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[2]Passed away on June 11, 2007
[3]Passed away on June 14, 2008

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Contents

Contents

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Contents

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EDITORIAL

A systematic review of the efficacy and safety of herbal medicines used in the treatment of obesity

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Abstract

This review focuses on the efficacy and safety of effective herbal medicines in the management of obesity in humans and animals. PubMed, Scopus, Google Scholar, Web of Science, and IranMedex databases were searched up to December 30, 2008. The search terms were "obesity" and ("herbal medicine" or "plant", "plant medicinal" or "medicine traditional") without narrowing or limiting search elements. All of the human and animal studies on the effects of herbs with the key outcome of change in anthropometric measures such as body weight and waist-hip circumference, body fat, amount of food intake, and appetite were included. *In vitro* studies, reviews, and letters to editors were excluded. Of the publications identified in the initial database, 915 results were identified and reviewed, and a total of 77 studies were included (19 human and 58 animal studies). Studies with *Cissus quadrangularis* (CQ), *Sambucus nigra*, *Asparagus officinalis*, *Garcinia atroviridis*, ephedra and caffeine, Slimax (extract of several plants including *Zingiber officinale* and Bofutsushosan) showed a significant decrease in body weight. In 41 animal studies, significant weight loss or inhibition of weight gain was found. No significant adverse effects or mortality were observed except in studies with supplements containing ephedra, caffeine and Bofutsushosan. In conclusion, compounds containing ephedra, CQ, ginseng, bitter melon, and zingiber were found to be effective in the management of obesity. Attention to these natural compounds would open a new approach for novel therapeutic and more effective agents.

Key words: Animal; Herbal medicine; Human; Obesity

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INTRODUCTION

The prevalence of obesity is increasing worldwide[1] resulting in an association with major health problems such as type 2 diabetes, ischemic heart disease, stroke, and cancer. It is necessary to treat obese individuals by both lifestyle interventions and/or pharmacological therapy. Pharmacologic treatment and surgical interventions used in some circumstances are not always appropriate[2]. Unfortunately, drug treatment of obesity despite short-term benefits, is often associated with rebound weight gain after the cessation of drug use, side effects from the medication, and the potential for drug abuse[3]. Pharmacologic options include sibutramine, orlistat, phentermine, diethylpropion, and fluoxetine or bupropion. Phentermine and diethylpropion have potential for abuse and are only approved for short-term use. Approved medications for long term use in the treatment of obesity are sibutramine and orlistat, however, these agents should be used with caution in patients with a history of cardiovascular disorders[4]. The general public uses many other methods for weight

loss including herbs, vitamins, nutritional supplements, and meal replacement preparations. Rigorous scientific studies have not been carried out on these products, and in many cases safety and efficacy take a back seat to marketing.

Complementary and alternative therapies have long been used in the Eastern world but recently these therapies are being used increasingly worldwide[5]. When conventional medicine fails to treat chronic diseases and conditions such as obesity efficaciously and without adverse events, many people seek unconventional therapies including herbal medicine[6]. Although the number of randomized trials on complementary therapies has doubled every 5 years and the Cochrane library included 100 systematic reviews of unconventional interventions[7], none of these studies specifically mentioned herbal therapy in obesity.

This review aimed to evaluate the current science on the efficacy and safety of herbal medicines in the management of obesity.

DATA SOURCES AND STUDY SELECTIONS

PubMed, Scopus, Google Scholar, Web of Science, and IranMedex databases were searched up to December 30, 2008 for all human and animal studies investigating the effects (both harmful and beneficial) of treating obesity with herbal medicines. The search terms were "obesity" and ("herbal medicine" or "plant", "plant medicinal" or "medicine traditional") without narrowing or limiting search elements. Only publications with available abstracts were reviewed. The main outcome measures sought at the end of treatments as anti-obesity effects, were body weight, body fat including fat mass/fat weight or fat percentage/visceral adipose tissue weight, triceps skin fold thickness, waist or hip circumference, and appetite or amount of food intake.

