and develop obesity and fatty liver, as do *ob/ob* mice. Loffreda isolated and cultured macrophages from the peritoneum and bone marrow in *db/db* mice (with abnormality in the leptin receptor) and *ob/ob* mice (deficient in the leptin gene), added *Candida parapsilosis*, and examined the phagocytic activity of macrophages in the groups with and without addition of leptin. Phagocytic activity was augmented in the *ob/ob* mice but unchanged in the *db/db* mice by addition of leptin, which suggested the possibility that leptin interacted directly with KCs to regulate phagocytic activity[21].

Sakaida *et al*[22] examined lipopolysaccharide (LPS)-induced phagocytic activity and production of TNF-α in

the KCs isolated from *fa*/*fa* rats, with a mutation in the leptin receptor gene, and reported that both were reduced compared to the control (*fa*/- rats) and therefore leptin potentially affected the function of KCs. It has been presumed that leptin activated KCs and their phagocytic activity, and phagocytic activity of KCs and serum leptin concentrations were indeed decreased in MCDD rats in this study. In other words, because the amount of leptin secretion was low in MCDD rats, activation of KCs by leptin was attenuated and, thereby, the phagocytic activity of KCs was decreased. Although stimulation with leptin may be closely associated with the phagocytic activity of KCs, it is one of our experimental challenges to isolate and culture KCs from MCDD rats and determine whether addition of leptin will improve their phagocytic activity.

Although there is a report that contrast enhancement by contrast ultrasound was decreased in NASH patients compared to NAFLD and type C chronic hepatitis patients[5], its pathogenesis has yet to be elucidated. By downregulating KCs and provoking leptin resistance, persistence of high concentrations of serum leptin levels decreases the activity of leptin and thereby reduces phagocytic activity in NASH patients. In this study, serum leptin concentrations were low and reduced leptin activity was one potential factor responsible for the pathogenesis. It should be possible to clinically diagnose NASH patients non-invasively, without liver biopsy, by quantifying contrast enhancement in contrast ultrasonography[3]. Sensitivity and specificity for diagnosis of NASH differentiation from NAFLD are more than 90% and decrease in Levovist accumulation is the early stage of NASH.

In MCDD rats, pathological findings revealed fatty liver without inflammation or necrosis two weeks after the beginning of the MCDD, and the phagocytic activity of KCs was attenuated. These results suggest that it may be possible to diagnose NASH in human NASH patients at an early stage when inflammation, necrosis, and fibrosis specific for NASH have not developed. In addition, should early diagnosis of NASH be possible, early treatment would be feasible with prevention of progression to liver cirrhosis, and improvement of prognosis might be expected. It is necessary to accumulate clinical cases examined by a combination of contrast ultrasound and liver biopsy.

In conclusion, contrast enhancement in the late phase of contrast ultrasound was compared in MCDD rats and rats taking a regular chow. In the MCDD rats, accumulation of the contrast medium was attenuated compared to the control rats. As for the cause, a reduction in phagocytic activity by KCs was suspected, but activation by serum leptin might also be involved in the reduction of phagocytic activity.

COMMENTS

Background

Nonalcoholic steatohepatitis (NASH) is a disease that exhibits inflammation and fibrosis, and NASH may progress to liver cirrhosis and hepatocellular carcinoma. Early clinical diagnosis is important and difficult, and histological diagnosis based on liver biopsy is the only diagnostic method of NASH at the present time.

Research frontiers

Liver biopsy is somewhat invasive and may cause some complications, such as bleeding in the abdominal cavity or infection. Thus, a non-invasive method to diagnose NASH needs to be developed. It has been previously reported that liver-specific Kupffer images of contrast ultrasound can be used for differential diagnosis between NASH and nonalcoholic fatty liver disease (NAFLD).

Innovations and breakthroughs

The authors carried out a comparison of contrast ultrasonography between a NASH model and control rats. In the NASH model group, the intensity showed a significant decrease.

Applications

Contrast ultrasonography can be easily used to differentially diagnose NASH from NAFLD without liver biopsy.

Peer review

In the study, the authors demonstrated that phagocytic function of Kupffer cells from NASH liver is decreased. In this animal study, the authors also found that late phase contrast enhanced-ultrasound may be helpful in diagnosing NASH. Late phase contrast enhancement of NASH liver by ultrasound was lower compared to the simple fatty liver. The use of objective contrast quantification software appears promising. This might have implications in the diagnosis of NASH patients and in the evaluation of focal liver lesions in NASH patients; however further human studies are needed to evaluate this method.

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BRIEF ARTICLE

Comparative outcome of stapled trans-anal rectal resection and macrogol in the treatment of defecation disorders

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Abstract

AIM: To prospectively assess the efficacy and safety of stapled trans-anal rectal resection (STARR) compared to standard conservative treatment, and whether preoperative symptoms and findings at defecography and anorectal manometry can predict the outcome of STARR.

METHODS: Thirty patients (Female, 28; age: 51 ± 9 years) with rectocele or rectal intussusception, a defecation disorder, and functional constipation were submitted for STARR. Thirty comparable patients (Female, 30; age 53 ± 13 years), who presented with symptoms of rectocele or rectal intussusception and were treated with macrogol, were assessed. Patients were interviewed with a standardized questionnaire at study enrollment and 38 ± 18 mo after the STARR procedure or during macrogol treatment. A responder was defined as an absence of the Rome Ⅲ diagnostic criteria for functional constipation. Defecography and rectoanal manometry were performed before and after the STARR procedure in 16 and 12 patients, respectively.

RESULTS: After STARR, 53% of patients were responders; during conservative treatment, 75% were responders. After STARR, 30% of the patients reported the use of laxatives, 17% had intermittent anal pain, 13% had anal leakage, 13% required digital facilitation, 6% experienced defecatory urgency, 6% experienced fecal incontinence, and 6% required re-intervention. During macrogol therapy, 23% of the patients complained of abdominal bloating and 13% of borborygmi, and 3% required digital facilitation.No preoperative symptom, defecographic, or manometric finding predicted the outcome of STARR. Post-operative defecography showed a statistically significant reduction (P < 0.05) of the rectal diameter and rectocele. The post-operative anorectal manometry showed that anal pressure and rectal sensitivity were not significantly modified, and that rectal compliance was reduced (P = 0.01).

CONCLUSION: STARR is not better and is less safe than macrogol in the treatment of defecation disorders. It could be considered as an alternative therapy in patients unresponsive to macrogol.

Key words: Constipation; Obstructed defecation; Rectocele; Rectal intussusception; Stapled trans-anal rectal resection

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INTRODUCTION

Functional constipation affects up to 20% of the population, and about 50% of constipated patients consulting a tertiary referral practice complain of difficult evacuation[1,2], such as straining at stools, sensation of incomplete evacuation, or ano-rectal obstruction[3,4], and may require digitation to facilitate defecation. Rectocele and intra-rectal intussusception are frequent findings in patients with functional constipation, and are thought to play a relevant role in defecatory alterations[5]. Surgical repair of the recto pelvic anatomy has been proposed to improve defecation. Recently, an international consensus conference[6] proposed that "the combination of the characteristic history of disordered defecation and the anatomical finding of one or more of the following: rectocele, rectal intussusception, perineal descent, mucosal prolapse may lead a surgeon to offer the stapled trans-anal rectal resection (STARR)[7-9] procedure, provided that the individual has failed medical management".

The STARR procedure consists of the trans-anal resection of the distal rectum using a double-stapler. Semicircular purse-string sutures are applied on the prolapsed rectal wall, including mucosa, submucosa, and rectal muscle wall, at 2 cm above the hemorrhoidal apex so as to include the rectocele and the internal rectal prolapse.

So far both favorable[10] and unfavorable results[11] of the procedure have been reported. However, no study has assessed the efficacy and safety of the STARR procedure in comparison with evidence based conservative treatment for the management of functional constipation and defecatory disorders.

The main aim of the present study was to assess the efficacy and safety of the STARR procedure in the treatment of patients with chronic constipation complaining of defecatory disorders and with defecographic evidence of rectocele and intra-rectal intussusception. An additional aim of the study was to assess whether preoperative symptoms, and manometric or defecographic findings, can predict the long-term outcome of the STARR procedure.

MATERIALS AND METHODS

Population

Consecutive outpatients referred for refractory chronic constipation in a 48-mo period by two surgical and one gastroenterological centers, underwent a diagnostic work up, including anorectal manometry and defecography. Patients with refractory chronic constipation were identified as those who did not respond to the usual conservative treatment and still complained of difficult and/or incomplete evacuation, despite the use of high daily doses of contact laxatives, enemas, or digital evacuation. Inclusion criteria were the following: (1) diagnosis of functional constipation according to the Rome Ⅲ criteria during the preceding three months, with onset at least 6 mo prior to the diagnosis (in the absence of laxatives and/or enemas) of two or more of the following complaints: less than three bowel movements (BM) per week; straining at defecation and/or sense of incomplete evacuation and/or hard stools and/or sensation of anorectal obstruction/blockage and/or manual maneuvers to facilitate defecation on at least 25% of occasions; (2) difficult evacuation defined as either straining or sensation of obstruction/blockage; (3) age between 18 and 75 years; (4) no previous anorectal surgery; (5) no abnormality at barium enema or colonoscopy; (6) normal laboratory routine tests; (7) evidence of rectocele and/or intra-rectal intussusceptions at defecography; (8) no previous treatment with oral macrogol solution; (9) no pregnancy and efficacious birth control methods; (10) absence of systemic disease; and (11) absence of therapy affecting intestinal function. Chronic anxiolytic and antidepressive treatment were admitted provided the dosage was not modified during the study period. Exclusion criteria were the following: (1) no diagnosis of functional constipation; (2) previous anorectal surgery; (3) age less than 18 and above 75 years; (4) absence of rectocele and intra-rectal intussusception at defecography; (5) abnormality at barium enema or colonoscopy; (6) abnormal laboratory tests; (7) previous treatment with oral macrogol solution; (8) pregnancy and no use of efficacious birth control methods; (9) presence of systemic disease; and (10) presence of therapy affecting intestinal function.

Study protocol

At referral, all patients were interviewed with a standardized questionnaire, and had a physical examination. The questionnaire inquired about bowel habit: frequency of defecation, straining, stool consistency, sensation of incomplete evacuation, sensation of anal obstruction/blockage, digital facilitation to evacuate, anal pain, and anal incontinence.

On a different day, a colonoscopy was performed in patients over 50 years of age who had not had a colonoscopy or a barium enema in the previous five years. Patients were then submitted on different days for defecography and anorectal manometry.

Thirty consecutive patients, reporting an unsatisfactory response to the conservative treatment of constipation with different types of laxatives, were enrolled by the two surgical units; sixteen by AC and fourteen by

MM, and were then submitted to the STARR procedure according to a standardized and previously published method[10]. In the same time period, thirty additional and consecutive patients referred to the gastrointestinal center were assigned conservative treatment with oral macrogol solution. These patients were instructed to consume 1 sachet (8.75 g) of macrogol dissolved in 125 mL of water bid., with the option to either reduce the dose to od or increase it up to qid. to obtain evacuation of soft stools.

After the STARR procedure patients, were subjected to a second rectoanal manometry and defecography.

All patients were re-assessed at least 24 mo after the surgical procedure or the medical prescription.

At follow-up, the patients were interviewed, either face to face or by telephone, with the same standardized questionnaire used at referral, which included additional items related to treatment satisfaction and adverse events. Patients were invited to declare whether they were "totally dissatisfied with the treatment", or "partially satisfied with the treatment" when at least one symptom of constipation and/or side effects were present, or "fully satisfied with the treatment".

The degree of constipation was evaluated with the Wexner score[12]. The study was approved by the local ethics committee.

Defecography

A cleansing non-medicated water enema was performed the night before the radiological examination. About 200 milliliters of barium paste were injected into the rectum, through an anal catheter. Continuous injection of the contrast during slow withdrawal of the catheter rendered the anal canal opaque. Patients were then seated on a radiolucent commode. The entire evacuation sequence was recorded on videotape (JVC SR-VS30E Mini DV/S-VHS). Latero-lateral radiograms were taken at rest and during mid-evacuation, as previously reported[13].

Anorectal manometry

After a cleansing enema, rectal sensitivity and anorectal manometry were evaluated using a multilumen polyethylene catheter with four open tips disposed radially, and 0.5 mm apart longitudinally, continuously perfused (0.5 mL/min) with bubble-free distilled water by means of a pneumohydraulic infusion system (Arndorfer, Milwaukee, Wisconsin, United States), and connected to Beckman 611 external transducers. A fifth lumen ended in a latex balloon attached to the tip of the catheter. Intraluminal pressure variations were transmitted from the transducers to a polygraph (R612 Dynograph Recorder Sensor-Medics Italia srl) for recording.

The resting pressure profile of the anal canal was recorded with a pull-through technique. Thereafter, the manometric probe was positioned in the anal canal with the recording holes and the deflated balloon in the rectum. The patient was then asked to squeeze and to strain. Lastly the intra-rectal balloon was intermittently inflated with progressive volumes of air to elicit the recto-anal inhibitory reflex (RAIR). To assess the threshold of rectal sensitivity, patients were instructed to refer the first sensation of rectal distension and/or the urge to defecate during the incremental intrarectal balloon inflations[14].

Analysis of data

The primary endpoint of the study was relief of constipation, i.e., when the patient no longer met the Rome Ⅲ criteria for functional constipation and was considered a responder.

Secondary endpoints were: the assessment of (1) the constipation improvement by means of the Wexner score; and (2) symptoms, defecographic, and manometric findings in predicting the outcome of the STARR procedure.

Analysis of defecographic data: **Frame by frame** analysis of the sequences recorded on the videotape assessed timing and dynamics of evacuation, rectal emptying, and presence of anatomical alterations of the rectal wall. Anorectal angle (ARA) widening, anal canal opening, pelvic floor (PF) location at rest, and its mobility were assessed on the latero-lateral radiograms. Rectocele was defined as an outpouching of the anterior rectal wall into or across the rectovaginal septum, rectal intussusception was defined as an enfolding of the rectal wall, which may (intra-anal) or may not (intra-rectal) protrude through the anal canal. Pelvic floor dyssynergia was defined as: anorectal angle (ARA) widening < 10° and/or the opening of anal canal < 10 mm, and/or anal canal opening > 10 mm in more than 30 s or interrupted by repetitive squeezing contractions. Contrast rectal residue was assessed by a semi-quantitative evaluation of the rectal residue as small (< 40%), intermediate (40%-70%), and abundant (> 70%).

Analysis of manometric data: **Maximal resting and** squeezing pressures were identified as the maximal steady value observed for 20 s in basal condition and during maximal voluntary anal contraction. The thresholds of RAIR and rectal sensitivity were defined as the smallest inflated volumes of the intrarectal balloon inducing, respectively, a fall in anal pressure of at least 12 mmHg[14], and the urge to evacuate. Rectal compliance was calculated as the ratio between intrarectal balloon volume inflated with 100 mL of air and intrarectal pressure. During straining, a decrease of anal pressure of more than 20% was considered a normal relaxing pattern; the absence of a decrease of less than 20%, or the increase, of the anal pressure were considered as dyssynergic patterns.

Statistical analysis

Results are reported as means and standard deviation (mean ± SD). Descriptive statistical techniques were used to compare the two groups of patients. A χ^2 test and Fisher's exact test were used to compare the frequency of symptoms in the different study groups; Student's *t* test was used to compare the two groups for

continuous variables.

RESULTS

Sixty patients were enrolled: 30 patients underwent STARR, and 30 patients were treated with macrogol. The two study groups were comparable for gender, age, and symptom presentation (Table 1).

Macrogol group: The follow up period was 44 ± 11 mo. Twenty-two (75%) patients were classified as responders; constipation by the Wexner score decreased significantly (13.9 ± 1.5 *vs* 5.9 ± 4.5; $P < 0.001$). Seven patients (27%) had discontinued macrogol and were using either contact or other osmotic laxatives; two patients still required digital facilitation (Figure 1).

STARR group: The follow up period was 38 ± 18 mo. Sixteen (53%) patients were classified as responders. No outcome difference was observed between patients of the two surgical units (responders: 8/16 *vs* 6/14). Constipation by the Wexner score decreased significantly (13.4 ± 3.2 *vs* 7.32 ± 5.76; $P < 0.001$); nine (30%) patients still used laxatives and four (13%) digital facilitation (Figure 1). After the surgical procedure, 14 patients reported at least one side effect and two (6%) required re-intervention; the first for relapse of rectocele and the second for fecal incontinence. The presenting symptoms at referral did not differ between responders and non-responders (Table 2).

Comparison between the study groups: The two groups did not differ statistically for response to the treatment according to the Rome criteria for functional constipation, for improvement of constipation evaluated by means of the Wexner score, and for degree of satisfaction (Table 3). Bowel symptoms at follow-up were similar in the two study groups (Figure 1).

Adverse events in the STARR procedure group, at the end of follow up, were: staining/leakage (13%), fecal incontinence (6%), urgency (6%), intermittent anal pain (17%), and re-intervention (6%). Adverse effects in the macrogol therapy group were abdominal bloating (23%), and borborygmi (13%).

Defecography

Defecography was performed at referral and at 7 ± 4 mo after STARR in 16 patients. In comparison to the pre-surgical condition, defecographic variables did not vary after surgery, except for a significant reduction in rectal diameter (7.5 ± 1.2 cm *vs* 5.6 ± 1.2 cm; $P < 0.001$), and size of rectocele (3.9 ± 1.3 cm *vs* 1.4 ± 1.5 cm; $P < 0.001$). The size of intussusceptions was reduced, but the variation was not statistically significant (3.2 ± 1.7 cm *vs* 2.4 ± 1.3 cm; ns).

Responders and non-responders did not statistically differ for any defecographic variable assessed before surgery (Table 3). Before the operation, the mean ARA variation during evacuation was not statistically different between non-responders and responders (Table 4). After the operation, it was significantly less in the non-responders compared to the responders (28 ± 16 *vs* 8 ± 20 degrees; $P < 0.05$).

Before STARR, defecographic evidence of pelvic floor dyssynergia was detected in three patients; equally represented in responders (one patient) and non- responders (two patients). After STARR, defecographic evidence of pelvic floor dyssynergia was detected in three patients; equally represented in responders (one patient) and non responders (two patients).

Ano-rectal manometry

Ano-rectal manometry was performed at referral and 24 ± 4 mo after STARR in 12 patients. After STARR, rectal compliance was significantly reduced (5.4 ± 1.9 *vs* 3.7 ±

Table 1 Clinical presentations of the two study groups *n* (%)

	STARR *n* = 30 (F28)	Macrogol *n* = 30 (F30)	*P* value
Mean age (yr)	51 ± 9	53 ± 13	
Duration of constipation > 10 yr	24 (80)	26 (87)	0.7
Straining	28 (93)	29 (97)	1
Hard stools	27 (90)	29 (97)	0.6
Incomplete evacuation	27 (90)	27 (90)	0.7
Anal blockage	27 (90)	27 (90)	0.7
Digital facilitation	11 (37)	8 (27)	0.6
< 3 BM/wk	23 (77)	26 (87)	0.5
Laxatives	27 (90)	30 (100)	0.2
Rectal bleeding	13 (43)	7 (23)	0.2
Rectocele > 3 cm	23 (77)	15 (50)	0.06
Rectal intussusception	15 (50)	20 (67)	0.3

BM: Bowel movements; STARR: Stapled trans-anal rectal resection.

Table 2 Presenting symptoms in responders and non-responders to stapled trans-anal rectal resection *n* (%)

	Responders	Non-responders	*P* value
Straining	16 (100)	12 (86)	0.4
Hard stools	14 (87)	13 (93)	0.9
Incomplete evacuation	16 (100)	11(78)	0.2
Anal blockage	15 (94)	12 (86)	0.9
Digital facilitation	4 (25)	7 (50)	0.3
< 3 BM/wk	13 (81)	10 (71)	0.8
Laxatives	14 (87)	13 (93)	0.9

BM: Bowel movements.

Table 3 Number of responders, degree of satisfaction, and Wexner constipation score variation during macrogol and after stapled trans-anal rectal resection treatment *n* (%)

	Macrogol	STARR	*P* value
Responders	22 (73)	16 (53)	0.2
Satisfaction			0.4
Total	18 (60)	16 (53)	
Partial	5 (17)	9 (30)	
Not satisfied	7 (23)	5 (17)	
Δ Wexner score, mean ± SD	8 ± 5	6 ± 5.2	0.1

STARR: Stapled trans-anal rectal resection; Δ: Difference.

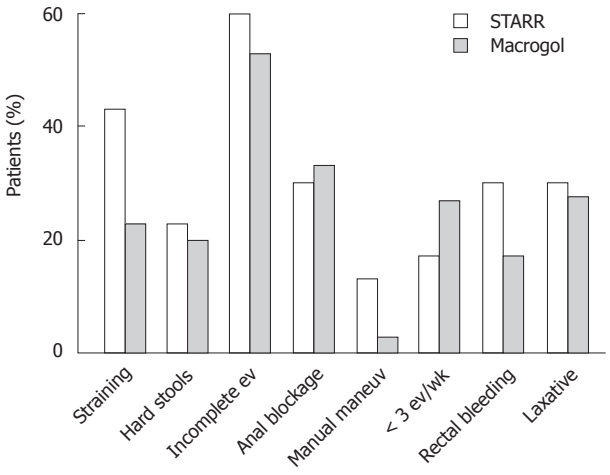

Figure 1 Percentage of patients with constipation symptoms, rectal bleeding, and use of laxatives (other than macrogol in the macrogol treatment group) after stapled trans-anal rectal resection and during macrogol treatment. No statistical difference was observed between the two study groups. STARR: Stapled trans-anal rectal resection. ev: Evacuations.

1.2 mL/mmHg; $P = 0.01$), all other manometric variables did not vary. Responders and non-responders did not differ for any manometric findings before and after the procedure.

Before STARR, manometric evidence of pelvic floor dyssynergia was detected in four patients, equally represented in responders (two patients) and non-responders (two patients). After STARR, manometric evidence of pelvic floor dyssynergia was detected in seven patients, equally represented in responders (four patients) and non-responders (three patients).

DISCUSSION

No previous study has compared the STARR procedure with conservative pharmacological therapy. This study evaluated, over a long time period, the outcome of STARR treatment for constipation, defined with standardized and validated criteria. The surgical procedure was performed following the standardized and previously published procedure[9] by two experienced coloproctology units that obtained comparable postsurgical outcomes. The patients submitted to the STARR procedure reported an unsatisfactory response to a usual, but not standardized, laxative treatment.

Previous studies have reported the postsurgical outcomes of the STARR procedure or of other modified techniques, but no study has compared the efficacy and safety of STARR procedure versus the standardized conservative treatment.

In the present investigation, we compared the outcome of STARR procedure with the gold standard treatment of constipation based on macrogol in a prospective, parallel group, longitudinal study. This therapy for functional constipation is supported by level 1 evidence and grade A recommendation[15]. This is a high molecular weight (3350 or 4000) non-absorbable, non-metabolized soluble polyethylene, which forms hydrogen bonds with water in the gut; it is used with orthograde whole-gut irrigation in preparing for colon investigation, and in small-volume daily doses (125-250 mL) to treat functional constipation. Macrogol therapy is reported to be effective in about 80% of constipated patients, is well tolerated, and devoid of serious side effects, even in long term treatment[16,17].

Table 4 Defecographic findings in responders and non-responders submitted to stapled trans-anal rectal resection (mean ± SD)

Variables	Before		After	
	Non-responders	Responders	Non-responders	Responders
Rectal diameter (cm)	7.7 ± 1.4	7.3 ± 1	5.5 ± 0.9	5.8 ± 1.5
Δ ARA evacuation (°)	16 ± 8	25 ± 18	8.1 ± 20.3	28 ± 16.2[a]
PF at rest (cm)	5.1 ± 1.5	4.8 ± 1.3	4.3 ± 1.5	4.6 ± 1
PF during sqeezing (cm)	1.4 ± 0.7	1.3 ± 0.9	1.8 ± 0.9	1.2 ± 0.8
Δ PF evacuation (cm)	3.1 ± 1.6	3 ± 1.3	3.6 ± 1.1	3.1 ± 1.5
Rectocele (cm)	3.8 ± 1.8	4.1 ± 0.6	1.1 ± 1.6	1.7 ± 1.5
Anal diameter (cm)	1.4 ± 0.6	1.4 ± 0.8	1.3 ± 0.8	1.1 ± 0.4

ARA: Ano-rectal angle; PF: Pelvic floor; Δ: Difference; [a]$P < 0.05$ *vs* non-responders.

All the patients of these study groups met the Rome Ⅲ criteria for functional constipation and had defecographic evidence of rectocele and or intrarectal intussusception. Patients were evaluated with a standardized questionnaire before and after treatment, with a mean follow-up of 38 mo, which is, to our knowledge, one of the longest reporting STARR outcome[18-31]. Some long-term studies reported sustained improvement of defecation score, but provided conflicting results about side effects, relapse, and complications[32-34].

The present study demonstrated that the surgical treatment was less, but not statistically so, efficacious than the conservative one, as indicated by the finding that 75% of the macrogol group and 53% of the STARR group did not present any Rome criteria for functional constipation. In addition, after the STARR operation, about 30% of the patients still consumed laxatives and 13% were using digital manipulation to evacuate.

Of note is that during a mean follow up of three years after the STARR operation, intermittent bleeding was present in 24% of the patients, anal pain in 17%, and anal incontinence of variable severity in 25%. Furthermore, two patients required a re-intervention. The observed prevalence of these complications in the present study is similar to that reported in other studies[11,25]. The European STARR register reports perioperative and postoperative complications in about 36% of the patients, and defecatory urgency in 20% of the cases at one year of follow-up[35]. In our study, a few adverse events of STARR persisted in this long-term follow up with possible detrimental effects on daily living.

The degree of satisfaction expressed by patients parallels that of the improvement of constipation achieved by the two treatments. Only 15% of the patients were

not satisfied with the STARR procedure, despite the presence of some persistent symptoms or the previously mentioned complications.

Defecography was performed in all patients before and, in a subgroup of them, after the STARR procedure. No defecographic finding before surgery predicted the outcome of the STARR operation. As expected, after surgery there was a significant reduction of rectal diameter and the size of the rectocele, but such variations were no different between responders and non-responders.

After STARR, the mean value of ARA variation during evacuation in non-responders was significantly reduced in comparison to the preoperative value, and was significantly less than in responders. This finding indicates that the STARR procedure may affect the relaxation pattern of the puborectalis muscle during evacuation. It remains to be established how the STARR procedure induces this effect on the puborectalis. It is reasonable to assume that a reduced ARA variation during defecation may negatively affect evacuation, nonetheless it is not possible to conclude from this study whether it has any role in the poor clinical outcome of non-responders.

According to the STARR consensus conference, the inclusion criteria for surgical treatment are based on clinical presentation of difficult evacuation and/or straining, in the presence of rectocele and/or intussusception. In this study, the inclusion criteria were based on the consensus conference recommendations; however, no presenting symptom was predictive of the outcome of STARR, nor of the procedure adverse events.

In addition, anorectal manometry was not useful in predicting the outcome of STARR. A previous study reported that altered compliance could be predictive of positive outcome[10]. This observation was not confirmed by the present study; the discordance could be due to the small sample in this study or to the different method used to evaluate compliance. In the previous study, compliance was assessed by the ratio between volume and pressure at the threshold of rectal sensitivity, whereas in this study, it was calculated using the fixed volume of 100 mL of air, to avoid possible subjective differences of rectal sensitivity. However, a study designed to investigate whether rectal compliance is altered in females with obstructed defecation, showed that the compliance of the rectal wall is normal[36].

Several structural and functional alterations of the rectum and/or pelvic floor are considered to markedly impair the act of defecation; however, the findings of this study indicate that stool consistency is a major factor in chronic constipation. Indeed, the favorable response to macrogol treatment in the non-surgical group indicated that reducing stool consistency, reported at referral to be hard by more than 90% of the patients, effects resolution of constipation, despite the persistence of the structural and functional alterations. Thus, it seems reasonable to consider a surgical procedure only after the failure of a standardized macrogol treatment.

In conclusion, the results of this prospective study suggest that STARR is not better and is less safe than conservative therapy in the treatment of defecation disorders in functional constipation patients. Preoperatively, no presenting symptom, or defecographic, and manometric variables were useful to indicate STARR and predict its results. Postoperatively, a reduced widening of ARA during evacuation was associated with an unfavorable outcome of the procedure. The STARR procedure could be considered as an alternative treatment in patients with constipation and defecatory disorders who are unresponsive to conservative macrogol treatment.

COMMENTS

Background

Constipation is a common problem. It is not clear whether a defecation disorder commonly known as obstructed defecation syndrome (ODS) is due to anatomical abnormalities. However, some surgeons propose a new type of surgery [stapled trans-anal rectal resection (STARR)], which, by correcting the anatomical changes, should solve the constipation. Surgery should be considered for those patients who do not benefit from conservative treatment. The gold standard pharmacological treatment of constipation is based on macrogol, which is also effective in patients with altered defecation.

Research frontiers

Ideally, a randomized double blind clinical trial would have more properly assessed the efficacy and safety of the STARR procedure, but the comparison between a non-invasive treatment and an invasive procedure is an objective obstacle to plan a proper protocol, and such studies have not been previously performed. No previous study has so far compared the STARR procedure with conservative pharmacological therapy.

Innovations and breakthroughs

This study evaluated, over a long time period, the outcome of STARR treatment for constipation in comparison with macrogol therapy. The study has shown that STARR is not better, and is less safe, than macrogol therapy. In addition, no preoperative findings could predict the outcome of surgery.

Applications

The authors believe that macrogol should be used before considering surgery in cases of lack of response to conservative treatment

Terminology

Rectocele is a protrusion of part of the rectum into the vagina. Rectal intussusception is a protrusion of the rectal mucous membrane into the lower rectum.

Peer review

The manuscripts reports the comparative analysis of two strategies in managing functional constipation. The authors have compared outcomes of patients that underwent treatment with a conservative regimen that consisted of macrogol and patients that underwent the STARR procedure. This is a well done study; despite not being randomized it provides useful information and should be published.

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BRIEF ARTICLE

Comparison of Milan and UCSF criteria for liver transplantation to treat hepatocellular carcinoma

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Abstract

AIM: To assess the validity of the Milan and University of California San Francisco (UCSF) criteria and examine the long-term outcome of orthotopic liver transplantation (OLT) in patients with hepatocellular carcinoma (HCC) in a single-center study.

METHODS: This study is a retrospective review of prospectively collected data. Between 1998 and 2009, 56 of 356 OLTs were performed in patients with HCC. Based on pathological examination of liver explants, patients were retrospectively categorized into 3 groups: Milan + (n = 34), Milan -/UCSF + (n = 7) and UCSF - (n = 14).

RESULTS: Median follow-up period was 39.5 (1-124) mo. The 5-year overall survival rates in the Milan +, Milan -/UCSF + and UCSF-groups were 87.7%, 53.6% and 33.3%, respectively (P < 0.000). Within these groups, tumor recurrence was determined in 5.8%, 14.3% and 40% of patients, respectively (P < 0.011). Additionally, the presence of microvascular invasion within the explanted liver had a negative effect on the 5-year disease free survival (74.7% *vs* 46.7%, P < 0.044).

CONCLUSION: The Milan criteria are reliable in the selection of suitable candidates for OLT for the treatment of HCC. For cases of OLT involving living donors, the UCSF criteria may be applied.

Key words: Hepatobiliary radiology; Hepatobiliary surgery; Hepatobiliary pathology; Hepatocellular carcinoma; Liver malignancy; Liver transplantation; Living donor liver transplantation; Living related liver transplantation; Oncologic surgery; Survival; Transplant

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world, and is associated with the third highest number of cancer-related deaths[1]. Moreover, for 70%-90% of HCC cases, HCC develops on a background of cirrhosis or chronic liver inflammation[2]. Currently, there are three potentially curative therapeutic options for HCC, liver resection, orthotopic liver transplantation (OLT), and local ablative therapies[3]. Although liver resection treats localized HCC, it is not optimal for treating multifocal HCC, and has no efficacy in preventing *de novo* HCC that can develop in the remnants of a cirrhotic liver. Alternatively, liver transplantation is an established therapy which offers the potential advantage of removing both the tumor and the organ at risk for developing future malignancies[4].

In order to identify the best candidates for OLT, a set of criteria were proposed, referred to as the "Milan" criteria. According to these guidelines, patients with cirrhosis and a solitary tumor with a diameter less than 5 cm, or patients who have up to 3 tumor nodules, each of which is smaller than 3 cm and are not characterized by vascular invasion or extrahepatic metastasis (according to preoperative radiologic findings), are patients that have a higher probability of obtaining a successful outcome following OLT. For example, the 5-year recurrence-free survival rate for a set of patients who fulfilled the Milan criteria was reported to be 83%[5]. The "Milan criteria" were subsequently adopted by the United Network for Organ Sharing (UNOS) in 2002 as the optimal criteria for determining the use of OLT to treat HCC[6]. However, an expanded set of criteria proposed by the University of California San Francisco (UCSF), referred to here as the "UCSF" criteria, allows patients with a solitary tumor smaller than 6.5 cm, or patients having 3 of fewer nodules, with the largest lesion being smaller than 4.5 cm or having a total tumor diameter less than 8.5 cm without vascular invasion, to undergo OLT. Based on the comparable success of this set of criteria in selecting patients for OLT, it has been suggested that the Milan criteria may be too stringent[7]. Therefore, the aim of this study was to examine the long-term outcome of patients undergoing liver transplantation to treat HCC, and to compare the use of the current criteria (both the Milan and UCSF) for the selection of HCC patients for possible OLT.

MATERIALS AND METHODS

Between 1998 and 2009, 56 of 356 (15.7%) OLTs were performed in patients with HCC at the Dokuz Eylul University Hospital (Izmir, Turkey). Of these, 50 were diagnosed with HCC prior to transplantation, and 6 (10.7%) were diagnosed during OLT. According to pre-OLT imaging and post-OLT pathological evaluation, 56 patients were retrospectively classified into 3 groups: Milan +, Milan -/UCSF + and UCSF - (Table 1).

Following the pathological examination of liver explant specimens, 14 (25.0%) patients were reclassified due to underestimates of tumor size, and 7 (12.5%) patients were reclassified due to the tumor number being greater than expected (false negative rate: 25%) (Table 1). For the applied Milan and UCSF criteria, false negative rates of pre-OLT radiological evaluations were 22.7% (10/44) and 16.3% (8/49), respectively. In summary, 8 patients met the UCSF criteria prior to undergoing OLT, and exceeded the UCSF criteria following pathologic evaluation of the explants obtained.

Table 1 Number of patients associated with each criteria depending on pre-orthotopic liver transplantation imaging and post-orthotopic liver transplantation pathology results *n* (%)

Diagnostic approaches	Milan +	Milan -/UCSF +	UCSF -
pre-OLT imaging	44 (78.0)	5 (8.9)	7 (12.5)
post-OLT pathology	34 (60.7)	7 (12.5)[1]	15 (26.8)[2]

[1]6/7 patients were classified as "Milan +" based on pre-OLT imaging; [2]4 patients each were classified as "UCSF +/Milan -" and "Milan +" based on pre-OLT imaging. UCSF: University of California San Francisco; OLT: Orthotopic liver transplantation.

Pre-OLT workup

All patients included in this study had cirrhosis due to various etiologies. A pre-operative diagnosis of HCC was based on a patient's medical history, a physical examination, laboratory studies, α-fetoprotein (AFP) levels, and the results of one or more imaging studies [i.e., abdominal ultrasonography, contrast-enhanced computed tomography (CT), angiographic CT, or abdominal magnetic resonance imaging (MRI)]. Tumor biopsies were not performed to confirm each diagnosis. Chest CT, cranial CT, and technetium-99 m bone scintigraphy were used to detect the potential incidence of extrahepatic disease, and distant or lymph node metastases were not detected in any of the patients. Pre-OLT adjuvant therapies, including radiofrequency ablation (RFA), transarterial hepatic chemoembolization (TACE), percutaneous ethanol injection (PEI), and liver resection were performed in selected patients. In the absence of medical contraindications, patients who fulfilled the Milan criteria in pre-OLT evaluations were qualified to receive a transplant from either a living or deceased donor. Alternatively, for patients who did not fulfill the Milan criteria, these patients were qualified to receive organs from living donors only. In our series, 31 (55.3%) living and 25 (44.7%) deceased donor liver grafts were utilized. In the latter group, 3 marginal liver grafts from deceased donors were transplanted to recipients who did not fulfill the Milan criteria.

Pre-OLT locoregional therapy

Thirteen out of 50 (26%) patients received locoregional treatment prior to OLT, which included either TACE (n = 9), liver resection (n = 2), PEI (n = 1), or RFA (n = 1). Moreover, complete radiological regression was associated with all patients who underwent TACE, PEI, or RFA.

Table 2 Patient characteristics according to Milan and University of California San Francisco criteria determined from post-orthotopic liver transplantation imaging and post-orthotopic liver transplantation pathological evaluations *n* (%)

Variables	Milan +	Milan -/UCSF +	UCSF -	*P* value
No. of patients	34	7	15	
Gender (M/F)	29/5	7/0	14/1	
Age (yr)	55.1 ± 6.6	51.0 ± 4.5	56.4 ± 5.5	0.222
CTP (A/B/C)	8/18/8	3/1/2003	10/4/2001	
MELD	13.3 ± 4.9	12.7 ± 4.3	13.4 ± 4.3	0.803
AFP (ng/dL)	4.9	6.1	11.9	0.953
No. nodules	1.4 ± 0.6	2.4 ± 1.3	5.7 ± 4.4	0.000
Max diameter of largest nodule (mm)	22.5 ± 11.6	45.9 ± 11.5	47.9 ± 23.8	0.000
Grade				
Well	17 (73.9)	2 (28.6)	4 (26.7)	
Moderate	17 (53.1)	5 (71.4)	10 (66.7)	
Poor			1 (6.6)	
Microvascular invasion	6 (17.6)[a]	2 (28.6)	7 (46.7)[a]	0.034[a]

M: Male; F: Female; UCSF: University of California San Francisco; CTP: Child-Turcot-Pugh; MELD: Model of End-stage Liver Disease; AFP: α-fetoprotein. [a]$P < 0.05$.

Furthermore, two patients were successfully downstaged to the Milan criteria following treatment with TACE. For the two patients who underwent curative resection for HCC, both suffered intrahepatic recurrences one year later and were scheduled to undergo OLT. Due to the use of local ablative procedures, the incidence of major morbidity was 0%.

Immunosuppressive regimen and antiviral prophylaxis

OLTs involving deceased or living donors were performed by the same surgical team, and standard techniques were used. Briefly, patients received an immunosuppressive regimen of calcineurin inhibitors (i.e., cyclosporine A or tacrolimus), mycophenolic acid, and corticosteroids in the early post-operative period. The latter were tapered and eventually discontinued during the second month following each OLT. For patients with hepatitis B virus (HBV), peri- and post-operative hepatitis B immunoglobulin and an antiviral were administered. Lamivudine-resistant patients were treated with tenofovir. During the follow-up period, serum hepatitis B antibody levels were kept above 200 IU/L, and interferon and ribavirin treatments were initiated if hepatitis C recurred.

Pathological evaluation

All explants were examined by an experienced hepatopathologist (Sagol O), and were categorized depending on the size, number, distribution, HCC histologic grade, and vascular invasion associated with each tumor.

Post-OLT monitoring

Post-operative death was defined as death within 3 mo post-OLT. All patients underwent regular follow-up examinations in the outpatient clinic. Both the surgical team and an experienced hepatologist maintained surveillance for tumor recurrence or metastasis based on AFP levels and chest CT scans, as well as by contrast-enhanced abdominal CT scans performed once every 3 mo for the first year post-OLT, then once a year thereafter. The minimum follow-up period was 12 mo.

Statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Chicago, Illinois, United States). Data are expressed as the mean ± SD, and median and range values are provided when appropriate. Quantitative variables were compared using the Kruskal-Wallis test. Comparisons between groups with regard to qualitative variables were performed using the chi-square test and Fisher's exact test, if necessary. Survival was calculated using Kaplan-Meier estimates, with comparisons made using the log rank test. $P < 0.05$ was considered statistically significant.

RESULTS

The demographic characteristics of the patients included in this study are presented in Table 2. OLT was performed for patients who had been on a waiting list for a median of 62 d. Furthermore, the interval between when the patients were listed for transplantation and when the patients underwent transplantation was similar for both deceased and living donor transplantations (i.e., 60 *vs* 68 d, respectively). The average rates of graft weight/body weight for both OLT groups were also 1.09% (range, 0.69-1.8) and 1.82% (range, 0.76-2.58), respectively.

The mean hospital stay for patients was 31.2 ± 21.5 d, and complications associated with surgery were experienced by 10 (17.8%) recipients. Four (7%) recipients presented with biliary leak, with two of the cases resolving and two of the cases resulting in death due to sepsis. In addition, 3 (5.3%) recipients acquired pneumonia post-operatively. Two of these patients recovered, while the other died from respiratory arrest. One recipient died due to intra-abdominal sepsis and another developed intra-abdominal hemorrhage post-operatively and underwent a second operation. Only one patient experienced a wound infection. In contrast, a total of 4 (7%) patients died due to surgical complications, while another patient died from duodenal ulcer perforation with sepsis. The overall mortality for this study was 8.9% (5/56).

Pre-operative AFP levels ranged from 1.72 to 3630 ng/dL (median, 158.7 ng/dL), with the normal range being 0.5-5 U/L. Only 6/56 (10.7%) patients had an AFP level greater than 200 ng/dL. Furthermore, the mean AFP levels during the pre-OLT period for patients with incidental HCC was 15.5 ± 26.6 ng/dL (range, 2.45-63.1).

Tumor characteristics are described in Table 2. In particular, the number of nodules per patient and the diameter of the largest nodule were significantly lower in the Milan + group compared to the Milan -/UCSF + and UCSF-groups, respectively ($P < 0.000$).

Adjuvant chemotherapy was an option for 13 (23.2%) patients who were medically eligible for chemotherapy

Table 3 Follow-up data for patients who underwent orthotopic liver transplantation for hepatocellular carcinoma *n* (%)

Variables	Milan +	Milan -/UCSF +	UCSF -
No. of patients	34	7	15
Post-operative death	2 (5.9)	-	1 (4.5)
Death	5 (14.7)	2 (28.6)	11 (73.3)
HCC recurrence	2 (5.8)	1 (14.3)	6 (40.0)
Median follow-up	51.5 (1:124)	32 (1:66)	14 (3:66)

UCSF: University of California San Francisco; HCC: Hepatocellular carcinoma.

Table 4 Causes of mortality associated with cases in this study

Causes of death	*n* (%)
Sepsis (late postoperative period)	7 (36.8)
Lung metastasis	5 (26.3)
Sepsis (early postoperative period)	3 (15.8)
Recurrent fulminant hepatitis B	2 (10.5)
Duodenal ulcer perforation	1 (5.3)
Intracranial hemorrhage	1 (5.3)

and was administered post-OLT according to pathological tumors with a diameter of > 2 cm and/or microvascular invasion or post-OLT in case of recurrence. These patients received 5-fluorouracil in combination with epirubicin, mitomycin C, or cisplatin. None of the patients died as a result of chemotherapy-related complications. However, for three patients, grade 4 hematologic toxicity was reported, two patients experienced grade 3 gastrointestinal toxicity (i.e., excessive nausea and vomiting), and one patient exhibited grade 3 neurotoxicity.

Recurrence

Tumor recurrence was experienced by 9 (16%) patients (Table 3). Recurrence rates were 5.8%, 14.3% and 40% in the Milan +, Milan -/UCSF + and UCSF-groups, respectively. Five patients presented solely with distant metastasis in the lung ($n = 3$), in both the lung and bone ($n = 1$), and in the bone and skin ($n = 1$). The remaining 4 patients suffered intrahepatic tumor recurrence with ($n = 2$) or without ($n = 2$) extrahepatic metastasis (i.e., lung, adrenal gland, bone). None of the 6 recipients with incidental HCC recurred. For the treatment of tumor recurrence, chemotherapy was the only therapeutic option administered.

Survival

The median follow-up period was 39.5 mo (range, 1-124), and at this point, 19 (33.9%) patients had died. Causes of death are listed in Table 4. Correspondingly, the 1-, 3- and 5-year overall survival (OS) rates of the whole series were 80.4%, 68.9% and 65.3%, respectively. The disease-free survival rates for the same categories were 78.6%, 67.1% and 67.1%, respectively.

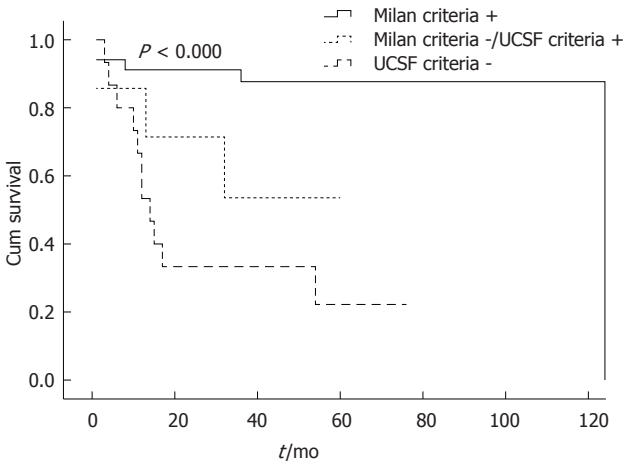

Figure 1 Kaplan-Meier overall survival curves for Milan +, Milan -/UCSF + and UCSF-patients (post-orthotopic liver transplantation). UCSF: University of California San Francisco.

Overall survival in the groups

When OS rates were calculated according to the criteria used, the 1-, 3- and 5-year OS rates for the Milan + group were 91.2%, 87.7% and 87.7%, respectively. The mean survival time was 110.3 ± 7.2 mo (95% CI: 96.1-124.4) for this group. In contrast, the 1-, 3- and 5-year OS rates for Milan -/UCSF + patients were 85.7%, 53.6% and 53.6%, respectively. The mean OS period was 39.8 ± 9.1 mo (95% CI: 22.1-57.6). The OS rates for UCSF-patients were 66.7%, 33.3% and 22.2%, respectively, with a mean survival time of 29.8 ± 7.4 mo (95% CI: 15.3-44.4) ($P < 0.000$) (Figure 1).

Disease-free survival in the groups

The rates of disease-free survival at 1-, 3- and 5-years post-OLT were 91.2%, 87.7% and 87.7%, respectively, for Milan + patients. Furthermore, the mean disease-free survival period was 109.3 ± 7.2 mo (95% CI: 95.2-123.1). In contrast, the 1- and 3-, and 5-year disease-free survival rates for Milan -/UCSF + patients were 71.4%, 53.6% and 53.6%, respectively, and the mean disease-free survival period was 39.6 ± 9.2 mo (95% CI: 21.6-57.5). The disease-free survival rates for the UCSF-group were 33.3%, 25.0% and 25.0%, respectively, and the mean disease-free survival period was 26.1 ± 7.8 mo (95% CI: 10.9-41.4) ($P < 0.000$) (Figure 2).

Microvascular invasion

When OS rates were calculated for patients with and without microvascular invasion, the 1-, 3- and 5-year OS rates for each category were 87.8%, 74.7% and 74.7%, and 73.3%, 53.3% and 35.6%, respectively ($P < 0.029$) (Figure 3). Furthermore, disease-free survival rates were 82.9%, 74.7% and 74.7% for patients without microvascular invasion, and 53.3%, 46.7% and 46.7% for patients with microvascular invasion ($P < 0.044$) (Figure 4). Moreover, we found that the presence of microvascular invasion was significantly higher in UCSF -than Milan +

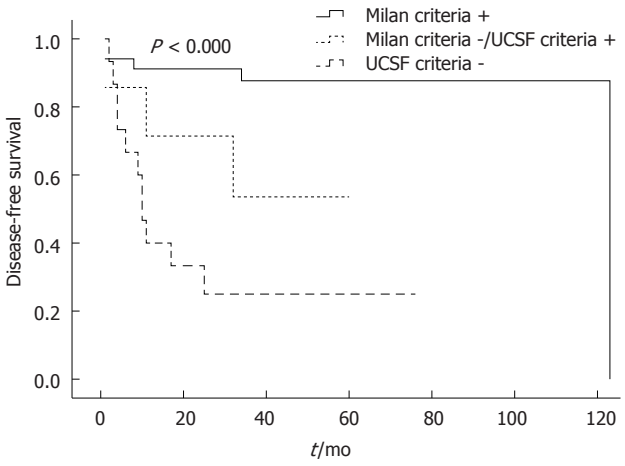

Figure 2 Kaplan-Meier disease-free survival curves for Milan +, Milan -/UCSF + and UCSF-patients (post-orthotopic liver transplantation). UCSF: University of California San Francisco.

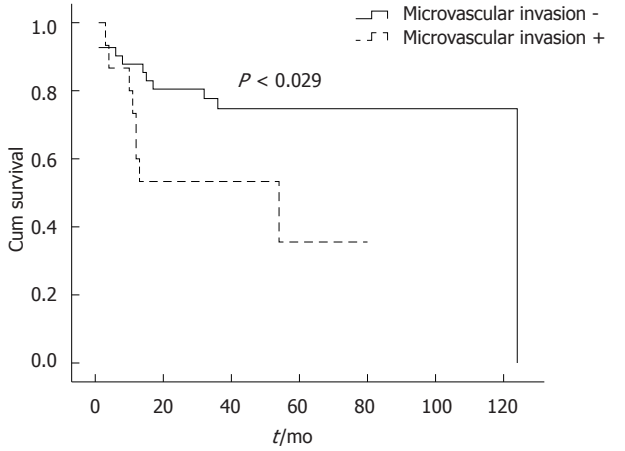

Figure 3 Kaplan-Meier overall survival curves for patients with microvascular invasion (post-orthotopic liver transplantation).

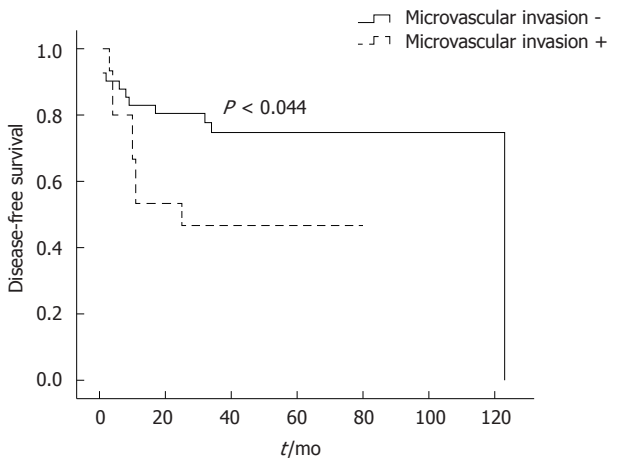

Figure 4 Kaplan-Meier disease-free survival curves for patients with microvascular invasion (post-orthotopic liver transplantation).