Herbal medicines are defined in this review as raw or refined products derived from plants or parts of plants (e.g. leaves, stems, buds, flowers, roots, or tubers) used for the treatment of diseases. The synonyms of herbal medicines are herbal remedies, herbal medications, herbal products, herbal preparations, medicinal herbs, and phytopharmaceuticals, *etc.*

All of the abstracts from human and animal studies with the main outcome of change in anthropometric measures such as body weight and waist-hip circumference, body fat (weight or mass of visceral adipose tissue, fat mass or percent), amount of food intake, and appetite in participants were included. Even studies on other relevant diseases such as diabetes were also reviewed and included if the appropriate outcomes were shown. *In vitro* studies, review articles, and letters to the editor were excluded. Unpublished data such as theses were also excluded. Two reviewers independently examined the title, abstract and references of each article meeting the inclusion criteria and eliminated duplications and those showing exclusion criteria.

FINDINGS

Of the publications identified from the initial database search, 915 results were identified and reviewed for inclusion or exclusion. A total of 77 studies were included (19 human and 58 animal studies). Human studies included 17 randomized clinical trials (RCTs) and two before-after clinical trials[8-26]. RCTs reported random allocation of humans to herbal medicines *vs* (placebo/another plant/combination of plants) with or without specific dietary and exercise programs outlined in Tables 1 and 2 as weight loss programs. Human subjects were healthy overweight, obese or with impaired glucose tolerance test volunteers. Animal studies included healthy, genetically or experimentally obese or diabetic mice, rats and other rodents. The route of administration of herbs in almost all studies was oral intake with the exception of some animal studies as indicated in Table 2.

HUMAN STUDIES

Change in human body weight

All studies showed loss of body weight except one[21] which seemed to have problems with the study design, and one other study[10] which showed a significant decrease only in body fat. Studies with *Cissus quadrangularis* (CQ)[26] or combined with *Irvingia gabonensis* (IG)[15], a combination of *Sambucus nigra* and *Asparagus officinalis*[16], calcium hydroxycitrate in *Garcinia atroviridis*[18], supplements containing ephedra and caffeine[9,13,20], and Slimax as an extract of several plants including *Zingiber officinale*[8] and Bofutsushosan[14] showed significant decreases in body weight.

Body fat

A significant decrease in body fat was shown with CQ[26], supplements containing ephedra and caffeine[9,13], a natural compound containing capsicum and some lipotropic nutrients[10], Bofutsushosan[14], and calcium hydroxycitrate in *Garcinia atroviridis*[18]. These phytopharmaceuticals showed a significant decrease in triceps skin fold thickness indicating significant loss of fat.

Waist and hip circumference

Efficient decreases in both waist and hip circumferences in trials with a supplement containing ephedra and caffeine[9] and Slimax (extract of several plants including *Zingiber officinale*[8] were shown whereas *Caralluma fimbriata*[19] and CQ with or without IG[15] significantly decreased waist size.

Food intake

Decreases in appetite or amount of food or energy intake with a supplement containing ephedra and caffeine[20] and *Caralluma fimbriata*[19] were shown (not significant) but hydroxycitric acid (HCA-SX) with or without *Gymnema sylvestre*[23] decreased the amount of food intake efficiently. A natural compound containing capsicum and other lipotropic nutrients[10] did not significantly change energy intake.