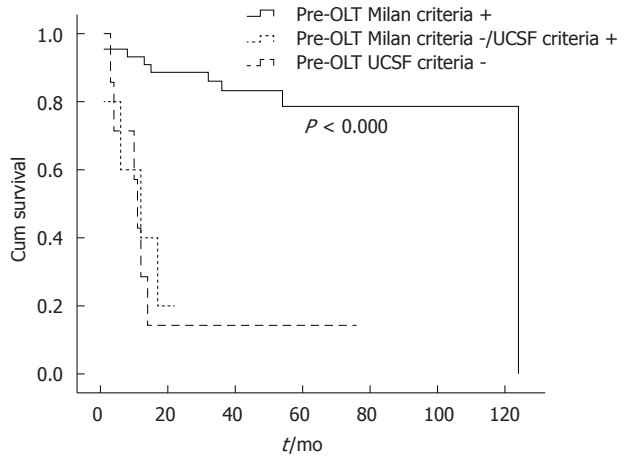

Figure 5 Kaplan-Meier overall survival curves for Milan +, Milan -/UCSF + and UCSF-patients based on pre-orthotopic liver transplantation imaging results. UCSF: University of California San Francisco. OLT: Orthotopic liver transplantation.

patients ($P < 0.034$).

Survival analysis based on pre-OLT imaging

In the three groups which were classified based pre-OLT imaging, OS rates are shown in Figure 5. Among these, the 1-, 3- and 5-year OS rates for Milan + patients were 93.2%, 83.3%, and 78.6%, respectively. The mean survival period was 102.7 ± 7.2 mo (95% CI: 88.5-116.9 mo). In contrast, the OS rates for UCSF-patients were 28.6%, 14.3%, and 14.3%, respectively, with a mean survival period of 18.6 ± 8.9 mo (95% CI: 1.0-36.2 mo) ($P < 0.000$). When the same evaluations were made for Milan -/UCSF + patients ($n = 5$), only the 1-, and 2-year OS rates were available and were 40% and 20%, respectively, and the mean survival period was 11.6 ± 3.4 mo (95% CI: 5.0-18.2 mo) (Figure 5).

DISCUSSION

This retrospective study sought to examine the overall reliability of the Milan and UCSF criteria as clinical tools for the selection of HCC patients to be treated with OLT. Currently, the best liver transplant outcomes for HCC are obtained using the Milan criteria. For these patients, the 5-year survival rates are greater than 70% and the recurrence rate is 15%[8-10]. In 2002, UNOS adopted the "Milan criteria" as the optimal criteria for selecting patients for possible OLT due to HCC[6]. However, it was subsequently proposed that the Milan guidelines be expanded based on the comparable survival rates that were being achieved for patients undergoing selection based on the UCSF criteria[7]. Therefore, to investigate whether the Milan criteria are too restrictive for the selection of patients who could otherwise benefit from OLT, a series of HCC cases, who were confirmed by pathology studies of explanted liver specimens, were analyzed. In particular, Milan + patients had significantly better 5-year OS rates than both Milan -/UCSF + and UCSF-patients (87.7% *vs* 53.6% and 33.3%; $P < 0.039$ and $P < 0.000$, respectively). Additionally, Milan -/UCSF + patients who would be expected to obtain the maximum benefit from the proposed expanded criteria had

no significant difference in survival rates compared to UCSF-patients (53.6% *vs* 33.3%, $P < 0.239$).

In most cases, patient selection criteria are based on radiological imaging performed to assess the extent of intrahepatic disease present, and to exclude extrahepatic spread. However, pre-OLT imaging studies have been shown to underestimate tumor stage in 20%-30% of cases[4,11,12]. Consistent with these results, pre-OLT imaging associated with the series of cases evaluated in this study underestimated either the size, or the number, of tumors present in 14/56 (25%) patients. As a result, 80% of patients identified as Milan -/UCSF + prior to OLT were reclassified as UCSF-following pathological evaluations of the explants obtained. In addition, the 5-year survival rate of these reclassified patients was 25%. Thus, the Milan criteria appear to provide a wider "safe" margin and reduce the negative influence of underestimates of tumor stage by pre-OLT imaging.

As the interval between imaging studies performed and the date of transplantation increases, the patient undergoing transplantation is potentially at a higher risk for tumor recurrence. This could be avoided by shortening the waiting time for a transplant by increasing the number of organ donors, or better utilization of living donors. In this series, OLT was performed a median of 62 d after the patient was placed on a waiting list. In addition, due to the limited number of deceased donors available in Turkey, transplant centers have agreed to allocate deceased donor liver grafts to HCC patients who meet the Milan criteria. Although living donors are currently utilized as a source of liver grafts for the treatment of HCC, the primary concern for transplant programs is minimization of donor morbidity and mortality. Today, living donor hepatectomies are performed safely, and for countries experiencing a shortage in deceased donors, OLTs from living donors shorten the time that patients spend on a waiting list[13]. In our study, 55.4% of the grafts used were obtained from living donors. However, despite all efforts, the morbidity and mortality of living donors following resection of the right lobe of the liver is approximately 0.5% and 35.0%, respectively[14-15]. Thus, considering the safety of living donors and the poor long-term survival rates associated with recipients exceeding the UCSF criteria, it is recommended that the UCSF criteria be followed in order to select HCC patients with the highest likelihood of survival following OLT.

Overall, the recurrence rate (16%) associated with this study was consistent with previous reports[16]. According to the patient groups, the recurrence rates were 5.8%, 14.3% and 40.0% for the Milan +, Milan -/UCSF +, and UCSF-patients, respectively. We hypothesize that these low recurrence rates are associated with the use of the Milan criteria in patient selection and especially for the allocation of deceased donor grafts.

Microvascular invasion is a key step in HCC metastasis. However, as a characteristic of tumor growth that must be determined pathologically, it is impossible to know pre-operatively if it exists. According to previous studies, the presence of microvascular invasion is considered a negative factor for OLT in the treatment of HCC. Correspondingly, in our study microvascular invasion was associated with a significant decrease in 5-year OS rates from 74.7% to 35.6% ($P < 0.029$). Moreover, we found that the presence of microvascular invasion was significantly higher in UCSF -than Milan + patients ($P < 0.034$).

There are cases where HCC is detected in explanted livers incidentally, and transplant centers worldwide have reported variable incidences of this situation. In particular, Chui *et al*[17] and Loinaz *et al*[18] reported the unexpected incidence of HCC to be 1.4% and 2.8%, respectively. In other series, slightly higher incidences of 7% and 8% have been reported[19,20]. This discrepancy could be partly due to the thickness of the liver sections used for pathologic examination. In the present series, the rate of unexpected HCC incidence was 10.7% (6/56). The mean tumor diameter associated with these cases was 14.7 mm, and the maximum nodule size was less than 20 mm. Furthermore, none of these tumors exhibited radiological features that are typically associated with HCC. Serum AFP levels for 5/6 of these patients were also within the normal range, while one patient had an AFP level of 63.1 ng/dL. However, previous studies have demonstrated that AFP levels are not a reliable indicator for the diagnosis of HCC[8], and this was consistent with our results. In addition, confirmatory biopsies were not performed for these cases since almost all of the patients had diagnostic findings identified in the imaging studies conducted. Therefore, our results indicate that the current guidelines of the American Association for the Study of Liver disease can provide a reliable diagnosis of HCC[8].

In conclusion, pre-OLT imaging continues to have a relatively high false negative rate for HCC patients considered for transplantation. The inaccuracy of imaging modalities for identification of tumor characteristics such as size and number may result in the selection of patients with unfavourable survival outcome for OLT. Based on the cases analyzed in this study, it would appear that the Milan criteria are very useful and safe in selecting recipients who will benefit from OLT. Therefore, given the limited number of deceased liver grafts available, the Milan criteria should be followed in the selection of suitable candidates for OLT for the treatment of HCC. In contrast, for cases of OLT involving living donors, the UCSF criteria may be applied. In addition, future advancement in imaging modalities may further improve the reliability and applicability of these selection criteria.

COMMENTS

Background

Liver transplantation offers the best long-term effective treatment for patients with hepatocellular carcinoma (HCC) in cirrhosis. Patients who fulfill the Milan criteria may have a 5-year survival of up to 88%. However, application of the

Milan criteria might lead to the exclusion of patients who otherwise would benefit from orthotopic liver transplantation (OLT). Several studies have evaluated more liberal criteria for tumor staging which could be adopted without significant impairment of patient survival or tumor recurrence. However, the expansion of tumor-specific criteria for transplantation raises concerns about rational use of scarce deceased donor organs and safety issues in living donation.

Research frontiers

The role of OLT in patients with HCC beyond the Milan criteria is a matter of debate. Several different selection criteria have been proposed to reach an optimum survival outcome after OLT. Improvements in imaging modalities and markers of aggressive tumor biology may help in selecting patients with better outcome after OLT.

Innovations and breakthroughs

In a substantial portion of HCC patients, pre-OLT imaging studies underestimate the extent of the disease. Patients who were reclassified to higher tumor stages after pathological evaluations of the explants had poor survival after OLT. The Milan criteria appear to provide a wider safe margin and reduce the negative influence of underestimates of tumor stage by pre-OLT imaging.

Applications

In the face of low deceased donor organ availability and safety issues in living donation, transplanting patients with HCC who meet the Milan criteria appears to have optimum benefit on patient survival. Better understanding of tumor behavior may help in selecting patients who may benefit from OLT even with higher tumor burden (expanded selection criteria).

Peer review

Despite several studies in the literature about the Milan and University of California San Francisco (UCSF) criteria, this study for the first time suggests Milan criteria for safer preoperative radiological staging over the UCSF criteria.

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BRIEF ARTICLE

Efficacy of premedication with activated Dimethicone or N-acetylcysteine in improving visibility during upper endoscopy

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Abstract

AIM: To assess the efficacy of N-acetylcysteine (NAC) and activated Dimethicone in improving endoscopic mucosal visibility.

METHODS: A total of 148 patients were randomly allocated into four groups to receive one of the following premedications: group A: 100 mL water alone; group B: activated Dimethicone plus water (up to 100 mL); group C: NAC plus water (up to 100 mL); and group D: activated Dimethicone and NAC plus water (up to 100 mL). A single endoscopist blinded to the patients group assessed the gastric mucosal visibility scores (range 1-4) at four sites. The sum of the scores from the four sites was considered as the total mucosal visibility score (TMVS).

RESULTS: The patients in group B showed a significantly lower TMVS than those of groups A and C ($P < 0.001$). The TMVS in patients of group D was significantly lower than that of groups A and C ($P < 0.001$). The TMVS did not significantly differ between groups B and D ($P > 0.05$). The difference between TMVS of groups C and A was not significant ($P > 0.05$).

CONCLUSION: Premedication with activated Dimethicone 20 min prior to the upper endoscopy leads to the best visibility. NAC does not improve visualization by itself.

Key words: Dimethicone; N-acetylcysteine; Simethicone; Upper endoscopy

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INTRODUCTION

Studies recently demonstrated a declining trend in gastric cancer incidence throughout the world; yet, it is still the second most common cause of mortality due to malignant diseases[1]. As detecting the cancer at the early stage has a great impact on its potential curability, mass screening programs are implementing in Japan with the highest rate of the disease. Although the real effect of this approach on mortality is said to be little by some, studies conducted in Japan favor endoscopic mass screening especially by the advent of new minimally invasive procedures such as endoscopic mucosal resection for cancers detected at early stages[2-5].

Foam, bubbles, and mucus accumulated in the upper gastrointestinal tract can interfere with clear mucosal visualization and pose potential risk of missing early or subtle lesions. That is why anti-foam and bubble-bursting

agents are widely used in gastrointestinal endoscopic centers particularly in Japan where it is common. This is a routine practice neither in the country where this study was conducted nor in the West, probably in fear of some presumed risk of pulmonary aspiration[6].

Simethicone [Dimethylpolysiloxane (DMPS) or activated Dimethicone] was proved to be a good defoaming agent for pre-endoscopic usage to remove bubble and mucus[7,8]. Pronase, a proteolytic enzyme isolated in 1962 from the culture filtrate of Streptomyces griseus, is another agent whose efficiency as a premedication for improving the visual field of endoscopy devoid of foam and mucus has been investigated and is now being used routinely in Japan's endoscopic centers. It is better to be used in combination with DMPS and bicarbonate to yield more improvement in visibility[9,10].

Other than upper endoscopy, Simethicone has been studied to be used in colonoscopy as an additive to other bowel preparations to eliminate bubbles[11,12], in capsule endoscopy as small bowel preparation for the same goal[13,14], and in endoscopic ultrasonography to reduce artefacts and increase the accuracy of the modality[15,16].

Currently, N-acetylcysteine (NAC), a mucolytic agent, in combination with DMPS has shown to be effective in elimination of gastric mucus and bubbles when used 20 min prior to endoscopy, improving visualization of the gastric mucosa[17]. Owing to the lack of any study surveying the efficiency of NAC independently, the present study aimed to compare the effect of this compound and activated Dimethicone (Simethicone) with placebo and together as premedications for gastroscopy.

MATERIALS AND METHODS

This double-blind, randomized, placebo-controlled study was carried out from April to August 2010. Amongst all the consecutive patients referred to our out-patient endoscopy clinic, 148 patients were enrolled in the study after giving a written informed consent. The patients with a history of upper gastrointestinal surgery, gastric cancer, gastrointestinal bleeding, caustic ingestion, pregnancy, diabetes mellitus, asthma, and allergic reactions were excluded from the study. This study was approved in the ethics committee of the local university.

The patients were randomly allocated into four different groups (using random blocks) with peculiar liquid premedication for each one: (1) group A,100 mL water; (2) group B, 100 mg, 2.5 mL, activated Dimethicone (Dimetin, Tolid Daru co., Tehran, Iran) plus water up to 100 mL; (3) group C, 600 mg N-acetylcysteine (ACC, Hexal AG, Holzkirchen, Germany) in water up to 100 mL; and (4) group D, 100 mg, 2.5 mL, activated Dimethicone and 600 mg N-acetylcysteine plus water up to 100 mL.

All the liquid solutions were prepared in the same opaque bottles and taken about 20 min prior to endoscopic procedure under supervision of an informed attendant nurse. All patients were unaware of their groups and the type of liquid solutions. Then the patient awaited endoscopy in sitting position in the endoscopy waiting room.

All the endoscopic procedures were performed by a single, experienced endoscopist blinded to the patient's group and premedication. The endoscopies were done at a relatively fixed period of time in a clinic affiliated with Shiraz University of Medical Sciences, using a video endoscope (EPK 1000 PENTAX, Japan).

During endoscopy, four distinct domains of the stomach including the antrum, the upper part of the greater curvature, the lower part of the greater curvature, and the gastric fundus were evaluated separately for mucosal visibility. Scoring from 1 to 4 for each domain, known as visibility score, was defined based on the modified form of Kuo *et al*[9] scoring system like the one used by Chang *et al*[17] as follows: (1) score 1, no adherent mucus on the gastric mucosa; (2) score 2, little amount of mucus on the gastric mucosa, but no obscuring vision; (3) score 3, large amount of mucus on the gastric mucosa, with less than 50 mL of water to clear; and (4) score 4, large amount of mucus on the gastric mucosa, with more than 50 mL of water to clear.

The sum of visibility scores of all four domains is considered as the TMVS for each patient.

Statistical analysis

The demographic characteristics were assessed using a χ^2 test, ANOVA, or one-way analysis of variance. The visibility scores of all the four groups were analyzed using Kruskal-Wallis and Mann-Whitney pairwise comparisons. P value < 0.05 was considered statistically significant.

RESULTS

From a total of 148 patients enrolled in the study, 77 (52%) were male and 71 (48%) female. Then, 38, 37, 37 and 36 patients were randomly assigned to groups A, B, C and D, respectively. The mean (± SD) age was 42.2 ± 13.9 in group A, 44.3 ± 18 in group B, 44.6 ± 16.4 in group C, and 41.8 ± 17.5 in group D. The mean age in the whole study population was 43.2 ± 16.4. The most common reason for endoscopy in all the groups and also in the total population was dyspepsia (65.5% in total). Moreover, the second most common cause in all the patients was acid reflux (12.8%). All demographic data encompassing sex distribution per group and reason for endoscopy are shown in Table 1. There was no statistically significant difference ($P > 0.05$) among groups regarding age and gender.

The mean of TMVS in group A was 9.50 ± 2.55, in group B 5.11 ± 1.28, in group C 8.41 ± 2.10, and in group D 5.39 ± 1.71. The total mean ranks in groups A, B, C and D were 109.96, 41.69, 98.39 and 46.24, respectively (the lower the rank, the better the visibility). The difference among the mean ranks was statistically significant ($P < 0.001$). Group B showed the least visibility scores at different locations of the stomach and also the least

Table 1 Demographic characteristics of patients in each group

Group	A	B	C	D
Number	38	37	37	36
Age(yr; mean ± SD)	42.2 ± 13.9	44.3 ± 18.0	44.6 ± 16.4	41.8 ± 17.5
Female: Male (*n*)	18:20	19:18	16:21	18:18
Cause of endoscopy				
Dyspepsia	25	28	21	23
Reflux	6	5	4	4
Screening for cancer	7	0	5	4
Others	0	4	7	5

No significant difference between each two groups regarding age and gender. Group A received water, group B received activated Dimethicone plus water, group C received N-acetylcysteine plus water, and group D received activated Dimethicone and N-acetylcysteine plus water.

Table 2 The mean rank[1] of any group of patients in distinct domains of stomach

Group	A	B	C	D
Antrum	102.82	48.91	91.74	53.19
Lower part of the greater curvature	103.43	51.24	88.00	53.99
Upper part of the greater curvature	100.17	48.49	92.43	55.71
Fundus	102.21	51.43	90.96	52.04

[1]The lower the mean rank, the better the visibility.

Table 3 Mean mucosal visibility score at different sites and total mean mucosal visibility scores in any group separately (mean ± SD)

Group	A	B	C	D
Antrum	2.39 ± 0.94	1.22 ± 0.53[a,b]	2.05 ± 0.78	1.28 ± 0.51[c,d]
Lower part of the greater curvature	2.26 ± 0.89	1.14 ± 0.34[a,b]	1.89 ± 0.87	1.19 ± 0.46[c,d]
Upper part of the greater curvature	2.47 ± 0.79	1.38 ± 0.54[a,b]	2.35 ± 0.94	1.53 ± 0.69[c,d]
Fundus	2.37 ± 0.75	1.38 ± 0.54[a,b]	2.11 ± 0.65	1.39 ± 0.54[c,d]
Total (TMVS)	9.50 ± 2.55	5.11 ± 1.28[a,b]	8.41 ± 2.10	5.39 ± 1.71[a,b]

[a]$P < 0.001$ *vs* group A; [b]$P < 0.001$ *vs* group C; [c]$P < 0.05$ *vs* group A; [d]$P < 0.05$ *vs* group C; a, b, c, d: Kruskal-Wallis and Mann-Whitney pairwise comparisons. TMVS: Total mucosal visibility score.

mean TMVS which were all significantly lower than those of groups A and C ($P < 0.001$). The patients in group D had significantly lower visibility scores for separate gastric domains ($P < 0.05$) and showed lower mean TMVS than group A and C too ($P < 0.001$). Groups B and D did not differ significantly in scores ($P > 0.05$). Despite the fact that patients in group C achieved lower scores than group A patients, the difference was not significant at all ($P > 0.05$). The mean rank, the mean mucosal visibility scores at separate sites, and the mean TMV scores in distinct groups are depicted in Table 2 and 3, respectively. No adverse reaction was detected during the study in any group.

DISCUSSION

Esophagogastroduodenoscopy or upper endoscopy remains commonplace for the evaluation of upper gastrointestinal tract disorders. One of the major applications of this modality is to discern gastric cancer at early stages. This is of paramount importance because of the direct effect of early diagnosis of gastric cancer on patients' future survival, quality of life, and management. A case series from Britain, reported by Sue-Ling *et al*[18] showed a 5 year survival rate of 98% for patients detected at early stages of gastric cancer and survived operation. On the other hand, rapid diagnosis is not guaranteed by doing upper endoscopy alone even in a wide range. Suvakovic *et al*[19] in 1997 remarked that open-access gastroscopy by itself was not sufficient to increase early gastric cancer pick-up; moreover, more awareness from general practitioners, more experience in endoscopy, and high sensitivity for biopsying are important among others too. Besides, foam, bubbles, and mucus accumulated on gastric mucosa are postulated to play a role by blurring visual field during endoscopy. So, it seems prudent to make use of some agents before endoscopy to eliminate these troubles and enhance the precision and accuracy of endoscopy in showing subtle abnormalities.

Simethicone (activated Dimethicone or activated Methylpolysiloxane), commonly used for relief of bloating and gas with no significant adverse reaction or interaction[20], is a safe adjunct to endoscopic premedications. It works via decreasing the surface tension of bubbles of air and dispersing them without remarkable absorption in the gastrointestinal system[20]. The effectiveness of Simethicone has already been proved in some other trials as a defoaming agent[7,8]. Recently, Keeratichananont *et al*[22], though using a different grading scale and including the esophagus and duodenum in their study, concluded that 133.3 mg (2 mL) of liquid Simethicone in 60 mL water 15-30 min prior to procedure could improve the visibility and reduce the number of flushings required for removing the mucus significantly. They also showed that using Simethicone prior to endoscopy would cut down the duration of the procedure and consequently lead to more satisfaction to both physician and patient. Similarly in our study, those patients in group B who received 100 mg activated Dimethicone in water showed better visualization compared to group A that received only simple water as placebo. The amount of water to be given with Simethicone had been a matter of debate. We used of a fixed volume of water in all our patient groups to remove the possible role thereof; however, in two clinical trials it was shown that there was no significant difference in visibility between those who received Simethicone alone or with 100 mL water[9,17].

Pronase is a proteolytic enzyme commonly used in Japan as a premedication in combination with bicarbonate and Gascon (Simethicone)[6]. Fujii *et al*[10] came to the conclusion that the solution of 100 mL water, 20 000 units Pronase, 1 gm bicarbonate, and 80 mg DMPS was more effective than DMPS alone in improving visibility during conventional endoscopy and chromoendoscopy. They showed that this would decrease duration of endoscopy. Kou *et al*[9] in a similar study proved that Pronase would

improve visualization much better than DMPS only when used in combination with bicarbonate and DMPS. They vividly concluded that Pronase without DMPS was of no use. Pronase is not routinely used in this country and was not the scope of the study.

NAC is a mucolytic and antioxidant agent acting via its free sulfhydryl group to lower the mucus viscosity[21]. Nor significant interaction neither adverse reaction has been reported with oral preparations. In this study, those patients with a history of asthma and Diabetes Mellitus were excluded. This study is the only one in which the effect of NAC alone has been investigated and compared to Dimethicone and placebo. The patients in group C who received 600 mg NAC in 100 mL water did not show any betterment in visibility scores (8.41 ± 2.10 *vs* 9.50 ± 2.55 in group A). Combination of NAC and Dimethicone in group D demonstrated better visualization than simple water in group A. But this combination was not superior to Dimethicone alone in group B. We supposed that this was the effect of Dimethicone appearing in group D as in group B and NAC had no effect. In contrast to our results, Chang *et al*[17] concluded that the mixture of 400 mg NAC and 100 mg DMPS plus water up to 100 mL is more effective than DMPS alone or DMPS in water in a significant manner. They also recommended that NAC could be a substitute for Pronase where it was unavailable. In their study, the mean of the total visibility score in the patients who received NAC plus DMPS was 6.5 ± 2.2 (*vs* 5.39 ± 1.71 in this study) and in those receiving DMPS with water 7.6 ± 2.6 (*vs* 5.11 ± 1.28 in this study). The scoring system was exactly similar in the two studies though performed by different endoscopists. All these compounds were proved not to affect the result of rapid urease tests using Campylobacter-like organism tests[9,17].

In conclusion, regarding the lower cost of Dimethicone (activated) (one third that of NAC per patient herein) and lack of Pronase, we suggest the routine use of 100 mg activated Dimethicone in water up to 100 mL twenty min prior to upper endoscopy here and all other areas where Pronase is not available. To clarify the exact benefits of NAC requires further trials.

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COMMENTS

Background

Upper endoscopy is a routine and widely-used procedure to evaluate upper gastrointestinal tract. Since foam and air bubbles can impair visibility, some anti-foam agents such as Simethicone and Pronase are used as premedications prior to endoscopy.

Research frontiers

Activated Dimethicone (Simethicone) has been shown to be effective in reducing foam and bubbles in the stomach. N-acetylcysteine (NAC) is a mucolytic drug that is supposed to be efficacious too. This study aims to compare the efficacy of these agents in improving visibility.

Innovations and breakthroughs

Activated Dimethicone was shown to be effective. In contrast to prior findings our study showed that NAC was not able to improve visibility when used alone. Furthermore, combination of NAC and Dimethicone did not differ from Dimethicone alone in providing more clear visualization. This is the first study in which the efficacy of NAC was investigated independently as a premedication.

Applications

Usage of activated Dimethicone prior to upper endoscopy is of benefit for improving mucosal visibility; however, N-acetylcysteine seems not to be effective if used alone. Thus, activated Dimethicone should be considered as a premedication before upper endoscopy especially in areas where other agents are lacking.

Peer review

This is a nice study. It is well composed, balanced, documented and the English spelling is good.

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BRIEF ARTICLE

Usefulness of fecal lactoferrin in predicting and monitoring the clinical severity of infectious diarrhea

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Abstract

AIM: To explore the value of fecal lactoferrin in predicting and monitoring the clinical severity of infectious diarrhea.

METHODS: Patients with acute infectious diarrhea ranging from 3 mo to 10 years in age were enrolled, and one to three stool samples from each subject were collected. Certain parameters, including white blood cells /differential count, C-reactive protein, fecal mucus, fecal pus cells, duration of fever, vomiting, diarrhea and severity (indicated by Clark and Vesikari scores), were recorded and analyzed. Fecal lactoferrin was determined by enzyme-linked immunosorbent assay and compared in different pathogen and disease activity. Generalized estimating equations (GEE) were also used for analysis.

RESULTS: Data included 226 evaluations for 117 individuals across three different time points. Fecal lactoferrin was higher in patients with *Salmonella* (11.17 μg/g ± 2.73 μg/g) or *Campylobacter* (10.32 μg/g ± 2.94 μg/g) infections and lower in patients with *rotavirus* (2.82 μg/g ± 1.27 μg/g) or *norovirus* (3.16 μg/g ± 1.18 μg/g) infections. Concentrations of fecal lactoferrin were significantly elevated in patients with severe (11.32 μg/g ± 3.29 μg/g) or moderate (3.77 μg/g ± 2.08 μg/g) disease activity compared with subjects with mild (1.51 μg/g ± 1.36 μg/g) disease activity ($P < 0.05$). GEE analysis suggests that this marker could be used to monitor the severity and course of gastrointestinal infections and may provide information for disease management.

CONCLUSION: Fecal lactoferrin increased during bacterial infection and with greater disease severity and may be a good marker for predicting and monitoring intestinal inflammation in children with infectious diarrhea.

Key words: Lactoferrin; Diarrhea; Generalized estimating equations; Vesikari scores; Clark scores

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INTRODUCTION

Infectious diarrhea caused by pathogens may induce gastroenteritis, bloody stool, or severe intraabdominal infections, which spreads disease, especially among infant and child populations, and increases the economic burden. Viral infection is a leading cause of diarrhea among children in developed and developing countries. Moreover, several bacterial pathogens, including *Salmonella spp.*, *Campylobacter spp.*, and *Shigella spp.*, can cause invasive diarrhea. These pathogens have the capacity to invade the mucosa of the distal small intestine and colon, stimulate local and systemic inflammatory responses, and may sometimes cause hemorrhaging and ulceration of the mucosa. Although acute infectious diarrhea is a common clinical disease in children, few reliable and noninvasive diagnostic tools have been used as biological markers in patients with acute infectious diarrhea or persistent digestive symptoms.

Lactoferrin, an 80 kDa iron-binding glycoprotein produced by many exocrine glands, is a major whey protein with a major constituent in the secondary granules of neutrophilic leukocytes. Lactoferrin displays diverse biological activities, ranging from the activation of innate immunity[1,2], microbicidal effects[3], and anti-cancer cell responses[4]. Exposure of host cells to lactoferrin may modulate subsequent cellular functions, such as cytokine gene activation[5], cytotoxicity[6], and T cell[7] or B cell[8] maturation. Lactoferrin may affect innate immunity by stimulating macrophages through interaction with toll-like receptor pathways[2]. Because diarrheal illnesses are extremely common in communities and hospitals throughout the world, a noninvasive inflammatory marker may be helpful for disease management. The presence of lactoferrin in bodily fluids, including intestinal lumen, is proportional to the flux of neutrophils, and its assessment can provide a reliable biomarker for inflammation. Neutrophils have been shown to be involved in the perpetuation of inflammation in the gut in acute infections caused by *Shigella* and *Salmonella* and inflammatory bowel disease (IBD)[9-11]. Guerrant *et al*[12] confirmed increased fecal lactoferrin in 96% (25/26) of samples from patients with shigellosis and concluded that fecal lactoferrin was a useful marker for fecal leukocytes.

Few scales are available for evaluating gastroenteritis disease severity. The most commonly used scoring scales are the Vesikari 20-point scale, in which an episode of gastroenteritis with a score $\geqslant$ 11 is considered severe[13] ($\geqslant$ 11 moderate, $\geqslant$ 16 severe), and the Clark 24-point scale, in which an episode of gastroenteritis with a score $\geqslant$ 16 is considered severe[14]. Our present prospective study was conducted to explore the role of fecal lactoferrin in gastrointestinal infection, including (1) predicting bacterial or viral infection; (2) ascertaining the extent to which values may be associated with the severity of gastroenteritis in the above scales; and (3) monitoring the severity and course of gastrointestinal infection, which may provide information for disease management.

MATERIALS AND METHODS

This prospective study enrolled and analyzed children being treated in Chang Gung Children's Hospital located in Northern Taiwan. All subjects provided written informed consent, and three fecal samples were collected from each subject.

Enrollment was conducted between September 2008 and May 2010. Diarrhea was defined as three or more outputs of loose or liquid stools per day. Inclusion criteria were 3 mo to 10 years of age and hospitalization with infectious diarrhea. Exclusion criteria were immunodeficiency and history of IBD or gastrointestinal tract surgery. The study protocol was approved by the Institutional Review Board of Chang Gung Memorial Hospital. Informed consent was obtained from the parents of all eligible children. The study was performed in accordance with the Declaration of Helsinki.

Upon entering the study, hospitalized patients received treatment consisting of intravenous fluid and oral rice water or half-strength formula. The severity of diarrhea was evaluated according to the following parameters: volume of stools, fecal consistency and frequency. Other clinical symptoms, including fever, vomiting, abdominal pain, daily intake, and appetite, were also assessed. All participants underwent first-step hematology and biochemistry tests [including blood cell counts, serum C-reactive protein (CRP), and electrolytes] as well as fecal pus cell and mucus analysis. Disease severity was recorded using the severity scoring methods of the Vesikari 20-point scale and Clark 24-point scale. In the Vesikari 20-point scale, an episode of gastroenteritis with a score $\geqslant$ 11 is considered moderate or severe ($<$ 11 mild, $\geqslant$ 11 moderate, $\geqslant$ 16 severe)[5], and in the Clark 24-point scale, an episode of gastroenteritis with a score $\geqslant$ 16 is considered severe. Fecal samples of some patients were collected at three different time points, including the initial stage of infectious diarrhea, 3-5 d later and 7-10 d later. Series follow-ups of fecal lactoferrin were measured by enzyme-linked immunosorbent assay. Their control group comprised 15 children (mean age, 3.7 years; range, 1-10 years) without diarrhea. We compared and analyzed the levels of fecal lactoferrin collected from the different patients at the same time point.

Identifying pathogens

To assess the etiology of infectious diarrhea, fecal specimens were collected to detect *Salmonella, Shigella* or *Campylobacter* colonies on specifically prepared agar plates.

Table 1 Patient characteristics

Gender	
Female	52
Male	65
Age (mean), yr	3.23 (3 mo-10 yr)
Pathogen identified	
Rotavirus	41
Norovirus	28
Salmonella	31
Campylobacter	17
Disease severity (Vesikari scoring scale)	
Mild	42
Moderate	50
Severe	25
Duration of diarrhea (median), h	73.8 (14-169)
Vomiting, (d)	2.1 (0-5)
Fever, (d)	2.9 (0-7)
WBC counts (10^6/L)	12 658 ± 2364
CRP (mg/L)	34.7 ± 22.1
Hemoglobin (g/dL, n = 117)	11.6 (8.2-15.3)
Platelets (10^9/L, n = 117)	232 (134-585)

Range in brackets. WBC: White blood cells; CRP: C-reactive protein.

The fecal specimens were also sent for evaluating *rotavirus* antigen levels by ELISA and *norovirus* RNA by real-time polymerase chain reaction.

Lactoferrin assay

The stool samples were prepared and analyzed for lactoferrin according to the manufacturer's instructions (AssayMax Human Lactoferrin ELISA Kit, St. Charles, MO, United States). This assay employs a quantitative sandwich enzyme immunoassay technique that measures lactoferrin in 4 h. A polyclonal antibody specific for lactoferrin was pre-coated onto a microplate. Lactoferrin standards and samples were sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for lactoferrin recognized by a streptavidin-peroxidase conjugate. Absorbance was read at A_{450} nm. Lactoferrin was expressed as μg/g of feces.

Statistical analysis

Simple univariate correlation coefficients (Spearman rank correlation) were calculated using baseline data only. Independent associations between the variables of interest were investigated by generalized estimating equations (GEE). GEE is a regression technique that allows the investigation of longitudinal data while adjusting for within-patient correlations. GEE requires a predefined working correlation structure for the dependent variable (lactoferrin), and based on first level and follow-up data, an exchangeable correlation structure was chosen here. The GEE approach was developed to correct for repeated outcomes within the same subject[15]. When using data from more than two time points, the GEE analysis was employed for longitudinal analysis (associations).

A univariate comparison between groups was performed with a t test for repeated measures, and the χ^2 test and Fisher's exact test were used with categorical data. Analyses were performed on the intention-to-treat population. A P value less than 0.05 was considered significant, and the statistical tests were two-tailed. The GraphPad Software Prism 3.03 (GraphPad Software, Inc., San Diego, CA, United States) and SPSS Software, version 15.0 (SPSS Inc., Chicago, IL, United States), were used for the statistical analysis.

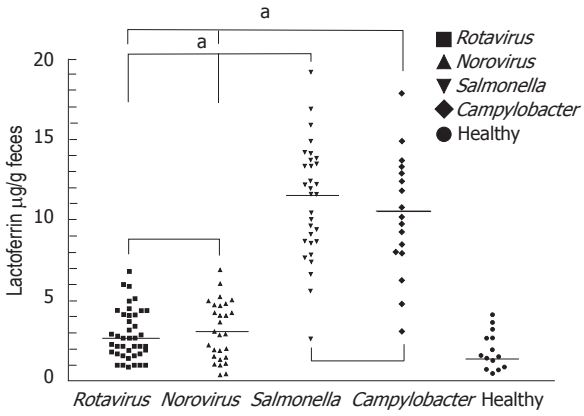

Figure 1 Grouped samples of fecal lactoferrin (μg/ g feces) in healthy children and children with gastroenteritis caused by different pathogens, including *rotavirus*, *norovirus*, *Salmonella* and *Campylobacter* infection. The mean level of fecal lactoferrin was higher in patients with *Salmonella* or *Campylobacter* infections but lower in patients with *rotavirus* or *norovirus* infections. Horizontal line: Mean; $^aP < 0.05$.

RESULTS

Description of samples

A total of 154 participants were screened between September 2008 and May 2010. From that cohort, 37 patients were excluded from further study because no definite pathogen was identified from the stool examination. Among the individuals included in the study, *rotavirus* infection was diagnosed in 41 patients, and *norovirus* infection was diagnosed in 28 patients. In addition, *Salmonella* infection was diagnosed in 31 patients and *Campylobacter* infection in 17 patients. Demographic details are shown in Table 1.

The data include a total of 226 evaluations for 117 individuals across three different time points. The pattern of assessment was as follows: 43 subjects (36.9%) had three assessments, 23 (19.6%) had two assessments, and 51 (43.5%) had one assessment. The mean age of the subjects was 3.23 years (SD 2.15, range 3 m/o-10 y/o), and 65 (55.5%) were male.

Fecal lactoferrin

The mean ± SD of the fecal lactoferrin concentration was 11.17 μg/g ± 2.73 μg/g in patients with *Salmonella* infections, 10.32 μg/g ± 2.94 μg/g in patients with *Campylobacter* infections, 2.82 μg/g ± 1.27 μg/g in patients with *rotavirus* infections and 3.16 μg/g ± 1.18 μg/g in patients with *norovirus* infections (Figure 1). Concentrations of each fecal marker for patients with either form of bacterial infection were significantly elevated compared

Table 2 The Vesikari and Clark severity scoring scales for the evaluation of gastroenteritis in children

Severity scoring scales	Point value		
	1	2	3
Vesikari[5]			
Duration of diarrhea (d)	1-4	5	⩾ 6
Maximum number of diarrhea stools/24 h	1-3	4-5	⩾ 6
Duration of vomiting (d)	1	2	⩾ 3
Maximum number of vomiting episodes/24 h	1	2-4	⩾ 5
Temperature (℃)	37.1-38.4	38.5-38.9	⩾ 39.0
Dehydration	-	Mild	Moderate to severe
Treatment	Rehydration	Hospitalization	-
Clark[6]			
Diarrhea			
Number of stools/d	2-4	5-7	⩾ 8
Duration in days	1-4	5-7	⩾ 8
Vomiting			
Number of emeses/d	1-3	4-6	⩾ 7
Duration in days	2	3-5	⩾ 6
Rectal temperature			
Temperature (℃)	38.1-38.2	38.3-38.7	⩾ 38.8
Duration in days	1-2	3-4	⩾ 5
Behavioral symptoms/signs			
Description	Irritable/less playful	Lethargic/listless	Seizure
Duration in days	1-2	3-4	⩾ 5

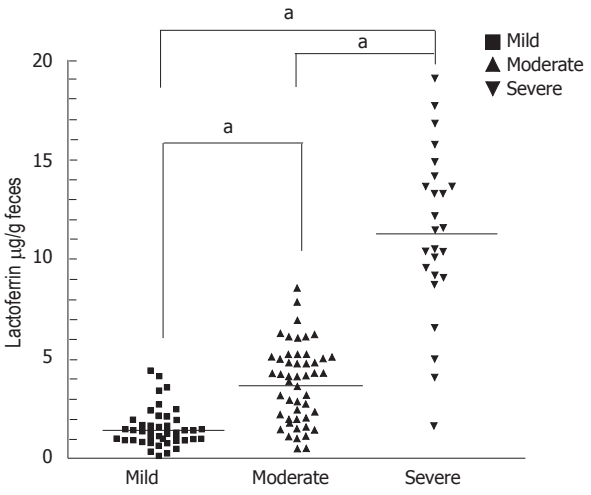

Figure 2 Fecal lactoferrin level (μg/ g feces) in children with mild , moderate or severe disease activity according to the Vesikari score (< 11 mild, ⩾ 11 moderate, ⩾ 16 severe). Levels of fecal lactoferrin were elevated in moderate and severe disease activities. Horizontal line: Mean; $^{a}P < 0.05$.

with those of virus-infected patients. The *P* values for lactoferrin were < 0.05. No statistical differences were observed in fecal lactoferrin concentrations between the clinically confirmed *Salmonella* and *Campylobacter* infections. The *P* values for lactoferrin was 0.71.

The mean ± SD of fecal lactoferrin concentration was 11.32 μg/g ± 3.29 μg/g in patients with severe disease activity (Vesikari score ⩾ 16, Table 2), 3.77 μg/g ± 2.08 μg/g in patients with moderate disease activity (Vesikari score ⩾ 11) and 1.51 μg/g ± 1.36 μg/g in patients with mild disease activity (Vesikari score < 11, Figure 2). Concentrations of each fecal marker for patients with either form (viral or bacterial) of severe or moderate disease activity were significantly elevated compared with those of mild disease activity. The *P* values for lactoferrin were < 0.05.

Univariate linear regression analysis

Certain parameters associated with the level of fecal lactoferrin, including white blood cells/differential count, C-reactive protein, fecal mucus, fecal pus cells, duration of fever, vomiting, diarrhea and severity (as indicated by Clark and Vesikari scores), were recorded and analyzed. To determine the correlation between these parameters and fecal inflammatory markers, we performed a univariate linear regression analysis.

The univariate linear regression analysis revealed that the Vesikari score, Clark score, fecal pus cells, CRP, vomiting and dehydration were all correlated with lactoferrin level (Table 3).

GEE analysis results

Table 4 reveals the results of the multivariate analysis of the predictive value of fecal lactoferrin with time variations. Subjects with higher Vesikari severity scores had higher fecal lactoferrin levels initially (when time = 0), and the levels of fecal lactoferrin may have decreased when followed-up at different time points (when time > 0).

(Lactoferrin = 3.9289 + 3.2257 × Vesikari score - 0.1835 × time - 0.1575 × time × Vesikari score)

However, there was no significant relationship between fecal lactoferrin and the Clark score with time variations. On the contrary, subjects with higher band form (%) had higher fecal lactoferrin levels initially (when time = 0), and the levels of fecal lactoferrin may have decreased when followed-up at different time points (when time > 0).

(Lactoferrin = 3.6654 + 1.9759 × Band + 1.627 × time - 1.5261 × Band × time)

Table 3 Univariate linear regression outcome: Lactoferrin ($y = \alpha + \beta x$)

	β	Standard error	95% CI		*P* value
			Lower	Upper	
WBC	-0.05	0.10	-0.25	0.14	0.581
Segment	0.00	0.02	-0.05	0.05	0.941
Band	0.47	0.27	-0.07	1.01	0.089
CRP	0.02	0.01	0.00	0.04	0.043[a]
Fecal pus cell					
None	0.00				
Present	0.38	1.55	-2.74	3.49	0.809
Fecal occult blood					
None	0.00				
Present	1.71	1.22	-0.73	4.15	0.165
Fecal mucus					
None	0.00				
Positive	-2.21	1.46	-5.14	0.71	0.135
Vesikari scoring scale					
Non-severe < 11	0.00				
Moderate ⩾ 11	2.76		-1.50	7.02	0.191
Severe ⩾ 16	2.81	1.13	0.56	5.07	0.015[a]
Clark scoring scale					
Non-severe < 16	0.00				
Severe ⩾ 16	3.13	1.10	0.93	5.32	0.006[a]
Body temperature	0.29	0.30	-0.31	0.89	0.341
Abdominal pain	0.88	0.63	-0.39	2.15	0.169
Abdominal distension	-0.30	1.01	-2.32	1.71	0.764
Dehydration	3.13	1.38	0.37	5.89	0.027[a]
Oral intake	1.13	1.23	-1.33	3.59	0.362
Activity	1.13	1.00	-0.87	3.13	0.262
Fever day	0.09	0.28	-0.46	0.65	0.739
Diarrhea day	0.41	0.29	-0.60	1.41	0.423
Vomiting day	-0.99	0.53	-1.93	-0.04	0.041[a]

[a]$P < 0.05$. WBC: White blood cells; Band: Band form neutrophil; CRP: C-reactive protein.

Table 4 Generalized estimating equations analysis for series follow-up of fecal lactoferrin

Parameter	Estimate	Standard error	95% confidence interval		*P* value
Clark score					
Intercept	5.1523	0.7490	3.6843	6.6202	< 0.0001[a]
Clark score	2.0393	1.7397	-1.3704	5.4489	0.2411
Time	-0.3729	0.2530	-0.8688	0.123	0.1405
Time x Clark	0.5826	0.8342	-1.0525	2.2177	0.485
Vesikari score					
Intercept	3.9289	0.8437	2.2752	5.5825	< 0.0001[a]
Vesikari score	3.2257	1.3018	0.6741	5.7773	0.0132[a]
Time	-0.1835	0.2715	-0.7157	0.3487	0.4992
Time x Vesikari	-0.1575	0.5222	-1.1811	0.8660	0.0429[a]
Band (band form neutrophil)					
Intercept	3.6654	1.0750	1.5585	5.7723	0.0007[a]
Band	1.9759	0.6951	0.6135	3.3383	0.0045[a]
Time	1.6270	0.8206	0.0186	3.2354	0.0474[a]
Band x time	-1.5261	0.4677	-2.4428	-0.6094	0.0011[a]

[a]$P < 0.05$.

DISCUSSION

An intense intestinal infection involves intense infiltration of neutrophils, macrophages, mast cells, lymphocytes, natural killer cells, other inflammatory cells in the epithelial lining and the lamina propria of the colonic mucosa[16]. Cells in the innate immune system secrete various enzymes and metabolites, including myeloperoxidase and lactoferrin, produced by activated neutrophils. Lactoferrin is found mainly in the oral cavity and intestinal tract where it can come into direct contact with pathogens, such as viruses and bacteria. The noninvasive fecal marker lactoferrin may prove useful in screening for inflammation in patients with abdominal pain and diarrhea[17]. Our study demonstrates the usefulness of fecal lactoferrin for detecting colonic inflammation in children with gastrointestinal symptoms, such as enteritis or enterocolitis.

The most significant function of lactoferrin in mucosal defense is its antimicrobial activity. Lactoferrin can also amplify the actions of lysozyme and secretory immunoglobulin A[18]. *In vitro* studies have shown lactoferrin's bactericidal effects on *V. cholerae*, *Salmonella enterica* subsp. *enterica* serovar mutants, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans*[19]. Thus, increased stool levels of lactoferrin in acute shigellosis may suggest increased degranulation of neutrophils upon stimulation that may promote the killing of *Shigella* in the colonic mucosa. Interaction of lactoferrin with immune system cells induces a regulated release of cytokines, such as interleukin 6 and tumor necrosis factor alpha[19], which has also been observed during acute *Shigella* infection in adults[20,21] and children.

Fecal lactoferrin has been reported as a promising biomarker in active Crohn's disease[22] and ulcerative colitis[23], requiring the exclusion of patients enrolled with a history of the above IBDs. Indeed, in patients without known IBDs suspected of having a bacterial diarrheal illness, fecal lactoferrin may be useful in evaluating bacterial gastrointestinal infections in which antimicrobial therapy may be prescribed (e.g., *Salmonella*, *Shigella*, *Campylobacter*, and pathogenic *Escherichia coli spp.*) and aid in following the inflammatory activity of bacterial infection. Previous study has suggested that fecal lactoferrin could serve as a screening tool for deciding when to perform a stool culture[24]. Fifty-five patients were enrolled in the Choi *et al*[24] study, and the researchers reported that fecal lactoferrin was higher in invasive bacterial pathogens and might greatly enhance a cost-effective approach for evaluating infectious diarrhea[24]. Fecal lactoferrin could be a more sensitive test than fecal leukocytes for evaluating patients with acute diarrhea. Scerpella *et al*[25] reported that 94% of travelers with invasive pathogens had positive fecal lactoferrin, while only 69% had fecal leukocytes. The other study has also found that fecal lactoferrin was better than methylene blue for detecting invasive pathogens[26]. According to our study, the fecal lactoferrin level is higher in bacterial gastrointestinal infections, such as *Salmonella* and *Campylobacter*, but lower in patients with *rotavirus* or *norovirus* infections. The above results are similar to the findings of Choi *et al*[24] (higher fecal lactoferrin in *Salmonella, Campylobacter* and *Shigella* infections but lower in *rotavirus* infections).

In some meta-analyses of the sensitivity and specific-

ity of different markers of intestinal inflammation associated with invasive pathogens (e.g., fecal leukocytes, occult blood in stool, and fecal lactoferrin), fecal lactoferrin was recommended as having the best diagnostic accuracy[27-29]. In enteroaggregative *Escherichia coli* infectious diarrhea, mucosal inflammation included heavy mucus formation, intimate cell adherence, and secretion of toxins, and the common finding was higher fecal lactoferrin, which suggests a diffuse colonic inflammatory process[30,31]. Our study has demonstrated that fecal lactoferrin is higher in infections caused by *Salmonella* and *Campylobacter* and in moderate or severe disease severity.

In our study, the data include 226 evaluations for 117 individuals across three different time points. Concentrations of fecal lactoferrin were significantly elevated in patients with severe or moderate disease activity compared with those with mild disease activity ($P < 0.05$ for each marker). Univariate linear regression analysis revealed that the Vesikari and Clark scores, fecal pus cells, CRP, vomiting and dehydration were all correlated with the lactoferrin level. The parameters of the Vesikari and Clark scores included body temperature, severity of dehydration, and the number of instances and duration of diarrhea and vomiting. Fecal pus cells are usually positive in bacterial infection. Increased CRP may be related to intestinal mucosal inflammation caused by pathogens. Taken together, fecal lactoferrin might correlate with disease activity, which may include the number of instances and duration of diarrhea or vomiting, severity of fever or dehydration, fecal pus cells and CRP.

GEE is a regression technique that allows the investigation of longitudinal data and corrects for the repeated outcomes within the same subject. GEE requires a predefined working correlation structure for the dependent variable (lactoferrin) and is based on first level and follow-up data. In our study, we found that fecal lactoferrin on the first evaluation and follow-up levels were highly associated with Vesikari scores. The above results indicate that fecal lactoferrin may be useful in monitoring the severity of infectious diarrhea during the course of the disease and may provide information for the management of gastrointestinal infection. In addition, fecal lactoferrin levels at the first evaluation and at follow-up were also associated with the band-form percentile. This result suggests that fecal lactoferrin may play a role in monitoring the disease activity and providing guidance for treating infectious diarrhea. According to our study, the measurement of fecal lactoferrin may be a useful noninvasive test for evaluating intestinal infectious or inflammatory situations. For children with persistent diarrhea or recurrent digestive symptoms after one episode of gastrointestinal infection, fecal lactoferrin could be a helpful tool for providing treatment and management information for physicians.

In conclusion, the non-invasive marker fecal lactoferrin was able to predict bacterial or viral infection, and the relative values may be associated with the severity of gastroenteritis, corresponding to Vesikari and Clark scores. Furthermore, fecal lactoferrin may be useful in monitoring the severity and course of gastrointestinal infections, which may provide information for disease management and follow-up.

ACKNOWLEDGMENTS

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COMMENTS

Background

This study provides increasing evidence that acute gastrointestinal infection is a common clinical disease in children. Few reliable, noninvasive and painless diagnostic tools have been used as biological markers in patients with acute gastroenteritis.