Table 1 Human studies considering the anti-obesity effects of herbal medicines

Authors	Target	Herbs (scientific name)	Study	Dose/duration	Groups	Main outcome	Other relevant effects & complications
Ignjatovic *et al*[8] 2000	Healthy volunteers	Slimax: extract of several plants: Hordeum vulgare, Polygonatum multiflorum, Dimocarpus longan, Ligusticum sinense, Lilium brownie, and *Zingiber officinale*	RCT	6 wk	C: Placebo I: Compound	Sig. decrease in body wt. & waist & hip Cir. & BMI	Modification of lipid metabolism with sig. effect on the accumulation & the release of lipid from adipose tissue
Boozer *et al*[9] 2001	Over wt. (n = 35)	An herbal supplement: (*Ma Huang* & Guarana)	RCT (double-blind)	72 mg (ephedra) 240 mg (caffeine)/8 wk	C: Placebo (n = 24) I: Compound (n = 24)	Sig. decrease in body wt. & total body fat & sig. greater reduction in hip & waist Cir.	Greater reduction in serum TG, potentially treatment-related dropouts (23%) in the active group and none in the placebo group. Dry mouth, insomnia & headache were reported
Hoeger *et al*[10] 1998	Healthy	A natural dietary compound of chromium picolinate, inulin, capsicum, L-phenylalanine, and other lipotropic nutrients	RCT (double-blind)	4 wk	C: wt. loss program (n = 67) I: wt. loss program + compound (n = 56)	Sig. decrease in body fat percent, fat mass & FFM, but no sig. difference in body wt. BMI and energy intake	
Ziauddin *et al*[11] 2004	Hhyperlip-idemic (n = 30)	Terminalia arjuna Roxb	Before-after CT			Sig. improvement in obesity. Reduction in body wt. in some cases	Sig. decrease in serum total lipid levels. Sig. relief of palpitation, dyspnea, chest & joint pain. Reduction in BP in some cases
Abidov *et al*[12] 2006	Obese non-diabetic women (n = 32)	A compound of Aralia mandshurica (A) and Engelhardtia chrysolepis (E) extracts named ARALOX	RCT	450 mg (A) & 450 mg (E)/d	C: Diet + placebo I: Diet + compound	Decrease in total body wt. & fat wt.	Reduction in perilipin content in adipocytes and plasma TG. Stimulate activity of hormone sensitive lipase
Greenway *et al*[13] 2004	Human (obese & over wt.) healthy	Herbal supplement containing caffeine and ephedra	RCT (double-blind)	210 mg (e) & 72 mg (c)/12 wk	C: Placebo I: Compound	Sig. decrease in body wt. & the percentage of fat	No differences in lipid levels, or BP were shown. No serious adverse effect
Hioki *et al*[14] 2004	Obese women with IGT (n = 80)	Bofu-tsusho-san containing (Ephedrae Herba, Glycyrrhizae Radix, Forsythiae Fructus, Schizonepetae Spica &…)	RCT (double-blind)	Equivalent of (24 mg/ephedrine & 280 mg caffeine/24 wk)	C: wt. loss program I: wt. loss program + compound	Compared to baseline the I group lost significantly more body wt. & abdominal visceral fat & the placebo group lost sig. body wt. & had no sig. change in abdominal visceral fat	No decrease in RMR. Sig. improvement in insulin resistance compared to week 0. Loose bowel movements resulted in three withdrawals
Oben *et al*[15] 2008	Human (obese & over wt.)	A combination of *Cissus quadrangularis* (CQ) & *Irvingia gabonensis* (IG)	RCT (double-blind)	300 mg (CQ) & 500 mg (IG) per day/10 wk	C: Placebo I: CQ CQ + IG	Sig. decrease in body wt. & body fat percent & waist size in both I groups but the combination group (CQ + IG) resulted in larger reductions	Sig. decrease in Chol & LDL of plasma and fasting blood glucose levels
Chrubasik *et al*[16] 2008	Healthy (n = 80)	A combination of *Sambucus nigra* (S) and *Asparagus officinalis* (A)	Before-after CT	(S): 1 mg anthocyanin, 370 mg flavonol, 150 mg hydroxycinnamate (A): 19 mg saponin per day	-	Sig. decrease in mean of the wt.	Sig. improvement of BP, physical and emotional well-being and quality of life