Research frontiers

How to predict the infectious pathogens (virus or bacteria) that caused acute diarrhea has not been fully clarified. The use of fecal leukocytes, pus cells and serum C-reactive protein has been attempted but was not fully effective. Fecal lactoferrin could be involved in the inflammation caused by the intestinal infectious pathogen. We attempt to investigate a useful noninvasive fecal marker for predicting and monitoring intestinal inflammation in children with infectious diarrhea.

Innovations and breakthroughs

The study design measures the level of fecal lactoferrin during acute infectious diarrhea. The authors also investigated the clinical information and certain parameters of patients, as well as used univariate linear regression analysis and generalized estimating equations to (1) predict bacterial or viral infection; (2) ascertain the extent to which values may be associated with the severity of gastroenteritis; and (3) monitor the severity and course of gastrointestinal infection, which may provide information for disease management.

Applications

This study found that fecal lactoferrin is higher in patients with *Salmonella* infection or *Campylobacter* infections but lower in patients with *rotavirus* infection or *norovirus* infections. Fecal lactoferrin increased during bacterial infection and with greater disease severity and may be a good marker for predicting and monitoring intestinal inflammation in children with infectious diarrhea.

Peer review

The study by Chen and colleagues presents the value of using fecal lactoferrin to predict and monitor the clinical severity of infectious diarrhea. The study is well planned, includes a robust sample size, is well controlled and the results are clearly interpretable. Perhaps, one benefit, which they argue for the approach in using fecal lactoferrin is that it allows for diagnosis and monitoring without using invasive approaches.

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BRIEF ARTICLE

Aberrant methylation of the 3q25 tumor suppressor gene *PTX3* in human esophageal squamous cell carcinoma

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Abstract

AIM: To identify the novel methylation-silenced gene *pentraxin 3* (*PTX3*) in esophageal squamous cell carcinoma (ESCC).

METHODS: PTX3 mRNA expression was examined in six human ESCC cell lines, one human immortalized normal esophageal epithelial cell line, primary ESCC tumor tissue, and paired adjacent nontumor tissue using reverse transcription polymerase chain reaction (RT-PCR). Semi-quantitative immunohistochemistry was used to examine cellular localisation and protein levels. Methylation specific PCR and bisulphite genomic sequencing were employed to investigate the methylation of the candidate gene.

RESULTS: In the majority of ESCC cell lines, we found that PTX3 expression was down-regulated due to gene promoter hypermethylation, which was further confirmed by bisulphite genomic sequencing. Demethylation treatment with 5-aza-2′-deoxycytidine restored PTX3 mRNA expression in ESCC cell lines. Methylation was more common in tumor tissues (85%) than in adjacent nontumor tissues (25%) ($P < 0.01$).

CONCLUSION: PTX3 is down-regulated through promoter hypermethylation in ESCC, and could potentially serve as a biomarker of ESCC.

Key words: Tumor suppressor gene; Pentraxin 3; Microarray; DNA methylation; Esophageal squamous cell carcinoma

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INTRODUCTION

Esophageal cancer is the sixth most common cause of cancer death worldwide, with over 400 000 new cases diagnosed each year[1]. Esophageal squamous cell carcinoma (ESCC) has a high morbidity and mortality rate in

China. However, the molecular mechanisms underlying ESCC development remain poorly understood.

Human carcinogenesis is a multi-stage process in which genetic and epigenetic changes lead to oncogene activation and tumor suppressor gene inactivation[2]. Epigenetic changes, such as promoter DNA methylation, can induce the inactivation of tumor suppressor genes. DNA methylation plays a crucial role in the development of nearly all types of cancer[3]. Recently, a growing list of aberrantly methylated genes has been reported in ESCC, including esophageal cancer related gene 4[4], p16[5], adenomatous polyposis coli[6], transmembrane protein endothelial factor[7], deleted in liver cancer 1[8], ubiquitin carboxy-terminal hydrolase 1[9], testis-specific Y-like protein 5, and human protein phosphatase-1 regulatory subunit-14A[10]. Nevertheless, most of these tumor suppressor genes exhibit a relatively low frequency of methylation in ESCC. Thus, further studies of a greater number of genes involved in the disease pathogenesis and progression are needed to identify putative epigenetic biomarkers for this tumor type.

The *pentraxin 3* (*PTX3*) gene at 3q25 is a member of the pentraxin superfamily. PTX3 expression is induced in response to inflammatory signals, and is produced at the site of inflammation by several cell types, primarily monocytes/macrophages, dendritic cells (DCs), endothelial cells, smooth muscle cells (SMCs), and fibroblasts. PTX3 can combine with a variety of soluble receptor ligands, and plays multiple biological roles, such as immune defense, female reproductive fertility, atherosclerosis, apoptosis, and the regulation of angiogenesis[11-14]. To date, there has been no reported study concerning *PTX3* gene promoter methylation.

In the present study, we examined reactivation of epigenetically silenced genes using an oligonucleotide microarray in ESCC cell lines. We also investigated the gene expression profiles of tumor tissue and nontumor tissue in ESCC. The genes markedly up-regulated by 5-aza-2'-deoxycytidine (5-Aza-dC) treatment in an ESCC cell line and markedly decreased in tumor tissue compared with nontumor tissue were considered genes of interest. Bisulphite sequencing and methylation-specific polymerase chain reaction (MSP) analyses were carried out on these genes to confirm the presence of aberrantly methylated CpG dinucleotides. Using the methods mentioned above, we successfully identified PTX3 as a new epigenetically silenced hypermethylated gene in ESCC.

MATERIALS AND METHODS

Cell lines and tissue samples

Six human ESCC cell lines were utilized in this study (TE-11, KYSE-30, KYSE-410, KYSE-510, EC-109, and EC-9706) [from American type culture collection (ATCC) and Sciencell]. One human immortalized normal esophageal epithelial cell line (Het-1A) (from ATCC) was used as the "normal" control for ESCC. The ESCC cell lines were cultured in Roswell Park Memorial Institute 1640 supplemented with 10% fetal bovine serum (Hyclone, United States) and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin) at 37 ℃ in a humidified 5% CO_2 incubator. Het-1A cells were maintained in bronchial epithelial basal media with growth supplements (Clonetics, United States).

Twenty primary ESCC and paired adjacent nontumor tissues were obtained from the Beijing Friendship Hospital, Beijing, China. Specimens were snap-frozen in liquid nitrogen and subsequently stored at -80 ℃. Formalin-fixed, paraffin-embedded samples of 79 primary ESCC cancer tissue specimens and paired adjacent nontumor tissues were also obtained from the Beijing Friendship Hospital. All patients from whom we obtained the study specimens gave informed consent to participate in this study. All case samples were collected from the primary surgical resection in patients with no prior history of ESCC and adjuvant therapy. Pathological diagnosis was performed and confirmed in the Pathology Department. Tumors were histopathologically classified according to TNM (tumor node metastasis) criteria.

Treatments with 5-aza-2'-deoxycytidine

ESCC cell lines and Het-1A were treated with 10 μmol/L of the DNA demethylating agent 5-Aza-dC (Sigma-Aldrich, United States) for 4 d.

RNA extraction and reverse transcription-PCR

Total RNA was extracted from cell line pellets and tissues using Trizol (Invitrogen, United States). Reverse transcription was performed using total RNA (1 μg) with Reverse Transcription System (Applied Biosystems, United States). The PTX3 mRNA expression levels were detected by conventional RT-PCR with Taq polymerase (Takara, Japan). RT-PCR was performed for 35 cycles at an annealing temperature of 55 ℃. Glyceraldehyde-3-phosohate dehydrogenase (GAPDH) was used as an internal control of RNA integrity. Primer sequences for PTX3 are as follows: (1) PTX3-F: 5'- TCTCTGGTCTGCAGTGTTGG-3'; (2) PTX3-R: 5'- TGAAGAGCTTGTCCCATTCC-3'; (3) GAPDH-F: 5'-CGGAGTCAACGGATTGGTCGTAT -3'; and (4)GAPDH-R: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

Isolation and bisulphite modification of genomic DNA

Genomic DNA was extracted from cells and tissues by standard phenol–chloroform extraction. Bisulphite modification of DNA was produced with a Zymo DNA Modification Kit (Zymo Research, United States) according to the manufacturer's protocol.

MSP

Bisulphite-treated DNA was amplified with the methylation-specific primer set, PTX3-MF: 5'-CGTTTGCGGTTAGGAGTATTC-3', and PTX3-MR: 5-CAAAACGTCGTCCGTAACTTA-3', or the unmethylation-specific primer set, PTX3-UF: 5'-TGTGTTTGTGGTTAGGAGTATTTG-3' and GPX3-UR: 5'- CAAAACATCATC-

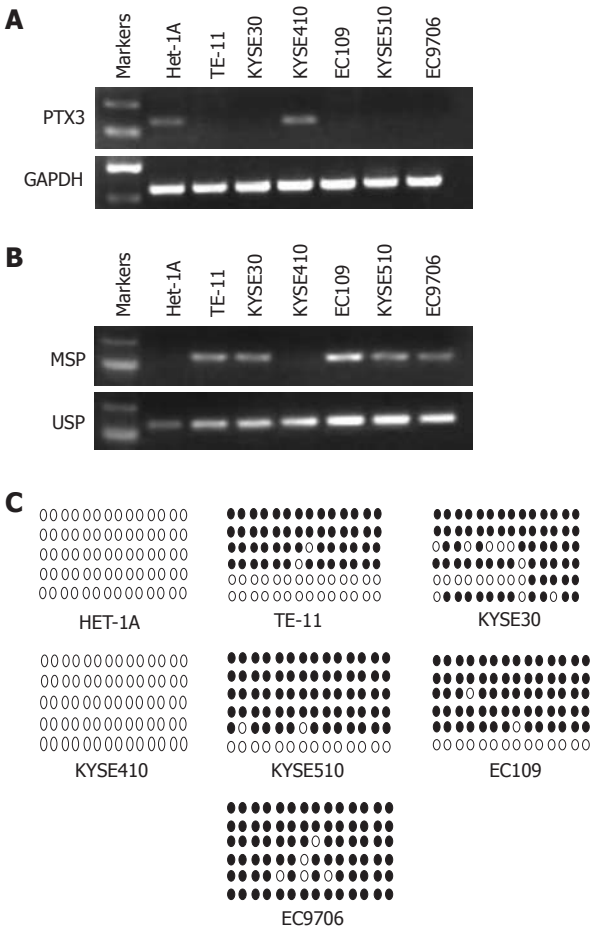

Figure 1 Promoter methylation contributes to pentraxin 3 downregulation in esophageal squamous cell carcinoma cell lines. A: Pentraxin 3 (PTX3) mRNA expression was detected by reverse transcription polymerase chain reaction; B: The methylation status of PTX3 promoter was determined by methylation-specific polymerase chain reaction. C: Representative high-resolution analyses of the methylation status of individual CpG sites in the PTX3 promoter by bisulphite genomic sequencing. Each row represents one bacterial clone with one circle symbolizing one CpG site. Methylated and unmethylated CpG sites were indicated by black and white circles, respectively. **PTX3: Pentraxin** 3; GAPDH: Glyceraldehyde-3-phosohate dehydrogenase; MSP: Methylation-specific polymerase chain reaction; USP:Unmethylation.

CATAACTTA-3', in a total volume of 20 μL using a 0.5 unit of hot-start Taq-polymerase (Takara, Japan) per reaction. The size of the unmethylated amplicon is 105 bp, and the methylated amplicon is 103 bp. Cycle conditions were: initial denaturation and hot start for 10 min at 95 ℃, then 35 cycles consisting of 30 s at 94 ℃, 30 s at 59 ℃ (for methylated reactions) or 50 ℃ (for unmethylated reactions), and 45 s at 72 ℃, and a final extension of 10 min at 72 ℃.

Bisulphite genomic sequencing

For the bisulphite genomic sequencing technique, the targeted fragment was amplified from bisulphite-treated DNA, cloned, and sequenced to obtain an accurate map of the distribution of CpG methylation. Forward: 5' TTTTTGAGATATTTATATGTTGTTTTT-3' and Reverse: 5'AAACACTAATCAACCTAACCTCTAC-3'. PCR was performed for 40 cycles at an annealing temperature of 54 ℃. The PCR products were then cloned into the pEasy-T1 vector (Transgene, China), and eight to ten colonies were randomly chosen and sequenced.

Immunohistochemistry

Expression of PTX3 was investigated in 79 primary ESCC cancer tissue specimens and in paired adjacent nontumor tissues using immunohistochemistry. The immunohistochemical expression of PTX3 was examined with light microscopy. The percentage of positive tumor cells was determined semi-quantitatively by assessing the entire tumor section, and each sample was assigned to one of the following categories: 0 (0%-4%), 1 (5%-24%), 2 (25%-49%), 3 (50%-74%), or 4 (75%-100%)[15]. The intensity of the immunostain was categorised as 0 (no stain), 1+ (weak stain), 2+ (medium stain), and 3+ (strong stain). Additionally, an immunoreactive score was calculated by multiplying the percentage of positive cells and the stain intensity. In instances of heterogeneous staining intensity within one sample, each component was scored independently and the results were summed. Using this system, the maximum score was 12. For statistical analysis, the criteria were combined as follows: 0, negative; 1 to 4, weakly positive; 5 to 8, moderately positive; and 9 to 12, strongly positive[16].

Statistical analysis

Statistical analysis was performed using the Student's *t* test and the χ^2 test; a value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Transcriptional silencing of PTX3 in most ESCC cell lines

Using RT-PCR with glyceraldehyde 3-phosphate dehydrogenase as a control, we tested the mRNA expression of PTX3 in the immortalized normal esophageal epithelial cell line Het-1A, and in 6 ESCC cell lines (TE-11, KYSE-30, KYSE-410, KYSE-510, EC-109, and EC-9706). The PTX3 transcript was silenced in 5 ESCC cell lines (TE-11, EC109, EC9706, KYSE30, and KYSE510), but not in KYSE-410 and Het-1A (Figure 1A).

As DNA methylation is an important regulator at the transcription level, we hypothesized that the silencing of PTX3 could be caused by methylation. Thus, we performed the MSP assay to measure the methylation level in these ESCC cell lines. The MSP results indicated that partial methylation was observed in the ESCC cell lines TE-11, EC109, EC9706, KYSE30, and KYSE510, whereas the unmethylated promoter was detected in KYSE-410 and in the immortalized normal esophageal epithelial cell line (Het-1A) (Figure 1B). The methylation results were in concordance with the transcription results of these ESCC cell lines.

We performed bisulphite genomic sequencing analysis to confirm the MSP result, and as shown in Figure 1C,

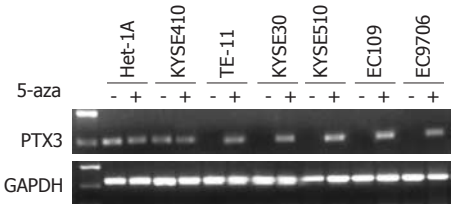

Figure 2 The mRNA expression of pentraxin 3 was restored after treatment with demethylation agent 5-aza-2'-deoxycytidine in esophageal squamous cell carcinoma cell lines. PTX3: Pentraxin 3; GAPDH: Glyceraldehyde-3-phosohate dehydrogenase.

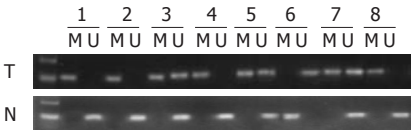

Figure 3 Representative methylation-specific polymerase chain reaction results of esophageal squamous cell carcinoma primary tumors (T) and paired adjacent nontumor tissues (N). Numbers1-8: Sample number. M: Methylation; U: Unmethylation.

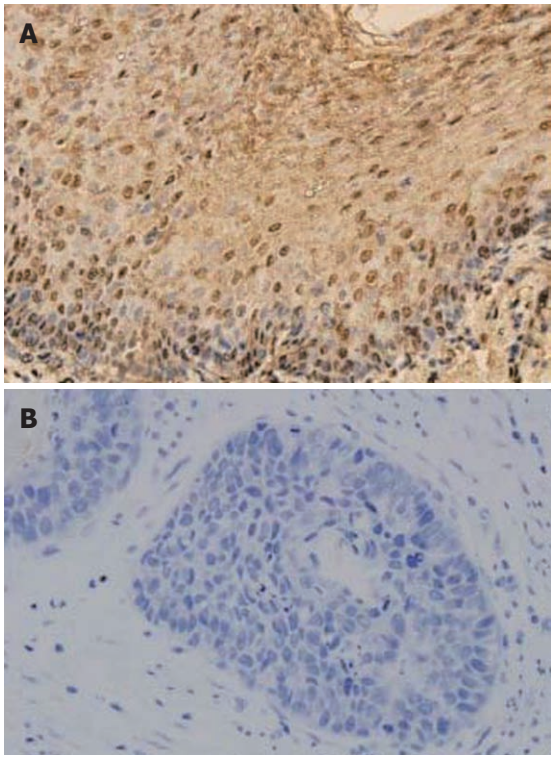

Figure 4 Pentraxin 3 expression assessed by immunohistochemistry staining in esophageal squamous cell carcinoma tumor tissues and adjacent nontumor tissues. A: Significant expression of PTX3 was detected in adjacent nontumor tissues (x 40); B: Negative or weak expression of pentraxin 3 was observed in esophageal squamous cell carcinoma (x 40).

the BGS result was consistent with our MSP data: no methylated CpG sites detected in KYSE-410 and Het-1A, but methylated CpG sites in the other ESCC cell lines (TE-11, EC109, EC9706, KYSE30 and KYSE510). The results indicated that transcriptional silencing of PTX3 was associated with methylation in ESCC cells.

PTX3 expression is up-regulated in ESCC cell lines treated with 5-aza-2-deoxycytidine

The results of RT-PCR indicated that five ESCC cell lines did not express PTX3 mRNA (Figure 1A). As mentioned above, there was a correlation between PTX3 silencing and DNA methylation in ESCC cell lines. To determine whether PTX3 expression could be reactivated by pharmacological demethylation of genomic DNA, all cell lines above were treated with the demethylating agent 5-Aza-dC. After treatment with 5-Aza-dC at 10 μmol/L for 4 d, the five silenced ESCC cell lines resulted in an obvious increase in PTX3 expression (Figure 2), further supporting the role of methylation as a primary mechanism of PTX3 silencing.

Methylation of PTX3 promoter is observed in most ESCC tumor tissues

To assess whether the PTX3 promoter hypermethylation observed in cell lines was relevant to ESCC, we further examined PTX3 methylation in 20 primary ESCC tumors with paired adjacent nontumor tissues using MSP. We found that 80% of ESCC tumor samples (16 of 20) exhibited statistically different methylation within the PTX3 promoter region, whereas the paired nontumor tissues exhibited only 20% (5 of 20) ($P < 0.001$).

The MSP results of ESCC primary tumors (T) and paired adjacent nontumor tissues (N) are shown in Figure 3.

Decreased expression of PTX3 protein in ESCC

We then analyzed 79 primary ESCC specimens and their corresponding adjacent nontumor tissues using immunohistochemical staining. PTX3 protein was detected in 24 of 79 (30.38%) ESCC specimens. In the non-malignant tissues, 67 of 79 (84.81%) samples showed positive detection of PTX3 protein.

In adjacent nontumor tissues, intense immunostaining for PTX3 was observed consistent with cytoplasmic distribution (Figure 4), whereas absent or weak immunostaining was detected in tumor tissues. Immunohistochemical results revealed that the expression of PTX3 protein in ESCC tumor tissues was significantly lower compared to adjacent nontumor tissues ($P < 0.01$).

The clinicopathological features of these patients and the results of PTX3 expression are summarized in Table 1. Statistical analysis indicated that PTX3 protein expression exhibited no correlation with the patients' age, gender, smoking habit, depth of invasion, or lymph node metastasis ($P > 0.05$). However, we found a higher frequency of promoter hypermethylation of PTX3 in the early stages of cancer (Ⅰ and Ⅱ) compared to advanced stages, suggesting that PTX3 hypermethylation occurs during a relatively early stage of the multi-step esophageal carcinogenesis.

DISCUSSION

Tumorigenesis is a multistep process caused by the accumulation and interplay of genetic and epigenetic alterations. DNA methylation is a key regulator of gene

Table 1 Clinical features of esophageal squamous cell carcinoma patients and their expression status for pentraxin 3 *n* (%)

Clinicopathological features	PTX3 expression		
	Positive	Negative	*P* value
Cases	24 (30)	55 (70)	
Gender			
Male	16 (30)	38 (70)	0.831
Female	8 (32)	17 (68)	
Age (mean, yr)	59.6	61.2	0.510
Smoker	12 (32)	26 (68)	0.823
Non-smoker	12 (29)	29 (71)	
Depth of invasion			0.315
T1, T2	16 (35)	30 (65)	
T3, T4	8 (24)	25 (76)	
Lymph node metastasis			0.311
Positive	4 (20)	16 (80)	
Negative	20 (34)	39 (66)	
TNM classification			0.012
I	9 (23)	20 (77)	
Ⅱa	10 (35)	19 (65)	
Ⅱb	2 (29)	5 (71)	
Ⅲ	1 (11)	8 (89)	
Ⅳ	2 (67)	3 (23)	

TNM: TNM classification of malignant tumours, T describes the size of the tumor and whether it has invaded nearby tissue, N describes regional lymph nodes that are involved, M describes distant metastasis.

transcription and genomic stability. Alteration of DNA methylation is one of the most consistent epigenetic changes that silence tumor suppressor genes in human cancers[17]. Additionally, aberrant methylation results in increased gene mutagenicity, due to the deamination of 5-methylcytosine to thymine[17].

We have previously analyzed expression microarray data prior to and post treatment using a demethylating agent in the EC9706 cell line, and we speculated that the *PTX3* gene is potentially down-regulated by promoter hypermethylation. PTX3 is involved in the regulation of innate resistance to pathogens, the inflammatory reaction, and possibly the clearance of self-components and female fertility. Transfection of PTX3 into breast cancer cells lacking expression led to a reduction in endothelial cell invasion and capillary tube formation, as well as prevention of tumor formation in athymic nude mice[18]. In this study, we examined the methylation status of the PTX3 promoter region in ESCC. As far as we understand, this is the first study to report on *PTX3* gene promoter methylation in ESCC.

Silencing of PTX3 in ESCC

Down-regulation of PTX3 mRNA and protein was detected by RT-PCR and immunostaining. We found that PTX3 was silenced in most ESCC cell lines (5 of 6 ESCC cell lines that we tested) and also silenced in tumor tissues. Reduced expression of PTX3 suggests that PTX3 plays a tumor suppressive role in ESCC.

Methylation of PTX3 promoter

PTX3 promoter hypermethylation was confirmed by methylation specific PCR and bisulphate genomic sequencing methylation in 83.3% of ESCC cell lines (5/6) and 80% of primary esophageal squamous cell carcinoma tissues (16/20), suggesting that promoter hypermethylation is a major, if not the only, mechanism for PTX3 down-regulation in ESCC.

Up-regulation of PTX3 expression after treatment with 5-Aza-dC

Treatment of ESCC cell lines *in vitro* with 5-Aza-dC, a nucleoside analogue inhibitor of DNA methyltransferase, reversed PTX3 CpG island hypermethylation and restored PTX3 expression; this confirmed that PTX3 hypermethylation serves as the principal mechanism for PTX3 downregulation in ESCC. PTX3 hypermethylation is involved in the development and progression of esophageal cancer.

Clinico-pathological significance of PTX3 protein expression

In our study, PTX3 protein expression was not associated with patients' age, gender, smoking habit, depth of invasion, and lymph node metastasis. We found a high frequency of promoter hypermethylation of PTX3 in the early tumor stages (Ⅰ and Ⅱ) of ESCC, indicating that aberrant methylation is a relatively early event in esophageal carcinogenesis.

Methylation-mediated inactivation is reversible, and up-regulation of PTX3 by 5-aza-dC may reverse the malignant phenotype of tumor cells. Therefore, PTX3 could serve as a novel target for gene therapy in ESCC treatment. In the future, further studies to elucidate the function of PTX3 in ESCC are warranted.

In summary, our study provides the first documentation that *PTX3* is a novel tumor suppressor gene epigenetically silenced in most ESCC tumors. Our results suggest that methylation of the PTX3 promoter region occurs at an early stage of ESCC pathogenesis and may provide a suitable biomarker for ESCC diagnosis.

COMMENTS

Background

Esophageal cancer is the sixth most common cause of cancer death worldwide, with > 400 000 new cases diagnosed each year. The molecular mechanisms underlying esophageal squamous cell carcinoma (ESCC) development remain poorly understood. DNA methylation plays a crucial role in the development of ESCC.

Research frontiers

Oligonucleotide Microarray Analysis can be used to identify novel genes that are aberrantly methylated in ESCC. Genes that were significantly upregulated after 5-aza-2'-deoxycytidine (5-Aza-dC) treatment in ESCC cells and significantly downregulated in tumor tissue compared with paired nontumor tissue were selected as hypermethylated candidate genes. Subsequently, bisulphite sequencing and methylation-specific PCR were performed to confirm the presence of aberrantly methylated CpG dinucleotides. In this study, the authors successfully identified *pentraxin 3* (*PTX3*) as a new methylation-silenced gene in ESCC.

Innovations and breakthroughs

The study provides the first documentation that *PTX3* is a novel candidate tumor suppressor gene epigenetically silenced in most ESCC tumors. The results

suggest that methylation of the PTX3 promoter region occurs at an early stage of ESCC pathogenesis and may be used as a biomarker for ESCC diagnosis.