Udani *et al*[17] 2007	Healthy (*n* = 25)	Proprietary fractionated white bean extract	RCT (double-blind)	2000 mg/14 wk	C: Placebo + wt. loss program I: Extract + wt. loss program	In both groups, decrease in body wt. & waist size from baseline was sig. but no sig value between groups	There were no adverse effect
Roongpisu-thipong *et al*[18] 2007	Obese women	Calcium hydroxycitrate in *Garcinia atroviridis*	RCT	2 mo	C: Diet I: Diet + extract	Sig. decrease in body wt. & greater reduction in BMI. Sig. decrease in the triceps skin fold thickness	
Kuriyan *et al*[19] 2007	Over wt. (*n* = 50)	Caralluma fimbriata	RCT	1 g/60 d	C: wt. loss program I: wt. loss program + extract	Sig. decrease in waist Cir. & hunger levels. Greater decrease in body wt., BMI, hip Cir., body fat & energy intake but not sig.	
Hackman *et al*[20] 2007	Obese & over wt. women (*n* = 41)	Multinutrient supplement containing ephedra (e) and caffeine (c)	RCT (double-blind)	40 mg (e) and 100 mg (c)/9 mo	C: Control supplement I: Multinutrient supplement	Sig. decrease in body wt. decrease in appetite	Sig. decline in serum chol, TG, glucose, fasting insulin & leptin levels & minor adverse effects like dry mouth, insomnia, nervousness and palpitation were reported
Garrison *et al*[21] 2006	Over wt. women	Proprietary extracts of Magnolia officinalis and phellodendron amurense	RCT	750 mg/6 wk	C: Placebo I: Extract	No sig. wt. gain for the I group but sig. wt. gain in C. groups	The I groups tended to have lower levels of cortisol in the evening
Coffey *et al*[22] 2004	Human (over wt. & obese) (*n* = 102)	Product containing ephedrine, caffeine & other ingredients.	RCT (double-blind)	12 wk	C: Placebo I: Compound	Additional wt. loss (1/5 kg) & greater reduction in BMI & waist Cir. No difference in body fat & fat mass percent was shown	No difference in pulse, diastolic & systolic BP & adverse events
Preuss *et al*[23] 2004	Obese (*n* = 60)	Hydroxycitric acid (HCA -SX) and a combination of HCA-SX and niacin-bound chromium (NBC) and Gymnema sylvestre extract (GSE)	RCT (double-blind)	HCA-SX: 4667 mg GSE: 400 mg NBC: 4 mg/8 wk	C: Placebo I1 = HCA-SX I2 = GSE + NBC + HCA-SX All groups had wt. loss program	5%-6% decrease in body wt. & BMI & sig. decrease in food intake in both I groups	Sig. decrease in serum lipids & leptin & increase in HDL & excretion of urinary fat metabolites in both I groups. There were mild adverse effects but not significant between groups
Udani *et al*[24] 2004	Obese (*n* = 24)	A proprietary fractionated white bean (Phaseolus vulgaris)	RCT (double-blind)	3000 mg/8 wk	C: Placebo I: Extract	Decrease of body wt. with 129% difference	Reduction of TG three times greater than C. group. No adverse effect was shown
Bhatt *et al*[25] 1995	Healthy (*n* = 58)	Guggulu (Medohar)	RCT	1/5, 3 g/30 d	C: wt. loss program I: wt. loss program + extract	Higher mean wt. reduction in I group. In I group, all patients > 90 kg lost wt. but 3 in C group did not lose wt.	
Oben *et al*[26] 2007	Over wt. & obese	*Cissus quadrangularis*	RCT (double-blind)	300, 1028 mg	C: Placebo I: Two extract formulation: CQR-300, CORE	Sig. decrease in body wt & body fat	Sig. decrease in serum lipids and glucose. Sig. increase in HDL-C plasma 5-HT and creatinine levels

Cir: Circumference; BP: Blood pressure; BMI: Body mass index; sig.: Significant; C: Control; I: Intervention; RCT: Randomized control trial; CT: Clinical trial; TG: Triglyceride; HDL: High density lipoprotein; LDL: Low density lipoprotein; Chol: Cholesterol; IGT: Impaired glucose tolerance.

Other effects

Anti-hyperlipidemic, antihyperglycemic, and other relevant anti-obesity effects of medicinal plants in human studies are summarized in Table 1.

Adverse effects

No significant adverse effects compared to controls were mentioned and no mortality was reported, except in studies with supplements containing ephedra and

Table 2 Animal studies on the anti-obesity effects of herbal medicines

Authors	Target	Herbs (scientific name)	Dose/duration	Groups	Main outcome	Other relevant effects & complications
Wang *et al*[27] 2000	Rat (obese)	Haidonghua powder: Laminaria japonica Aresch & Benincasa hispida (Thunb.) Cogn. *etc*	(2.5 g/kg)	-	Sig. decrease in Lee's index & size of fat cells	Did not influence the function of thyroid gland & metabolism of water & salt
Jeon *et al*[28] 2003	Mouse	Rhus vemiciflua Stokes	8 wk	C: HFD I: HFD + extract	Sig. suppression of body wt. gain and lower wt. of subcutaneous adipose tissue	Lowered plasma TG
Alarcon-Aguilar *et al*[29] 2007	Mouse	Hibiscus sabdariffa	120 mg/kg 60 d	C: Healthy & obese (by MSG) + placebo I: Same groups + extract	Sig. decrease in body wt. gain in obese mice & increased liquid intake in both groups	No sig. change in TG & Chol levels. Increase in ALT levels was shown but was not sig.
Urías-Silvas *et al*[30] 2008	Mouse	Fructans extracted from Agave tequilana (TEQ) and Dasylirion spp (DAS)	10% supplement	C: STD I: STD + Raftilose/ DAS/TEQ	Sig. decrease in body wt. gain & food intake. The (TEQ) group had the lowest value	Lower serum glucose & Chol level but Sig. decrease in TG levels was shown in Raftilose group. Higher concentration of GLP-1 & it's precursor & proglucagon mRNA in I groups
Park *et al*[31] 2007	Rat	Platycodon grandiflorum	150 mg/kg 7 wk	C: NLD/HFD I: Same groups + extract	Sig. decrease in body wt & subcutaneous adipose tissue wt. & adipocytes size in I group	Sig. decrease in plasma TG & Chol concentrations, up-regulation of FABP mRNA expression induced by HFD
Jongwon *et al*[32] 2005	Rat (obese by HFD)	Allium victorialis var. platyphyllum leaves	100 mg/kg 2 wk	-	Considerable reduction of retroperitoneal, epididymal and total abdominal fat pad wt.	Sig. decrease in hyperlipidemia and increased lipid content in feces
Kobayashi *et al*[33] 2001	Rat	Evodiamine an alkaloid of a fruit: Evodia rutaecarpa	0/02%, 0/03% of the diet 12 wk	C: Control I: Extract	Sig. decrease in perirenal fat wt. & decrease of epididymal fat mass	Sig. decrease of lipid in liver & serum FFAs. Sig. increase of lipolytic activity in perirenal fat tissue & specific GDP binding in brown adipose tissue mitochondria as the biological index of heat production
Jin *et al*[34] 1994	Rat	Jiang-zhi jian-fei yao: the refined Rhubarb	Injected intragastrically		No sig. increase in body wt. but reduction of food intake. Decreased size of abdominal adipose cells	Prolongation of stomach evacuation time and acceleration of intestinal movements
Kim *et al*[35] 2008	Rat	Juniperus chinensis	1% supplement /79 d	C: NLD/HFD I: HFD + extract	Sig. decrease in body wt gain & visceral fat pad wt.	Sig. decrease in blood lipid, leptin & insulin levels. Sig. reversal of the down-regulation of genes implicated in adipogenesis & increased gene expressions & phosphorylations related to FABO
Shih *et al*[36] 2008	Mouse (obese by HFD)	Momordica charantia (bitter melon)	4 wk	C: Control I: Rosiglitazone/ extract	Sig. decrease in epididymal white adipose tissue wt. & visceral fat wt.	Sig. improvement in blood glucose, leptin, and FFA. Influenced PPARα/ PPARγ expression
Pang *et al*[37] 2008	Rat (obese by HFD)	Ilex paraguariensis			Sig. decrease in body wt. of visceral fat-pad wt.	Sig. decrease in blood and hepatic lipid, glucose, insulin and leptin levels. Reversed the down-regulation of genes implicated adipogenesis, thermogenesis & enhanced expression of uncoupling proteins in adipose tissue
Bruno *et al*[38] 2008	Mouse	Green tea	0%, 1%, 2% (wt.:wt.)/6 wk	C: Obese/lean I: Same groups + extract	Sig. decrease in body wt. of both I groups	In obese I group, sig. decrease in hepatic steatosis was observed dose dependently. Liver enzymes decreased. 30%-41% and 22%-33% lower serum ALT and AST activities were shown, respectively