Applications

Up-regulating PTX3 by 5-aza-dC may reverse the malignant phenotype of tumor cells. Therefore, PTX3 could be used as a novel target for gene therapy in ESCC treatment. PTX3 functions extracellularly as a secreted protein. PTX3 plays a tumor suppressive role in ESCC; thus, PTX3 protein could potentially be used directly as an anticancer drug therapy.

Terminology

PTX3 gene at 3q25 is a member of the pentraxin super-family. PTX3 expression is induced in response to inflammatory signals. PTX3 is able to combine with a variety of soluble receptor ligands and play multiple biological roles, such as immune defense, female reproductive fertility, atherosclerosis, apoptosis, and regulation of angiogenesis.

Peer review

This study reports the newly recognized tumor suppressor features of the gene *PTX3*, a gene that encodes a protein referred to as pentraxin 3 that serves as a protector against pathogens in tissues and aids in the control of autoimmunity. As an acute phase reactant, its blood level increases significantly during sepsis and severe inflammation, correlating with the severity of the disease and making the protein a useful biomarker for many inflammatory diseases. The authors of this study suggest that PTX3 could also be utilized as a tumor marker in ESCC.

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BRIEF ARTICLE

Role of Kasai procedure in surgery of hilar bile duct strictures

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Abstract

AIM: To assess the application of the Kasai procedure in the surgical management of hilar bile duct strictures.

METHODS: Ten consecutive patients between 2005 and 2011 with hilar bile duct strictures who underwent the Kasai procedure were retrospectively analyzed. Kasai portoenterostomy with the placement of biliary stents was performed in all patients. Clinical characteristics, postoperative complications, and long-term outcomes were analyzed. All patients were followed up for 2-60 mo postoperatively.

RESULTS: Patients were classified according to the Bismuth classification of biliary strictures. There were two Bismuth Ⅲ and eight Bismuth Ⅳ lesions. Six lesions were benign and four were malignant. Of the benign lesions, three were due to post-cholecystectomy injury, one to trauma, one to inflammation, and one to inflammatory pseudotumor. Of the malignant lesions, four were due to hilar cholangiocarcinoma. All patients underwent Kasai portoenterostomy with the placement of biliary stents. There were no perioperative deaths. One patient experienced anastomotic leak and was managed conservatively. No other complications occurred perioperatively. During the follow-up period, all patients reported a good quality of life.

CONCLUSION: The Kasai procedure combined with biliary stents may be appropriate for patients with hilar biliary stricture that cannot be managed by standard surgical methods.

Key words: Kasai procedure; Hilar bile duct; Stricture; Surgery

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INTRODUCTION

Surgical management of hilar biliary strictures remains a great challenge. The etiology of hilar biliary stricture is diverse, including benign and malignant lesions. The most common benign lesion associated with hilar biliary stricture is secondary to intraoperative injury; most commonly after laparoscopic cholecystectomy. Benign biliary strictures can also occur after hilar bile duct trauma and cholangitis. Malignant hilar biliary stricture can be caused by primary hilar cholangiocarcinoma; a cancer that involves confluence by contiguous spread (e.g., gallbladder and liver cancer), and metastatic cancer

to hilar lymphatic nodes[1]. Surgery for hilar bile duct stricture is difficult and not without risk. Several repair procedures have been described; none of which are fully satisfactory. Surgical complications are frequent and life-threatening, primarily related to anastomotic leak in the early postoperative period, and biliary strictures in the long term[2-4]. We therefore recently implemented the Kasai procedure with the use of biliary stents to repair hilar bile duct strictures. We report our experience of using this approach for hilar biliary strictures.

MATERIALS AND METHODS

Patients

We retrospectively analyzed 10 patients (five male, five female) with hilar bile duct strictures who underwent the Kasai procedure in our department from January 2005 to January 2011. The mean age was 52 years (range: 37-64 years). Clinical characteristics are shown in Table 1. Postoperative variables included complications and mortality. Long-term outcomes were retrieved from follow-up visit information.

Operative technique

Radical resections were performed for all malignant hilar lesions. For benign lesions, hilar bile duct dissection was performed, and healthy, non-scarred ducts were exposed for reconstruction. The hepatic quadrate lobule was removed at the level of the hilar plate to expose adequately the bile ducts. A Roux-en-Y portoenterostomy was performed. The afferent limb was approximately 50 cm, which was secured to the hepatic parenchyma, which surrounded the transected hepatic ducts, with 4-0 absorbable braided suture. Fine silicone catheters were used as intrahepatic duct stents to minimize the risk of bile duct restenosis. These were externalized through the stump of the intestinal Roux-en-Y loop and left *in situ* for 5 mo. Intra-abdominal drainage catheters were routinely placed at the anastomosis.

RESULTS

Two lesions were classified as Bismuth Ⅲ, and eight as Bismuth Ⅳ[5]. Four patients had biliary strictures secondary to bile duct injury: three due to cholecystectomy, and one secondary to abdominal trauma. Four patients had malignant biliary strictures caused by hilar cholangiocarcinoma: one had an inflammatory hilar bile duct stricture secondary to cholangitis; and one had a hilar inflammatory pseudotumor (Table 2).

Of 10 patients, four underwent one or two prior biliary operations. Radiological modalities for evaluation of these patients included ultrasonography (n = 7), contrast-enhanced computed tomography (n = 1), magnetic resonance cholangiopancreaticography (MRCP) (n = 10), and endoscopic retrograde cholangiopancreatography (n = 1, Figure 1). All patients underwent Kasai portoenterostomy with biliary stenting. Other surgical operations were performed simultaneously, including hepatic quadrate lobectomy in 10 patients and hepatic left lobectomy in one.

Table 1 Patient demographics and clinical characteristics

Variables	*n* (%)
Sex	
Female	5 (50)
Male	5 (50)
Symptoms	
Jaundice	7 (70)
Abdominal pain	4 (40)
Fever	2 (20)
Symptom-free	2 (20)
Physical signs	
Jaundice	7 (70)
Tenderness	2 (20)

Table 2 Etiology and Bismuth classification of bile duct stricture

Etiology	Bismuth classification	
	Ⅲ	Ⅳ
Injurious biliary stricture		
Abdominal trauma		1
Laparoscopic cholecystectomy		2
Open cholecystectomy	1	
Inflammatory biliary stricture		1
Inflammatory pseudotumor		1
Hilar cholangiocarcinoma	1	3
Total	2	8

There were no perioperative deaths. One patient experienced a postoperative anastomotic leak and was successfully managed conservatively with drainage and antibiotics. The liver functions of patients were returned to normal postoperatively. Surgical margins at the bile duct cut surfaces were clear in all four patients with hilar cholangiocarcinoma. No other complications such as hemorrhage, abdominal abscess, and wound infection were noted during the perioperative period.

All patients were followed up for a median period of 25.3 mo (range: 2-60 mo). All patients reported a good quality of life. No recurrence or metastasis was found in patients who underwent the Kasai procedure for malignant lesions. Moreover, cholangitis, anastomotic stricture, and cholelithiasis were not observed in any patients.

DISCUSSION

Surgical treatment of hilar bile duct strictures is one of the most challenging areas for hepatobiliary surgeons due to the anatomic complexity and diversity of lesions. Moreover, inappropriately managed biliary strictures can predispose patients to recurrent cholangitis, jaundice, and biliary cirrhosis, which requires additional surgical procedures[6].

Roux-en-Y hepaticojejunostomy is the most common method for repairing hilar biliary strictures. The

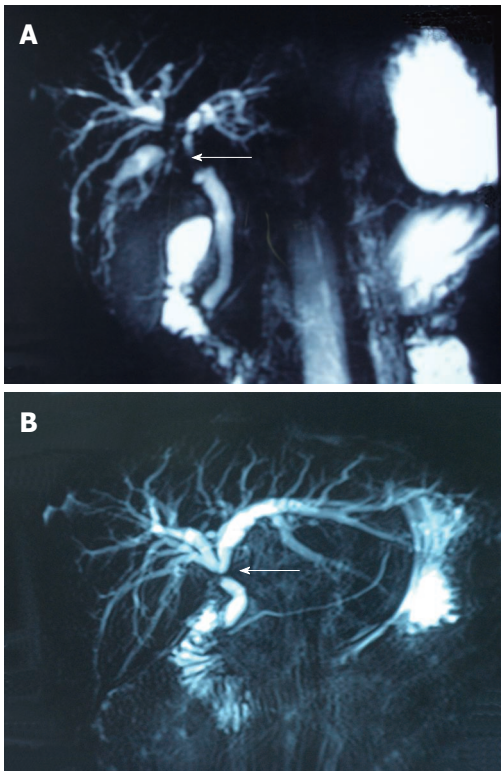

Figure 1 Magnetic resonance cholangiopancreaticography images. A: Magnetic resonance cholangiopancreaticography (MRCP) showing the hilar bile duct stricture (arrow) caused by cholangiocarcinoma; B: MRCP showing stricture at the hepatic duct confluence (arrow), due to post-laparoscopic cholecystectomy injury.

fundamental principle for repairing a biliary stricture at the hepatic hilum includes identification of healthy bile ducts proximal to the stricture, direct mucosa-to-mucosa anastomosis, a tension-free and wide anastomosis, and a 40-60 cm Roux-en-Y loop[7]. However, in some circumstances, it is difficult and risky to perform a standard Roux-en-Y hepaticojejunostomy due to the presence of edematous and fragile biliary wall tissues, or the presence of more than one small and thin duct, which cannot be reconstructed to one anastomotic opening. The Kasai procedure has been used extensively for infants with congenital biliary atresia since 1968[8]. However, there are few reports about the Kasai procedure performed in adults. Schlitt *et al*[9] performed the Kasai procedure for three adult patients with high ischemic-type biliary stricture after liver transplantation, but did not achieve satisfactory results. Pickleman *et al*[10] have reported five patients with bile duct injuries during laparoscopic cholecystectomy. These patients were managed by the Kasai procedure. All patients were symptom free and functioning normally for a follow-up period of 7-90 mo. In the present study, we applied the Kasai procedure to repair hilar biliary strictures in 10 patients. All patients had an uneventful recovery and have a good quality of life. Therefore, the Kasai procedure may be a good choice for the management of complex hilar biliary strictures that cannot be addressed by standard surgical methods.

Exposure of the proximal bile duct of hilar stricture remains the key to success in the repair of hilar biliary strictures. Some approaches are recommended to expose the hilar bile ducts, such as the hilar and transhepatic approaches[11,12]. The hilar approach involves lowering the hilar plate to expose the bile duct confluence, to manage the lesion extrahepatically. It is very difficult to expose the second- and third-order branches of the intrahepatic bile duct. The transhepatic approach involves exposing the hilar bile ducts by transecting the liver parenchyma between the left and right lobes of the liver. Although this approach can provide excellent surgical visualization, it requires more elaborate and complex skills, and is high risk. In this study, we performed a concomitant quadrate lobectomy to expose the hilar bile duct. We first dissected the hilar plate and hepatoduodenal ligament to evaluate the lower margin of the lesion, and assessed the portal vein and hepatic artery for tumor invasion. We routinely resected the base of quadrate lobe to visualize the bile duct confluence. Lastly, adequate exposure of the bile ducts could not be obtained, therefore, we resected more of the quadrate lobe between the gallbladder bed and the round ligament, to improve exposure of the hilar ducts, including the second- and third-order branches. Adequate exposure of the hilar bile ducts was obtained in all patients.

In recent years, biliary stenting has become a new technique for the treatment of biliary strictures. The major advantages are that the procedure used to place them is minimally invasive and well tolerated. It was first applied as palliative treatment in patients with unresectable malignant strictures. Previous studies have shown that patients undergoing stent placement for malignant strictures have a significant improvement in abdominal comfort, jaundice, and quality of life. The application of biliary stents as palliative treatment of biliary malignancies is a widely accepted practice[13,14]. With advances in stent material and the technical process of stent placement, many reports have described their use for treatment of benign biliary strictures[15-17]. However, complications of stent placement, such as stent occlusion and cholangitis limit their use in benign strictures. Both the requirement for and duration of stenting for benign strictures have been controversial for many years. Siriwardana and Siriwardena have reported a systematic appraisal of the current status of the use of metallic endobiliary stents in the treatment of benign biliary strictures[18]. They have demonstrated that, although stents can be deployed endoscopically or radiologically with relative ease and with a low procedure-related complication rate, there is a critical lack of data on long-term patency. Thus, currently, metallic endobillary stents should not be used for benign strictures in patients with a predicted life expectancy of > 2 years. In our study, six patients with benign hilar strictures underwent the Kasai procedure. All patients had a good outcome during follow-up. Therefore, the Kasai procedure may be a good alternative for patients with benign hilar strictures.

The Kasai procedure is a portoenterostomy performed by suturing a jejunal loop to the hepatic parenchyma that surrounds the transected hepatic ducts. Direct mucosa-to-mucosa anastomosis is not required. Anastomotic leak and stricture are the most common postoperative complications. To prevent or lessen the probability of postoperative stricture and bile leak, we routinely placed transanastomotic catheters in the bile ducts, which were externalized through the intestinal Roux-en-Y loop. In our study, all 10 patients underwent the Kasai procedure with transanastomotic stents, and no anastomotic stricture was observed during follow-up. Transanastomotic catheters not only limit the tendency to stricture, but also serve to decompress the biliary system and provide access for radiographic imaging in the perioperative period[19]. Innes *et al*[20] have suggested that a bilioenteric anastomosis to manage benign stenosis of the biliary tract might be undertaken without placing stents, which promises low postoperative morbidity and excellent obstruction-free long-term results. Although the use of postoperative transanastomotic stenting tubes is controversial, we recommend their use when the Kasai procedure is being performed.

In conclusion, the management of hilar biliary strictures is challenging. Surgical repair has been the preferred approach. A Roux-en-Y hepaticojejunostomy is a standard procedure to repair hilar stricture for most patients. The Kasai procedure may be a good choice for a small subset of patients who suffer from complex hilar biliary strictures that cannot be managed by standard surgical methods.

COMMENTS

Background

Surgical management of hilar bile duct stricture is a great challenge because of the complexity of the perihilar anatomy and the diversity of the lesions.

Research frontiers

The appropriate management of hilar bile duct strictures depends on the cause, type, and level of stricture. Roux-en-Y hepaticojejunostomy is a conventional procedure used to repair hilar bile duct stricture. However, in some circumstances, it is very difficult and high risk to perform a standard Roux-en-Y hepaticojejunostomy.

Innovations and breakthroughs

The authors investigated the role of the Kasai procedure in the surgical management of hilar bile duct strictures and demonstrated that the Kasai procedure is a successful method to treat complex hilar biliary strictures.

Applications

This study may help surgeons to choose the Kasai procedure as an appropriate procedure to deal with complicated hilar bile duct strictures.

Terminology

The Kasai procedure is a hepaticoportoenterostomy, which is performed by suturing a jejunal loop to the hepatic parenchyma that surrounds the transected hepatic ducts.

Peer review

This is a small case series that describes the use of hepatic portoenterostomy in adults for the reconstitution of biliary-enteric continuity when mucosal to mucosal hepaticojejunostomy is not feasible. Although this is not a new concept, it is an important technique that is occasionally used by hepatobiliary surgeons. Because the literature describing this technique is scarce, this article should be published.

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BRIEF ARTICLE

Three initial diets for management of mild acute pancreatitis: A meta-analysis

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Abstract

AIM: To compare non-liquid and clear-liquid diets, and to assess whether the latter is the optimal treatment for mild acute pancreatitis.

METHODS: The Cochrane Library, PUBMED, EMBASE, EBM review databases, Science Citation Index Expanded, and several Chinese databases were searched up to March 2011. Randomized controlled trials (RCTs) that compared non-liquid with clear-liquid diets in patients with mild acute pancreatitis were included. A meta-analysis was performed using available evidence from RCTs.

RESULTS: Three RCTs of adequate quality involving a total of 362 participants were included in the final analysis. Compared to liquid diet, non-liquid diet significantly decreased the length of hospitalization [mean difference (MD): 1.18, 95% CI: 0.82-1.55; $P < 0.00001$] and total length of hospitalization (MD: 1.31, 95% CI: 0.45-2.17; $P = 0.003$). The subgroup analysis showed solid diet was more favorable than clear liquid diet in the length of hospitalization, with a pooled MD being -1.05 (95% CI: -1.43 to -0.66; $P < 0.00001$). However, compared with clear liquid diet, both soft and solid diets did not show any significant differences for recurrence of pain after re-feeding, either alone [relative risk (RR): 0.95; 95% CI: 0.51-1.87; $P = 0.88$] and (RR: 1.22; 95% CI: 0.69-2.16; $P = 0.49$), respectively, or analyzed together as non-liquid diet (RR: 0.80; 95% CI: 0.47-1.36; $P = 0.41$).

CONCLUSION: The non-liquid soft or solid diet did not increase pain recurrence after re-feeding, compared with the clear-liquid diet. The non-liquid diet reduced hospitalization.

Key words: Acute pancreatitis; Diet; Nutritious supplement; Meta-analysis; Length of stay

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INTRODUCTION

Early enteral nutrition therapy is important for management of severe as well as mild acute pancreatitis[1]. The initial part of the necessity is to prevent bacterial infection, as well as energy supplementation[2]. This kind of nutrition is always preferable *via* the nasojejunal route with a nasal bowel nutrition tube[3]. However, it is not necessary for mild acute pancreatitis patients because of

longer length of hospitalization and discomfort[4,5].

In daily clinical care, 80%-90% of patients with acute pancreatitis demonstrate a mild clinical course of the disease[6]. The traditional initial treatment in mild acute pancreatitis has included: (1) fasting for the first few days; and (2) administration of parenteral fluids followed by clear-liquid-diet intake, orally until the abdominal pain has resolved and the levels of pancreatic enzymes have decreased[7-9]. It sounds reasonable that clear-liquid diet intake will shorten the presence of food in the duodenum, which reduces cholecystokinin release that stimulates pancreatic enzyme secretion[10]. Hospital discharge is usually planned on the basis of the patient's tolerance to solid diet[11].

Oral re-feeding has been recommended to start with small amounts of clear-liquid diet, rich in carbohydrates and proteins and low in fat, gradually increasing or shifting the intake to soft or solid diet during 3-7 d, to avoid abdominal pain and pancreatitis relapse[12,13]. Unfortunately, to date, evidence is sparse concerning when one kind of diet should be shifted to another, and what kind of diet is definitely optimal re-feeding[14].

Some recent studies have suggested that oral re-feeding with soft or solid diet instead of clear liquids can be considered safe for pain recurrence, and shorten the length of hospitalization. Some randomized trials in patients with mild acute pancreatitis have shown that non-liquid diets are feasible and safe[15-17]. However, the results of these studies were inconclusive. The aim of the present study was to perform a meta-analysis of current randomized controlled trials (RCTs) to evaluate non-liquid diet (including soft and solid diets) as an initial treatment in mild acute pancreatitis.

MATERIALS AND METHODS

Study selection criteria

The titles and abstracts of all citations identified by the literature search were reviewed. Selection criteria were then applied to all potentially relevant studies. Editorials and expert opinions, reviews without original data, case reports and studies lacking control groups were excluded. The selection criteria for inclusion in the meta-analysis were as follows: (1) only RCTs that compared non-liquid diet, including soft and solid diet, with clear-liquid diet were included; (2) diagnosis of mild acute pancreatitis was confirmed according to computed tomography scores, APACHE Ⅱ scores, and basic laboratory examination; (3) outcomes of length of hospitalization (LOH), total length of hospitalization (TLOH), and recurrence of pain after re-feeding were reported; and (4) no other nutritious supplement treatment was given to patients.

Search strategy for identification of studies

Trials were identified by searching the Cochrane Library (Issue 1 2011), PubMed (March 2011), EMBASE (March 2011), Science Citation Index Expanded, and CBM (Chinese Biomedical Literature Database). The query was constructed by using the combination of the following keywords: (mild pancreatitis or acute pancreatitis) and (diet or nutritious supplement or nutrition). Articles published in any language were considered. Reference lists from the trials selected by electronic searching were hand-searched to identify further relevant trials. Abstracts of the articles selected from each of these multiple searches were reviewed and those meeting the criteria were recorded. In the case of duplicate reports, or studies obviously reporting results from the same study population, only the latest published results were used.

Assessment of study quality

The quality of included studies was assessed independently by two authors (Meng WB and Li YM) without blinding to authorship or journal. Discrepancies were resolved by involving the third author, Xun Li. The quality of the studies was assessed using the scores proposed by Cochrane handbook 5 standards: randomization, allocation, concealment, blinding (participants, investigators, outcomes assessors, and data analysis), and completeness of follow-up.

Data extraction

Two investigators (Meng WB and Li YM) extracted the data from the studies that met the selection criteria (Tables 1-3). The outcomes were totalled from the three studies. There was > 98% agreement for data extraction between the two investigators.

Statistical analysis

We analyzed the data using Review Manager (version 5.0)[18] and pooled data for summary estimates. We expressed results for dichotomous outcomes as relative risk (RR), and mean difference (MD) with 95% CIs for continuous outcomes. We used the χ^2 test to assess heterogeneity between trials and the I^2 statistic to assess the extent of inconsistency. Statistical significance cut-off for the test of heterogeneity was set at 0.10. We used a fixed-effect model for calculations of summary estimates unless there was significant heterogeneity, in which case, results were confirmed using a random-effects statistical model.

RESULTS

Search results

The flowchart of reviews shows the detailed process of study selection (Figure 1). The comparison was made between non-liquid and clear-liquid diets[15-17]. Three trials fulfilled the inclusion criteria.

Quality and characteristics of included studies

Data regarding characteristics of the studies, including patients, baseline characteristics and quality assessment of the studies are summarized in Tables 1-3, respectively.

Group and subgroup arrangement

Groups for non-liquid diet *vs* clear-liquid diet were established first. We deemed both solid and soft diets as non-liquid diets to perform the analysis. In the study of Moraes *et al*[15] in Table 1, there were three arms with solid diet, soft diet, and clear-liquid diet, which were

Table 1 Baseline characteristics of the included studies

Study comparisons	Moraes *et al*[15]			Jacobson *et al*[16]		Sathlaraj *et al*[17]	
Type of diet	Solid	Soft	Liquid	Soft	Liquid	Solid	Liquid
No. of patients included	70	70	70	66	55	49	52
Mean age (yr)	53	49	51	51	47	37	39
Male/female	42/28	43/27	33/37	23/43	34/21	39/10	44/8
Mean body mass index (%)	ND	ND	ND	29	29	21.3	20.9
Cause							
Biliary system (*n*)	33	35	32	15	15	7	9
Alcohol (*n*)	17	14	16	14	19	26	25
Unknown and others (*n*)	20	21	22	26	32	16	18
Type of pain							
Acute (*n*)	ND	ND	ND	52	53	40	41
Acute or chronic (*n*)	ND	ND	ND	3	3	9	11
Time between admission and first meal (d)	3.4 ± 0.8	3.6 ± 1.0	3.5 ± 1.5	2	1	1.6	1.4
Total number of meals on study day 1 (*n*)	2	2	2	2	2	3	4
Calories in first meal on day 1 (kcal)	620	120	124	350	157	262	137
Fat in first meal on day 1 (g)	14	2	1	5	1	3	4
Total calories on first day (kcal)	1240	241	248	622	301	921	370
Total fat on first day (g)	28	4	2	13	2	15	8

ND: Not described.

Table 2 Quality assessment of randomized controlled trials included in the meta-analysis

Study	Methodological quality items			
	Randomization	Allocation concealment	Double blinding	ITT analysis
Moraes *et al*[15]	Yes	Yes	Yes	Yes
Jacobson *et al*[16]	Yes	Yes	Yes	Yes
Sathlaraj *et al*[17]	Yes	Yes	Yes	Yes

Based on Cochrane handbook 5. ITT: Intention-to-treat.

compared with each other simultaneously. We extracted the solid arm and placed it into the non-liquid diet group, and excluded the soft diet arm for good balance of the statistics. According to the type of control group compared, all the included studies were divided into two subgroups: subgroup A, soft diet *vs* liquid diet; and subgroup B, solid diet *vs* liquid diet.

Meta-analysis

Recurrence of pain: As shown in Figures 2-4, meta-analysis did not show any statistically significant difference between 174 patients in the non-liquid diet group and 188 in the clear-liquid diet group with regard to pain recurrence (RR: 0.80, 95% CI: 0.47-1.36; $P = 0.41$). There was no significant heterogeneity between them ($P = 0.74$, $I^2 = 0\%$). In the subcategory analysis, there was no difference between soft diet and clear-liquid diet (RR: 0.95, 95% CI: 0.51-1.87; $P = 0.88$), nor was heterogeneity ($P = 0.54$, $I^2 = 0\%$). Similarly, in subgroup B, RR was 1.22 (95% CI: 0.69-2.16, $P = 0.49$), and no significant heterogeneity was observed ($P = 0.46$, $I^2 = 0\%$).

LOH: Three trials comprising a total of 174 patients in the non-liquid diet group and 188 in the clear-liquid diet group reported LOH. There was a significant difference between the non-liquid and clear-liquid diet groups (Figure 2), with a pooled MD of 1.18 (95% CI: 0.82-1.55; $P < 0.00001$). There was significant heterogeneity between them ($P = 0.0001$, $I^2 = 89\%$). In subgroup A, there was heterogeneity between two trials ($P < 0.0001$, $I^2 = 94\%$), although a pooled MD was -0.30 (95% CI: -0.78 to 0.17, $P = 0.21$). In subgroup B, MD was -1.05 (95% CI: -1.43 to -0.66; $P < 0.00001$); However, significant heterogeneity was observed ($P = 0.0003$, $I^2 = 92\%$).