Lee *et al*[39] 2008	Mouse	A combination of Morus alba, Melissa officinalis and Artemisia capillaries	12 wk	-	Sig. decrease in body wt. gain & adipose mass	Decreased serum levels of TG, Chol & inhibited hepatic lipid accumulation, and increased hepatic mRNA levels of enzymes responsible for FABO
Choi *et al*[40] 2008	Mouse (obese by HFD)	Cucurbita moschata	500 mg/kg 8 wk	_	Sig. suppression of body wt. & fat storage increase but amount of food intake was not affected	
Huang *et al* 2008[41]	Rat	Momordira charantia L. (Bitter melon)	5%	C: HFD I1: HFD + plant I2: HFD + thiazolidinedione	Sig. decrease in the number of large adipocytes in both I groups. Sig. decrease in adipose tissue mass in I_1 group compared to I_2 group	Sig. decrease in enzymes of adipose tissue implicating reduction of insulin resistance in I group as compared to C group
Lemaure *et al*[42] 2007	Rat (obese)	Cyperus rotundus L. tubers	45, 220 mg/kg 60 d	_	Sig. decrease in wt. gain without affecting food consumption	
Lei *et al*[43] 2007	Mouse	Pomegranate leaf	400/800 mg per kilogram 5 wk	C: HFD/NLD I: Same groups + extract	Sig. decrease in body wt. & energy intake and adipose pad wt. percents in I. group. Sig. decrease in appetite of obese mice on NLD was shown	Sig. decrease in serum TG, Chol, glucose levels & Chol/ HDL ratio, inhibition of intestinal fat absorption
Aoki *et al*[44] 2007	Mouse (obese by HFD)	Licorice flavonoids oil (LFO)	0/5%, 1%, 2% 8 wk	C: Placebo I: Extract	Sig. decrease of abdominal white adipose tissue & body wt. gain with 1% & 2% LFO groups, decrease of adipocyte size	Improvement of fatty degeneration of hepatocytes and changes in genes implicating regulation of lipid metabolism with 2% concentration
Oluyemi *et al*[45] 2007	Rat	Garcinia cambogia seed (bitter cola)	200, 400 mg/kg 5 wk	C: Placebo I: Extract	Sig. decrease in body wt.	Sig. decrease in TG pool of adipose tissue & liver but sig. increase of HDL & decreased LDL
Han *et al*[46] 2006	Mouse (obese by HFD)	Kochia scoparia	1%, 3%/3 d	-	Prevented the increases in body & parametrial adipose tissue wt.	Sig. increase the fecal content & fecal TG levels in day 3
Goyal *et al*[47] 2006	Mouse (obese gold thioglucose)	*Zingiber officinale*	250 mg/kg 8 wk	C: Placebo I: Extract	Sig. decrease in body wt.	Sig. decrease in serum Chol, TG, glucose, and insulin
Kishino *et al*[48] 2006	Rat and mouse	Salacia reticulata	0/5% 8 wk in mice 0/2% 35 d in rats	C: HFD I: HFD + plant	Sig. decrease in the body wt. and visceral fat mass increase	Sig. decrease in plasma TG, 4 h after ingestion; Sig. decrease in energy efficiency, plasma leptin and adiponectine levels
Jayaprakasam *et al*[49] 2006	Mouse	Cornelian cherry (cornus mas) (Purified anthocyanins (A) & ursolic acid (u)	1 g/kg (A), 500 mg/kg (u) 8 wk	C: HFD I: HFD + A/A + u	24% decrease in wt. gain in (A) group	Elevated insulin levels; Sig. decrease of liver TG in A + u group
Moreno *et al*[50] 2006	Rat	Arachis hypogaea nutshell	1% (wt:wt) /12 wk	C: HFD I: HFD + extract	Sig. decrease in body wt. gain and liver size	Increased fecal lipid excretion. Reduced TG content of liver and serum glucose and insulin
Galisteo *et al*[51] 2005	Rat (obese)	Plantago Ovata	3/5% 25 wk	C: STD I: STD + extract	Sig. decrease in body wt. gain	Sig. improvement of lipid profile, FFA & insulin & TNF-α & hypoadinectinemia
Zhao *et al*[52] 2005	Mouse (obese by hyperalimentation)	Phillyrin (Fructose forsythia)			Sig. decrease in wet wt. of fat & fat index & diameter of fat cells & lee index	Decrease in jejunum microvillus area, and serum levels of TG & Chol
Chen *et al*[53] 2005	Rat	Bitter melon (Momordica charantia)	0/75% or 7/5 g per kilogram 7 wk	C: LFD/HFD I: LFD/HFD + extract	Lower energy efficiency and visceral fat mass after 4 wk in I group	Reduced plasma glucose and hepatic TG but higher serum FFA after 4 wk; Higher plasma catecholamine after 7 wk in I group; Sig. decrease in hepatic TG & steatosis and sig. increase of serum epinephrine & FFA in HFD group of I