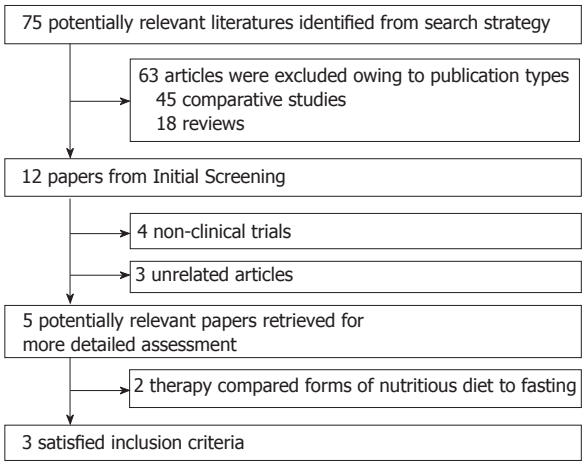

Figure 1 Flowchart of study selection.

TLOH: TLOH was reported by three trials comprising a total of 119 patients on non-liquid diet and 122 on clear-liquid diet. There was a significant difference between the two groups (MD: 1.31, 95% CI: 0.45-2.17; $P = 0.003$) (Figure 2). The subgroup analyses showed no significant difference for subgroup A (MD: -0.59, 95% CI: -1.33 to 0.14; $P = 0.11$) and subgroup B (MD: -0.70, 95% CI: -1.71 to 0.31; $P = 0.17$). No significant heterogeneity was found between the non-liquid and clear-liquid diet groups

Table 3 Results on length of hospitalization, total length of hospitalization and recurrence of pain

Study comparisons	Moraes *et al*[15]			Jacobson *et al*[16]		Sathlaraj *et al*[17]	
Type of diet	Solid	Soft	Liquid	Solid	Liquid	Soft	Liquid
LOH (d)	5.8 ± 1.1	7.4 ± 1.5	7.3 ± 1.6	1.71 ± 2.04	1.68 ± 1.85	4.18 ± 2.86	6.75 ± 3.37
TLOH (d)	7.5 ± 3.5	8.2 ± 2.4	8.2 ± 2.6	4 (3-6)	4 (3-5)	5.92 ± 2.978	8.71 ± 4.995
Recurrence of pain (*n*)	15	12	14	6	4	4	3

LOH: Length of hospitalization; TLOH: Total length of hospitalization.

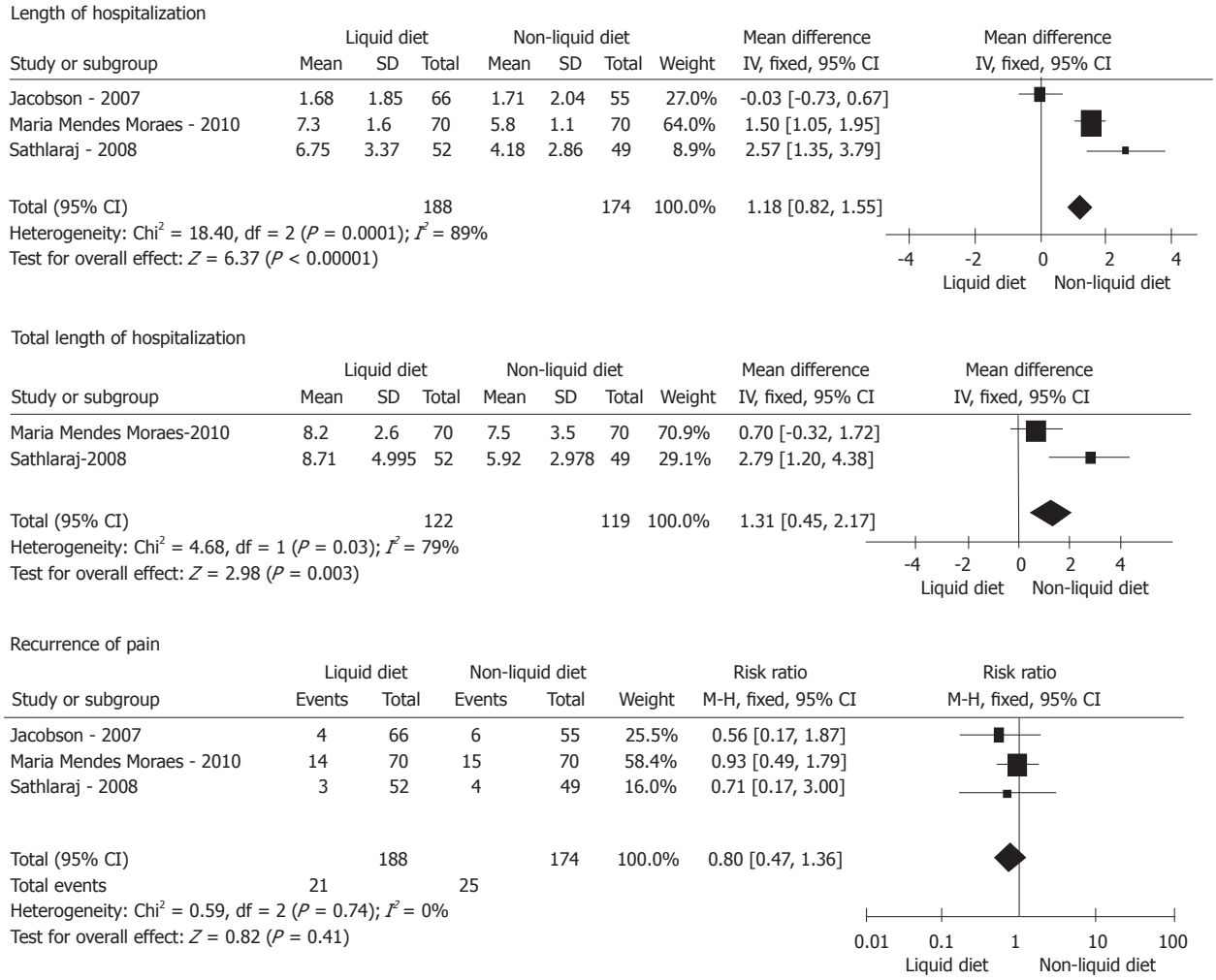

Figure 2 Outcomes in non-liquid diet *vs* liquid diet with length of hospitalization, total length of hospitalization and recurrence of pain. IV: Inverse-Variance; M-H: Mantel-Haenszel.

($P = 0.03$, $I^2 = 79\%$). However, significant heterogeneity was seen in subgroup A ($P = 0.002$, $I^2 = 89\%$).

DISCUSSION

The current meta-analysis demonstrated that, compared with clear-liquid diet, non-liquid diet did not increase the recurrence of pain after re-feeding in mild acute pancreatitis, and this finding was supported by the subgroup analyses. These outcomes totally challenged our belief that solid diet, even soft diet, would definitely induce the recurrence of abdominal pain and increase pancreatic enzyme secretion[19-21]. Physicians had previously hypothesized that oral re-feeding could promote inflammatory processes in the pancreas and increase production of enteral hormones (such as cholecystokinin, motilin and serotonin), which have a negative trophic effect on the pancreatic tissue, thus decreasing pancreatic blood flow and gastrointestinal motility[22-25]. However, the meta-analysis did not show any significant difference between non-liquid and clear-liquid diets (RR: 1.22, 95% CI: 0.69-2.16; $P = 0.49$), as well as the two subgroups. There have only been a few studies on diet in acute pancreatitis thus far, therefore, it is possible to speculate that most of the patients could tolerate non-liquid diet successfully. Another key point was that our analysis only selected data from mild acute pancreatitis, with potentially severe types being excluded. The inclusion criteria of the three

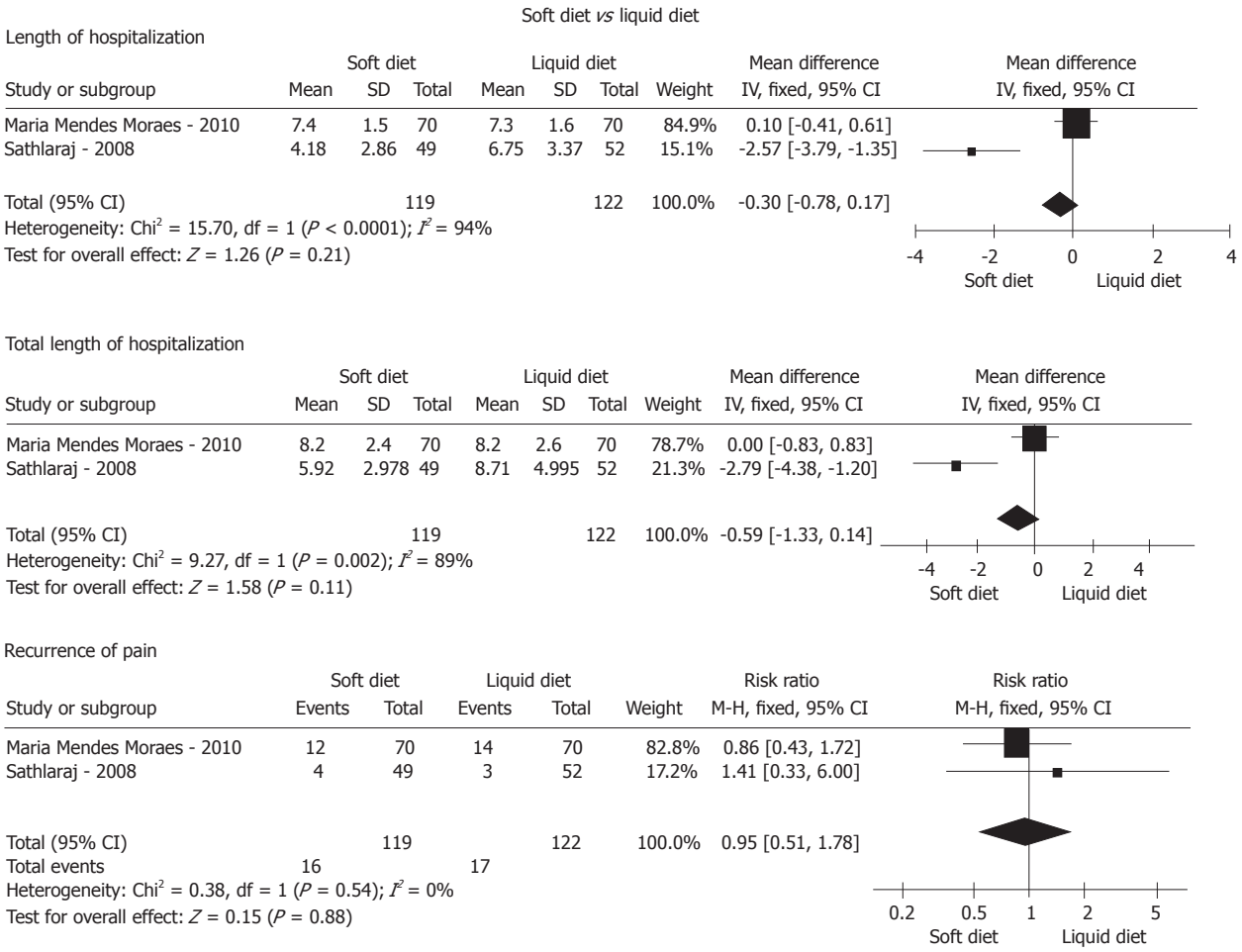

Figure 3 Outcomes in subgroup A: soft diet *vs* liquid diet with length of hospitalization, total length of hospitalization and recurrence of pain. IV: Inverse-Variance; M-H: Mantel-Haenszel.

RCTs were similar. There was no significant heterogeneity between any of the groups.

Non-liquid diet, especially solid diet, showed superiority over clear-liquid diet on the LOH in the meta-analysis. Consequently, as compared to soft diet, solid diet supplement showed significant beneficial effects on both LOH and TLOH in patients with mild acute pancreatitis. Significant heterogeneity was found in the non-liquid diet *vs* the clear-liquid diet groups ($P = 0.0001$, $I^2 = 89\%$) and in the comparison of solid diet *vs* clear-liquid diet ($P = 0.0003$, $I^2 = 92\%$). This heterogeneity could have originated from discrepancies in the criteria for hospital discharge and the limited number of RCTs. Although practice management guidelines have presented detailed information concerning the appropriate timing and form of nutrition in severe acute pancreatitis, little attention has been paid to optimizing the dietary management of mild pancreatitis[4,14,26,27]. Earlier studies were not able to explain the benefits of soft or solid diet with fat re-feeding, or the patients' tolerance. There is a viewpoint that the pancreas may be less responsive to stimulation by nutrients in normal digestive tract than when patients are suffering from pancreatitis[20,27].

With the respect to TLOH, a significant difference was only seen in the non-liquid diet *vs* the clear-liquid diet group. In the subgroup study, although outcomes favored the soft and solid diet, there was no significant difference. Significant heterogeneity was found in the non-liquid diet *vs* clear-liquid diet groups ($P = 0.03$, $I^2 = 79\%$) and soft diet *vs* clear-liquid diet groups ($P = 0.002$, $I^2 = 89\%$). Due to the lack of exact data on TLOH, the heterogeneity analysis could not be performed. The heterogeneity would also have derived from discrepancies in the criteria of discharge and the time zone difference from being hospitalized to re-feeding.

One of the disadvantages of this meta-analysis was that only three RCTs were included. All three studies had high methodological quality and generalizability, nonetheless, there may still have been bias in the final results. There was one study from Brazil[15] for which we could not obtain accurate data for TLOH. Additionally, another study[16] that showed shorter LOH may have been related to the fast discharge protocol, which could have led to heterogeneity. Therefore, more multicenter cooperative studies with prospective design are needed.

To the best of our knowledge, many diseases can cause mild acute pancreatitis. The tolerant form of the different diets should be projected separately by disease. That is probably why there are always some patients who cannot tolerate re-feeding, hence prolonging LOH

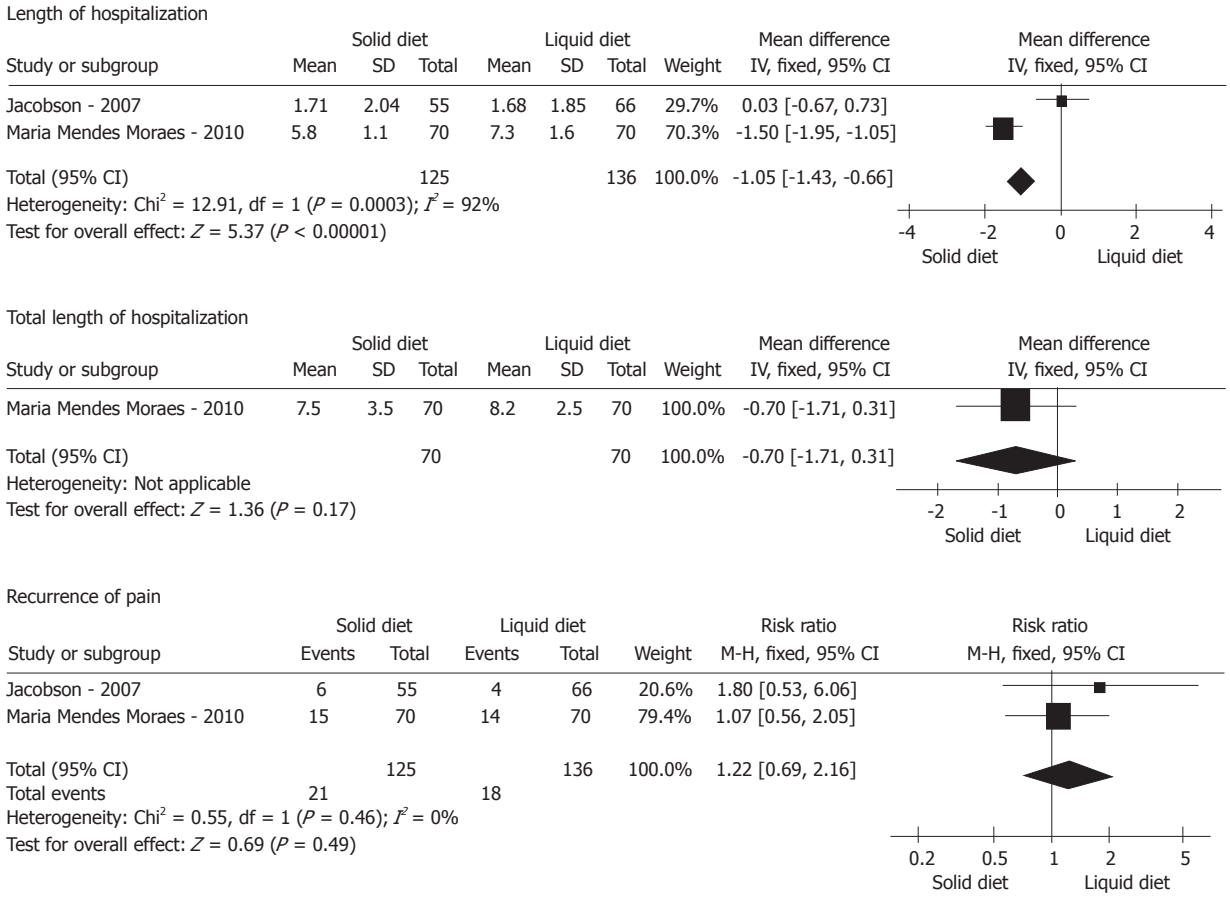

Figure 4 Outcomes in subgroup B: solid diet *vs* liquid diet with length of hospitalization, total length of hospitalization and recurrence of pain. IV: Inverse-Variance; M-H: Mantel-Haenszel.

in patients with mild pancreatitis[28]. For example, if patients suffer from bile duct obstruction or infection, high pressure in the bile duct can cause deterioration of pancreatitis by increasing inflammation if the pressure is not released immediately[29-31]. In that situation, even a small load with any kind of diet could lead to serious consequences[32-34]. Therefore, treatment directed against the causes of pancreatitis is still an essential step. It is advisable to try and cure pancreatitis as soon as possible after the cause has been established, therefore, we should focus therapeutic options on the pathogenesis, in addition to the necessary supporting treatments; not only to ameliorate abdominal pain, but also to recover the whole function of the gastrointestinal tract. This will in turn improve the tolerance of the patients to earlier application of enteral nutritional therapy, thus reducing LOH. According to our meta-analysis, we obtained novel results that will encourage us to promote further new protocols with regard to dietary management of pancreatitis secondary to different protopathies. Also, more multicenter cooperative studies with prospective design are needed for ultimate conclusions about this issue.

In conclusion, the encouraging outcomes in this analysis may demonstrate a different notion from our previous experience in nutritional supplementation of the patients who are diagnosed with mild acute pancreatitis. None of the soft or solid non-liquid diets showed greater recurrence of pain after re-feeding, compared to the clear-liquid diet. Non-liquid diet nutritional supplementation, especially with solid diet, could reduce LOH and TLOH. At this point, we cannot explain our findings with previous pathophysiological experiments performed on acute pancreatitis. One possibility is that the upper digestive tract is less responsive to stimulation by nutrients than we assumed. However, new dietary experiments with animal models of acute pancreatitis and more RCTs comparing roles of different diet forms in the recovery of mild acute pancreatitis are expected to resolve these issues.

COMMENTS

Background

Early enteral nutrition therapy is important for management of mild acute pancreatitis. Initial treatment includes fasting in the first few days and administration of parenteral fluids, followed by gradual clear-liquid diet intake until abdominal pain has resolved and levels of pancreatic enzymes have decreased. Hospital discharge is usually planned on the basis of the patients' tolerance to solid diet.

Research frontiers

Some recent studies have suggested that oral re-feeding with soft or solid diet instead of clear liquids can be considered safe for recurrence of pain, but it inconsistently shortens length of hospitalization (LOH). Some randomized trials in mild acute pancreatitis have shown that non-liquid diets are both feasible and safe.

Innovations and breakthroughs

To review systematically the outcomes of non-liquid diet including soft and solid diet compared with clear-liquid diet in mild acute pancreatitis. The meta-analysis demonstrated that none of the non-liquid soft and solid diets increased

recurrence of pain after re-feeding, compared with clear-liquid diet. Surprisingly, non-liquid diet nutritious supplement re-feeding reduced LOH and total length of hospitalization (TLOH). Furthermore, solid diet decreased LOH.

Applications

With the encouraging outcomes, non-liquid diet nutritional supplementation, especially solid diet, could reduce LOH and TLOH. It might potentially improve the management of mild acute pancreatitis. However more randomized controlled studies on dietary experiments comparing roles of different diet forms in the recovery of mild acute pancreatitis are expected.

Terminology

LOH means the minimum number of days that patients stay in hospital. TLOH is the total length of hospitalization.

Peer review

Nutrition management of mild acute pancreatitis was the focus of the study. In the literature, comparison between total parenteral and enteral nutrition have been performed but not among different types of oral feeding. This study was unique in describing a carefully conducted meta-analysis comparing liquid, soft and solid diets. The conclusions are intriguing but sound. It potentially can improve the management of mild acute pancreatitis as well as further understanding of gastrointestinal physiology.

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BRIEF ARTICLE

Integration of human papillomavirus 18 DNA in esophageal carcinoma 109 cells

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Abstract

AIM: To detect human papillomavirus (HPV) DNA in esophageal carcinoma (EC) 109 cells and investigate the relationship between HPV and EC.

METHODS: Genomic DNA and total RNA from EC109 cells were isolated. HPV DNA was detected by polymerase chain reaction (PCR) with the general primer sets of My09/11 and GP5 +/6 + for the *HPV L1* gene and type-specific primer sets for HPV18 E6 and HPV18 E6-E7. Reverse transcription (RT) of mRNA isolated from EC109 cells was performed to produce a cDNA. And then a PCR-based protocol for the amplification of papillomavirus oncogene transcripts was used to analyze HPV18 DNA and integrated transcripts of HPV18 in the chromosomes of EC109 cells. The final nested PCR products were cloned into a pMD-18T vector and sequenced to analyze the chromosomal location of HPV integration.

RESULTS: HPV18 DNA was detected in EC109 cells by PCR using the general primer sets of My09/11 and GP5 +/6 + for HPV L1 and the type-specific primer sets for HPV18 E6 and E6-E7 to generate products of 450 bp, 150 bp, 335 bp and 944 bp, respectively. Approximately 600 bp of integrated HPV18-specific transcript was identified. The final nested PCR product of integrated HPV18 DNA was cloned into a pMD-18T vector and sequenced to analyze the chromosomal location of HPV integration. Sequence alignment showed that the HPV18 sequence from EC109 cells was identical to that of the encoded early protein E7-E1 of the standard HPV18 strain X05015, and another partial gene sequence was identical to a partial sequence of human chromosome 8.

CONCLUSION: Integration of the HPV genome into the host cell chromosome suggests that persistent HPV infection is vital for malignant cell transformation and carcinogenesis.

Key words: Esophageal carcinoma; Human papillomavirus; Integration; Infection; Genome

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INTRODUCTION

The esophageal carcinoma (EC) cell line EC109 was established in 1976 by the Cell Biology Research Group at the Chinese Academy of Medical Sciences Institute of Cancer Research. The cell line was derived from esophageal cells that were surgically removed from a patient with a pathological diagnosis of EC[1]. The cells were determined to be positive for human papillomavirus (HPV) type 18[2].

EC is one of the major cancers in China. The etiology of EC has yet to be established despite extensive investigation of the contribution of environmental factors, lifestyle, and low levels of chemical elements. In 1982, Syrjanen formulated a hypothesis on the relationship between HPV infection and the development of EC; the hypothesis was based on the presence of papilloma-like tissues in EC specimens and other molecular evidence[3]. Thereafter, numerous clinical studies have supported this hypothesis[4-13]. However, the link between HPV infection and the etiology of EC remains inconclusive.

A link between HPV infection and squamous cell cancer of the cervix has been identified[14]. Currently, several oncogenic types of HPV are regarded as the etiological agents responsible for the development of cervical squamous cell carcinoma[15,16].

We further studied the association between HPV infection and carcinogenesis using HPV18 E6-E7–transfected stable cell lines derived from fetal esophageal epithelial tissue. Our results strongly supported the conclusion that the expression of HPV18 proteins E6 and E7 induced the transformation of the esophageal cells[17]. HPV18 was also detected in EC109 cells[2]. These results support a link between HPV infection and esophageal carcinogenesis.

Persistent infection with high-risk HPV and the integration of viral genomes into the host genome have been implicated in the etiology of malignant and premalignant disease of the female lower genital tract[18,19]. HPV is divided into low-risk (LR) and high-risk (HR) types according to the presumed degree of risk for the development of cancer. HR HPV types such as HPV16, HPV18, and HPV31 are associated with cancer, while LR HPV types such as HPV6, HPV11, and HPV40 are the causative agents of benign warts[20]. Episomal and integrated HPV can be distinguished using a polymerase chain reaction (PCR)-based protocol for the amplification of papillomavirus oncogene transcripts (APOT), developed by Klaes and his colleagues[21]. The same group hypothesized that HPV transcripts derived from the integrated HPV genome represent suitable molecular markers for a pre-neoplastic lesion at risk for progression to carcinoma. For EC, however, few data are available concerning HPV status and integration patterns. The aim of the present study was to assess high-risk HPV infection and HPV18 DNA integration into the host cell genome in EC.

MATERIALS AND METHODS

Cell lines

EC109 cells, the human embryonic kidney (HEK) 293 cell line, and the HeLa cell line were maintained by our laboratory. HeLa cells served as the HPV18-positive control, and HEK293 cells served as the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-positive control. The cell lines were cultivated in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin, the concentration of each antibiotic was 100 U/mL) under standard conditions. Cells were maintained as a subconfluent monolayer at 37 ℃ in a humidified atmosphere of 5% CO_2/95% air. Exponentially proliferating cells were harvested with 0.25% trypsin and 0.02% EDTA, resuspended in fresh medium, and seeded in new flasks. The cells (2×10^6) were collected and washed with phosphate-buffered saline. Then, the cells were separated into two cryotubes, immediately frozen in liquid nitrogen, or stored at -70 ℃ until further use.

Detection of HPV DNA

Genomic DNA was isolated from each cell line using the Easy-DNA kit ("BioTake", China) according to the supplier's instructions. HPV DNA was detected by PCR with the general primer sets of My09/11 (My09: 5'-CGTCCMARRGGAWACTGATC-3', MY11: 5'-GCMCAGGGWCATAAYAATGG-3', amplicon size 450 bp) and GP5 +/6 + (GP5 +: 5'-TTTGTTACTGTGGTAGATACTAC-3', GP6 +: 5'-GAAAAATAAACTGTAAATCATATTC-3', amplicon size 150 bp) for the *HPV L1* gene[22] and type-specific primer sets for HPV18 E6 (forward: 5'-GCGCTTTGAGGATCCAACAC-3', reverse: 5'-ATTCAACGGTTTCTGGCAC-3', amplicon size 335 bp) and HPV18 E6-E7 (forward: 5'-AACACACCACAATACTATGGCGCG-3', reverse: 5'-GCATTTTCGTCCTCGTCATCTG-3', amplicon size 944 bp). The type-specific primer sets for HPV18 E6 and HPV18 E6-E7 were designed according to the GenBank-provided *HPV18* gene sequences of X05015 (http://www.ncbi.nlm.nih.gov/nuccore/X05015).

PCR was conducted in a final volume of 25 μL containing 1× PCR buffer ("BioTake", China), 0.2 mmol/L dNTPs, 1 μL complexed recombinant Taq DNA polymerase, 0.2 mmol/L of each primer, and 100 ng DNA. An initial 5-min denaturation step at 95 ℃ was followed by 30 amplification cycles of 30 s at 95 ℃, 30 s at 55 ℃,

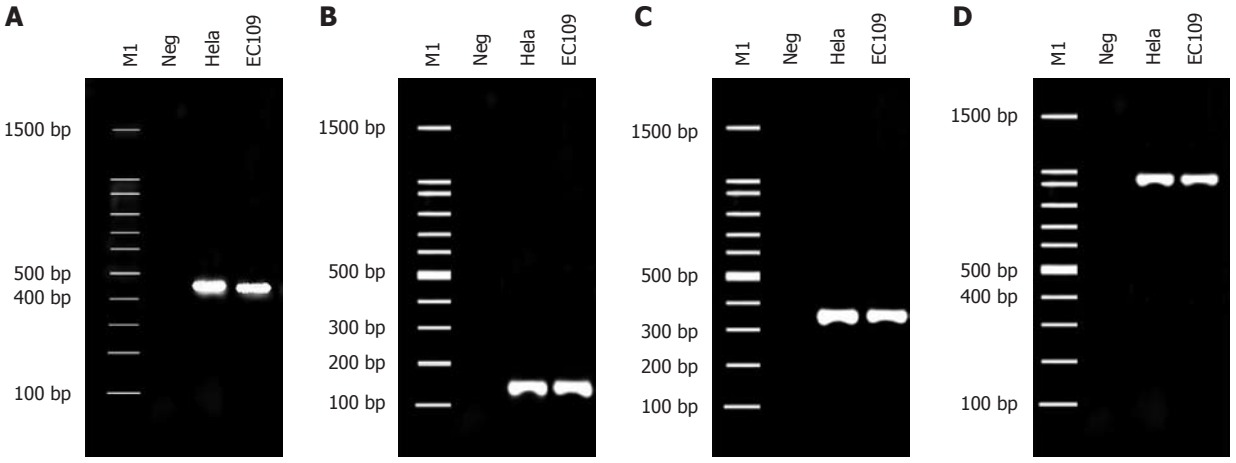

Figure 1 Detection of HPV18 DNA in EC109 cells. A-D: HPV18 DNA detection in EC109 cells using the primer pairs Y09/11, GP5 +/6 +, HPV18E6, and HPV18E6-E7, respectively. M1: 100 bp DNA ladder; Neg: Negative control (template of HEK 293 cell DNA); HeLa: Positive control (template of HPV18-infected cervical cancer cells; EC109: DNA from EC109 cells. HPV: Human papillomavirus; EC: Esophageal carcinoma.

and 1 min at 72 ℃, with a final extension step of 5 min at 72 ℃ using a block thermocycler (PeQLab Biotechnologie, Germany). A negative control (HEK293 cell DNA template) was included in each amplification step. DNA from HeLa cells was included as an HPV18-positive control. The PCR products were resolved on a 1.0% agarose gel.

Reverse transcription

Total RNA from the cell lines was isolated using the Micro-to-Midi Total RNA Purification System kit ("BioTake", China) according to the manufacturer's instructions. The RNA was quantified by spectrophotometry. Reverse transcription (RT) of mRNA isolated from EC109 cells was performed to produce a double-stranded DNA product that was then amplified by PCR. The final concentrations for the RT reaction were RNase-free H_2O (9.2 μL), 0.2 mmol/L dNTP mixture (1 μL), 10 pmol (dT)17-p3 (oligonucleotide primer: GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT; 1 μL), 5 × first-strand buffer ("BioTake", China; 4 μL), 200 U SuperScript reverse transcriptase ("BioTake", China; 1 μL), 40 U RNase inhibitor (1 μL), and total RNA (1-2 μg) in a total volume of 20 μL. The RNA in each reaction was reverse transcribed by heating at 42 ℃ for 50 min and inactivated by heating at 70 ℃ for 15 min. Samples were stored at 4 ℃.

To confirm that EC109 cells with detectable HPV18 E7 mRNA were indeed harboring HPV, mRNA for a human housekeeping gene was amplified by RT-PCR to ensure that mRNA isolated from EC109 cells was of sufficient integrity to be amplified by PCR. mRNA encoding human GAPDH was used as a target for the RT-PCR. PCR was carried out as described previously[19], using 10 pmol/L of each GAPDH primer (forward 5'-CATCACCATCTTCCAGGA-3'; reverse 5'-GTCTACCACCCTATTGCA-3') and 2 μL cDNA at a 52 ℃ annealing temperature for 30 s to generate a GAPDH product of 500 bp.

Detection of viral-cell fusion transcripts by nested PCR

HPV18 PCR reactions were prepared as described by Klaes *et al*[21] using forward primer p1-18 specific for HPV18 E7 (5'-TAGAAAGCTCAGCAGACGACC-3') and p3 (5'-GACTCGAGTCGACATCG-3') as reverse primer, 1 × buffer, 2.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 10 pmol/L primers, 1 U Ex Taq DNA polymerase, 3 μL cDNA product in a total volume of 25 μL. PCR was conducted as follows: 95 ℃ for 5 min, followed by 30 cycles of denaturation at 95 ℃ for 1 min, annealing at 56 ℃ for 1 min, and elongation at 72 ℃ for 3 min. The last cycle was followed by a final extension step at 72 ℃ for 5 min.

The amplification product (5 μL) was used for nested PCR under identical conditions using forward primers p2-18 specific for HPV18 E7 (5'-ACGACCTTCGAGCATTCCAGCAG-3') and $(dT)_{17}$-p3 as reverse primer, except that the annealing temperature was 67 ℃. The positions of the two primers were 814-835 for p1-18 and 830-853 for p2-18. To control for false-positives, a negative control (HEK293 cell DNA template) was included in each amplification. Electrophoresis was performed using a 1.2% agarose gel.

Cloning and sequence analysis

To confirm specific HPV18 oncogene transcription in EC109 cells, the final nested PCR products were cloned into a pMD-18T vector and sequenced to analyze the chromosomal location of HPV integration.

RESULTS

Detection of HPV DNA in EC109 cells

HPV18 DNA was detected in EC109 cells by PCR using the general primer sets of My09/11 and GP5 +/6 + for HPV L1 and the type-specific primer sets for HPV18 E6 and E6-E7 to generate products of 450 bp, 150 bp, 335 bp and 944 bp respectively (Figure 1).

Figure 2 Alignment sequencing results for esophageal carcinoma109 cells with human papillomavirus18. Query 288-388, sequencing results for esophageal carcinoma109 cells after polymerase chain reaction amplification with P2-18E7-specific primers; Sbjct 844-944, partial sequence of human papillomavirus18E7-E1 from X05015 in GenBank.

Figure 3 Alignment sequencing results for esophageal carcinoma109 cells with human chromosome. Query 227-483, sequencing results for esophageal carcinoma109 cells after polymerase chain reaction amplification with P2-18E7-specific primers; Sbjct 41562135-41562391, partial sequence of human chromosome 8.

HPV18 integration-derived transcript in EC109 cells

EC109 cells were used subsequently for HPV18 E7-specific nested PCR followed by gel electrophoresis. Approximately 600 bp of integrated HPV18-specific transcript was identified. The final nested PCR product of integrated HPV18 DNA was cloned into a pMD-18T vector and sequenced to analyze the chromosomal location of HPV integration. Sequence alignment showed that the HPV18 sequence from EC109 cells was identical to that of the encoded early protein E7-E1 of the standard HPV18 strain X05015 (Figure 2). Sequence alignment also showed that another partial gene sequence was identical to a partial sequence of human chromosome 8 (Figure 3).

DISCUSSION

The APOT assay is based on the structures of the 3' -ends of oncogenic HPV transcripts[21]. Integration of HPV genomes in carcinoma cells usually result in disruption of both the E1 and E2 open reading frames (ORFs), and transcripts derived from the integrated *E6* and *E7* oncogenes usually contain viral sequences at their 5' ends and flanking host cell-derived sequences at their 3' ends[21,23,24]. Klaes *et al*[21] applied the APOT assay to clinical samples of cervical dysplasia infected with HR-HPV types 16 and 18 and found a strong correlation between the detection of integration-derived transcripts and the stage of progression of the cervical dysplasia.

In the current study, HPV18 DNA and integration-derived transcripts were detected in EC109 cells, and HPV18 was found to be integrated into chromosome 8. Our results indicate that an episomal viral genome was broken for integration which usually leads to the disruption of the E1 and E2 ORFs. During the viral life cycle, E2-derived proteins act as important regulators of E6 and E7 ORF expression[24]. In most infected epithelia, E2 appears to inhibit transcription from E6 and E7 ORFs which helps to maintain the regulation of cellular proliferation[25,26]. Disruption of the E2 ORF with retention of the E6 and E7 ORFs could result in the unregulated expression of E6 and E7, which would lead to abrogation of cell-cycle activity and uncontrolled cell proliferation[27]. The high-risk HPV E6 and E7 gene are commonly integrated into the genome of cells in malignant tumors[28], when this occurs, longer incubation periods may be required for viral DNA integration into the appropriate host cell genomic location. When HPV DNA is integrated into the host nuclear genome, expression of *E6* and *E7* is elevated, and this leads to the occurrence of cancer[29]. After HPV infection of the esophageal epithelium, HPV DNA can be randomly integrated into human chromosomal DNA to produce a variety of genetic changes, leading to chromosomal instability and eventually malignant transformation.

COMMENTS

Background

Human papilloma virus (HPV) in patients with esophageal carcinoma (EC) has been studied previously, but the association of HPV with EC has not been firmly established. The authors hypothesized that integration of HPV DNA into host chromosomes is a critical step in the carcinogenesis of EC as a result of altered expression of two viral transforming genes, E6 and E7. The aim of this work was to study the relationship between HPV and esophageal tumors and to determine the chromosomal integration sites of HPV DNA in EC cells.

Research frontiers

EC is one of the major cancers in China, where the incidence and mortality rank first in the world. In 1982, Syrjanen hypothesized a relationship between

HPV infection and the development of EC. However, the role of HPV in the carcinogenesis of EC remains unclear. In this study, the authors demonstrate the integration of HPV DNA into host chromosomes, which could be a potential mechanism where by HPV infection leads to the development of EC.

Innovations and breakthroughs

Recent reports have highlighted the importance of HPV infection in EC. This paper is the first study to report that HPV18 integrated into one part of chromosome 8 in a cell line, EC109, derived from human EC cells. This study further suggests that HPV infection may be the cause of EC.

Applications

By understanding how EC is induced after HPV infection, this study may provide a future strategy for the diagnosis and prevention of EC.

Peer review

The authors examined HPV18 integration into one part of chromosome 8 in EC109 cells. Integration of the HPV genome into the host cell chromosome suggests that persistent HPV infection is a key factor in malignant cell transformation and carcinogenesis. The results may represent a molecular mechanism for the development of EC.

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CASE REPORT

Johanson-Blizzard syndrome

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Abstract

Johanson-Blizzard syndrome (JBS) is a rare autosomal recessive disease characterized by exocrine pancreatic insufficiency, hypoplastic or aplastic nasal alae, cutis aplasia on the scalp, and other features including developmental delay, failure to thrive, hearing loss, mental retardation, hypothyroidism, dental abnormalities, and anomalies in cardiac and genitourinary systems. More than 60 cases of this syndrome have been reported to date. We describe the case of a male infant with typical symptoms of JBS. In addition, a new clinical feature which has not previously been documented, that is anemia requiring frequent blood transfusions and mild to moderate thrombocytopenia was observed. A molecular study was performed which revealed a novel homozygous UBR1 mutation. Possible explanations for this new association are discussed.

Key words: Alae nasi aplasia; Anemia; Cutis aplasia; Exocrine pancreatic insufficiency; Johanson-Blizzard syndrome

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INTRODUCTION

Johanson-Blizzard syndrome (JBS) is a rare autosomal recessive disorder, first described in 1971 by Johanson and Blizzard[1]. The genetic defect causing the disease was unknown until 2005, when it was shown to result from mutations of the *UBR1* gene located on chromosome 15q15-21. UBR1 encodes one of at least four functionally overlapping E3 ubiquitin ligases of the N-end rule pathway, a conserved proteolytic system whose substrates include proteins with destabilizing N-terminal residues 20[2]. The precise pathophysiological link between altered protein degradation and the clinical anomalies observed in JBS remains to be determined.

The reported cases of JBS showed no difference in gender. Parental consanguinity is frequently observed. The typical clinical features of JBS are the following (with decreasing frequency): exocrine pancreatic insufficiency, hypoplasia/aplasia of the alae nasi, dental anomalies, congenital scalp defects, sensorineural hearing loss, growth retardation, psychomotor retardation, hypothyroidism, imperforate anus and genitourinary anomalies. A detailed list of observed clinical features is given in Table 1.

CASE REPORT

A 5-mo-old male infant of consanguineous Yemeni par-

Table 1 Clinical features of Johanson-Blizzard syndrome

Exocrine pancreatic insufficiency[1,3-5]
Hypoplasia/aplasia of alae nasi[1,4,6-8]
Scalp defect /aplasia cutis[1,6,8]
Sensory neural hearing loss[1,3,8,9]
Bilateral cystic dilation of cochlea, low set ears, and temporal bone defect[10]
Growth retardation, short stature[1,11]
Dental anomalies: oligodontia and absence of permanent teeth[1,6,7,11]
Anorectal anomalies: imperforate anus[4,11,12]
Hypotonia, microcephaly, and mental retardation sometimes normal intelligence[3,7,11]
lacrimal duct anomalies, coloboma of the lids, superior puncta absence, lacrimal cutaneous fistula, and congenital cataract[13]
Abnormal frontal hair pattern (upsweep)[7]
Vesicoureteric reflux, hypospadia, and duplex of uterine and vagina[8]
Congenital heart diseases such as myxomatous mitral valve, PDA, VSD, ASD, dextrocardia, complex congenital heart disease, and cardiomyopathy[13, 14]
Cholestatic liver disease (one case)[15]
Café au lait spots[16]
Hypothyroidism[1]
Growth hormone deficiency[5]
Hypopituitarism[17]
Impaired glucagon secretion response to insulin induced hypoglycemia[18]
Diabetes mellitus[19,20]

Italic letters show common features. PDA: Patent ductus arteriosus; VSD: Ventricular septal defect; ASD: Atrial septal defect.

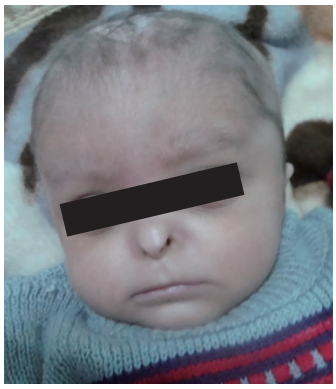

Figure 1 Typical facial appearance of this patient with Johanson-Blizzard syndrome, showing aplasia of alae nasi, scalp defect, and sparse hair.

ents was referred because of poor feeding. The history started when he was 2-mo old with recurrent attacks of pallor and edema in the feet and hands. In addition, the infant showed failure to thrive and greasy stools. He received 3 blood transfusions. There was a family history of two previous male siblings with the same facial features as the index case, who also received several blood transfusions and expired at 4 and 4 ½ mo, respectively

On physical examination, the patient was lethargic, hypotonic, and pale. His body weight was 3.3 kg (< 5% percentile), and body length was 52 cm (< 5% percentile). Head circumference was 37 cm (microcephaly), and the anterior fontanel was wide (6 cm × 3 cm). There was aplasia of the alae nasi, midline cutis aplasia and a small scalp defect on the occiput, the scalp hair was sparse with areas of alopecia (Figure 1), and eye lashes and eyebrows were sparse. Hypospadias was detected, and the anus was narrow and displaced anteriorly. There was also pitting edema on the feet and hands.

Routine laboratory tests revealed the following results: hemoglobin (Hb) was 4 g/dL, with reticulocytes 7%, mean corpuscular volume 85 fl and mildly decreased platelets (75 000/μL). Erythrocyte morphology showed anisocytosis and normochromia. Hb electrophoresis and bone marrow aspiration were normal (Table 2).

Serum pancreatic enzymes (amylase, lipase) were low. Total protein and albumin were low, and other liver function tests, renal function tests, serum electrolytes and blood sugar were within the normal range (Table 2).

Thyroid function tests revealed low free T4 and slightly increased thyroid-stimulating hormone. These results indicated hypothyroidism.

Echocardiography showed a small atrial septal defect. Whole body X-ray, abdominal ultrasound, brain computed tomography (CT) scan, and temporal bone CT scan were normal.

The patient received oral thyroxine, pancreatic enzyme replacement, multivitamins and strict monitoring to avoid complications. When the patient was seen last time at the age of 9 mo, his overall condition had significantly improved. He has gained weight, although still below the 3rd centile, and blood cell counts had normalized (Table 2). The child still showed muscular hypotonia and delay in motor milestones.

Genetic studies

After obtaining informed written consent from the parents for the genetic investigation, venous blood samples were taken from the index patient and his parents. DNA was extracted from blood leukocytes according to standard procedures. All 47 exons of the *UBR1* gene including the flanking intronic regions were analyzed by direct bidirectional sequencing as described previously[19]. Sequencing in the index patient revealed a homozygous mutation in exon 19. The nucleotide substitution c.2089 C > T predicts a missense change (p.S700P) affecting an

Table 2 Laboratory results of the patient at 5 mo and at 9 mo of age

	At presentation (5 mo)	At 9 mo	Normal range
Hemoglobin	4 g/dL Post transfusion: 12 g/dL,	11.7g/dL	10.5-12
Retics count	7%	1.20%	0.2-2%
MCV	85	85	70-86 fl
Leukocyte count	5600	16 000	6000-17 500/mm^3
Platelets	192 000, 79 000, 100 000	390 000	150 000-400 000/mm
RBC blood morphology	Anisocytosis and normochromia	Anisocytosis and normochromia	
Total bilirubin	3.5	3	0-24 mmol/L
Direct bilirubin	1.5	1.13	0-5.1 mmol/L
ALT	44	39	0-41 U/L
AST	45	55	0-35 U/L
ALP	235	410	180-1200 U/L
Total protien	35	56	60-87 g/L
Albumin	17.3	38.8	34-48 g/dL
Urea	1	1.7	3.3-6.4 mmol/L
Creatinine	13	19	62-106 mmol/L
Pancreatic amylase	2.96	15	13-53 U/L
Pancreatic lipase	10	20	13-60 U/L
FT4	9.28	11.55	13.9-26.1 pmol/L
TSH	17.8	6.43	1.4-8.8 ulU/mL
Serum iron	88.39	157	60-170 mg L/dL
Total iron binding capacity	120	106	100-400 mg/dL
Serum ferritin		1260 (repeated BT)	7-140 ng/mL
Serum folate level		20	15-55 ng/mL
Serum vitamin B12 level		252	197-866 pg/mL
Insulin		0.2	2.6-24.9 Uu/mL
Bone marrow results (normal)	Erythropoiesis, granulopoiesis, and lymphopoiesis are normal cellularity and maturity and megakaryocytes are present	The same result	
Hemoglobin electrophoresis	Normal		

MCV: Mean corpuscular volume; RBC: Red blood cell; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; FT4: Free Thyroxine; TSH: Thyroid-stimulating hormone; BT: Blood transfusion.

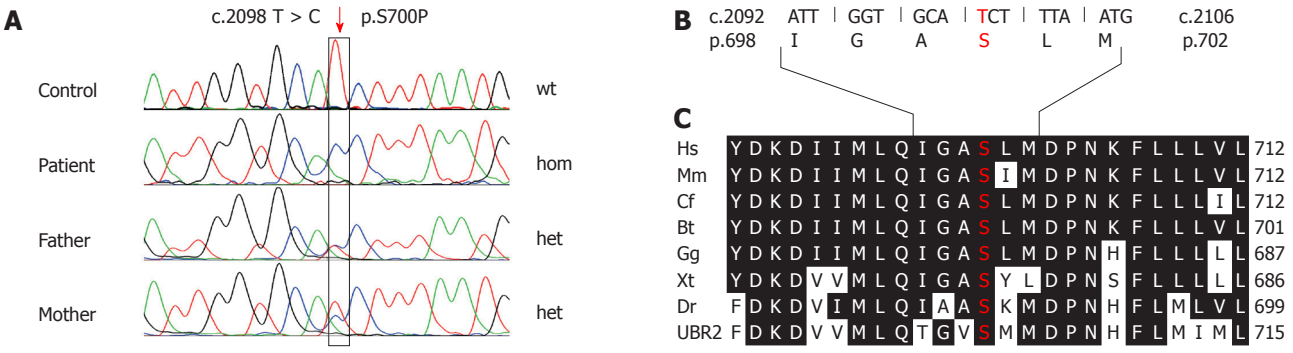

Figure 2 Demonstration and characterization of the familial mutation. A: Comparison of electropherograms around the T > C transition at position c.2098; B: Nucleotide and amino acid sequence; C: Multiple protein alignement (http://www.ebi.ac.uk/Tools/msa/clustalw2) of vertebrate UBR1. Black shading indicates identical residues. wt: Wild-type; Hom: Homozygous; Het: heterozygous; Hs: Homo sapiens; Mm: Mus musculus; Cf: Canis familiaris; Bt: Bos taurus; Gg: Gallus gallus; Xt: Xenopus tropicalis; Dr: Danio rerio; UBR2: Human UBR2 protein.

amino acid residue that is 100% conserved throughout vertebrate UBR1 and UBR2 proteins (Figure 2). To date, this change has not been known as a mutation or polymorphism. PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) predicts that this mutation is probably damaging with a score of 0.992 (sensitivity: 0.59; specificity: 0.96). Both parents were found to be heterozygous for the mutation. Based on this evidence we regarded p.S700P as the disease-causing mutation in this family.

DISCUSSION

JBS is a rare autosomal recessive disorder that affects many systems with a wide range of congenital abnormalities. A small beak-like nose (due to aplasia or hypoplasia of the alae nasi), and exocrine pancreatic insufficiency are considered the most consistent manifestations, while others features (Table 1) occur at varying frequencies in the affected patients. The patient presented here had typ-

ical facial features and exocrine pancreatic insufficiency, this combination is pathognomonic for JBS. Our patient also presented with scalp defects, developmental delay, and generalized hypotonia, which have been described in reported cases of JBS. Remarkably, our patient presented with an additional phenotypic feature, namely significant anemia, and required frequent blood transfusions from the age of 2 mo. The hematologic abnormalities also included thrombocytopenia and mild leukopenia. No definite etiology could be established. This feature has not been described in previous reports of JBS. Remarkably, two previous male siblings, who were assumed to have the same disease based on the report of similar facial features, also had significant anemia (Hb: 4 g/dL) and received frequent blood transfusions.

In addition, there was also mild to moderate thrombocytopenia in the other affected children of this family. The unusual and consistent association of JBS with a hematologic phenotype in this family may raise different speculations, such as a second autosomal recessive condition that might segregate JBS in this family or a specific function of the UBR1 protein carrying the novel missense mutation.

In infants, anemia caused by iron, vitamins and trace element deficiencies are unusual before the age of 6 mo, but in this patient the nutritional consequences of malabsorption might have appeared earlier due to many factors such as low birth weight, malnutrition in the mother and hypothyroidism in which normochromic, normocytic anemia may be secondary to reduced red blood cell production and reduced red cell survival.

Although we cannot exclude these possibilities, the fact that the hematologic disease resolved after efficient pancreatic enzyme and vitamin supplementation suggests a major contribution of malnutrition.

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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