Han *et al*[54] 2005	Rat	Coleus forskohlii	50 g/kg	C: Sham operated/ ovariectomized + control diet I: Same groups + extract	Reduced body wt.& food intake & fat accumulation	
Han et al[55] 2005	Mouse	Chikusetsu saponins isolated from Panax japonicus rhizomes	1%, 3%/9 wk	C: HFD I: HFD + extract	Prevented body wt. gain & increase of parametrial adipose tissue wt.	Sig. increase of the fecal content & TG level in day 3; reduction of plasma TG 2 h after oral lipid intake & inhibition of pancreatic lipase activity
Han *et al*[56] 2005	Mouse	*Zingiber officinale* Roscoe	1%, 3%/8 wk	C: HFD I: HFD + plant	Sig. decrease in body wt. gain at 2-8 wk with 3% & in final parametrial adipose tissue wt. with 1% concentration	
Cha *et al*[57] 2004	Mouse	Acanthopanax senticosus	0/5 g per kilogram 12 wk	C: NLD/HFD I: NLD/HFD + extract	HFD group of I had lower wt. gain but no difference in food consumption was shown	In HFD group of I, lower serum LDL and restoration of liver TG at the same level as fed by LFD was shown; No alteration in carnitine status
Kim *et al*[58] 2005	Rat	Crude saponin of Korean red ginseng	200 mg/kg 3 wk, ip	C: NLD/HFD I: NLD/HFD + extract	Reduced body wt., food intake & fat content in HFD group of I similar to those fed with NLD	Reduction of hypothalamic NPY expression and serum leptin level in HFD group of I
Yun *et al*[59] 2004	Mouse	Wild Ginseng	250, 500 mg/kg	C: HFD I: HFD + extract	Sig. inhibition of body wt. gain dose dependently. Decrease of white & brown adipocytes diameters	Sig. inhibition of FBG, TG, and FFAs dose-dependently; insulin resistance improved
Junbao *et al*[60] 2004	Rat (obese)	Semen cassiae	6%	-	Sig. decrease in body wt. & lee index	Reduction of fasting serum TG, insulin & malondialdehyde
Kim *et al*[61] 2004	Rat	Adlay seed (CoixLachrymajobi var. mayuen)	50 mg/100 g of body wt.	C1: NLD C2: HFD + saline (sham group) I: HFD + plant	Sig. decrease in body wt. & food intake & epididymal and peritoneal fat & white adipose tissue size as compared to sham group	Increase of brown adipocytes as compared to NLD group but not significant
Kwon *et al*[62] 2003	Rodent	Dioscorea nipponica Makino	5%/8 wk	C: HFD I: HFD + plant	Sig. decrease in body wt. & adipose gain	Suppression of time dependent increase of serum TG level after lipid intake
Lu *et al*[63] 1999	Rat (obese by hyperalimentation)	Inspissation tea (Guangdong kudingcha)		C: Control I1: Extract I2: Fenfluramine	Stronger modulation on lymphocytes hypertrophy and quantity was shown in I1 group	Only fenfluramine showed sig. difference in small intestine villus model
Yoshikawa *et al*[64] 2002	Rat (obese)	Salacia reticulata	125 mg/kg 27 d		Suppression of body wt. and periuterine fat storage increase in female rats but no effect on male rats	
Xie *et al*[65] 2002	Mouse	Ginseng berry	150 mg/kg 12 d, ip	C: Diabetic/lean diabetic + placebo I: Same groups + extract	Sig. decrease in body wt. as compared to day 0 in diabetic group of I. wt. loss in lean mice was shown	Sig. increase in glucose tolerance in diabetic mice but no sig. decrease of FBG in lean mice.
Yamamoto *et al*[66] 2000	Rat	CT-Ⅱ, an extract from Nomame Herba	8 wk, 12 wk, 6 mo	C: Lean/obese + HFD I: Same groups + HFD + plant	Sig. inhibition of body wt. gain dose dependently without affecting food intake in lean rats after 12 wk. Sig. decrease in body wt. gain in obese mice after 24 wk	Sig. inhibition of TG elevation
Han *et al*[67] 1999	Mouse	Oolong tea	10 wk	C: HFD I: HFD + extract	No sig. difference in food intake but prevented obesity & liver induced by a HFD	Enhancement of noradrenalin induced lipolysis & inhibition of pancreatic lipase activity

Pusztai *et al*[68] 1998	Rat	Kidney bean (Phaseolus vulgaris)	130, 150, 280 g/kg 10-70 d	C: Lean/obese + LFD/HFD I: Same groups + extract	The growth was retarded dose-dependently lower body fat	Sig. decrease of body protein in lean I group. Sig. decrease in plasma insulin levels in obese I group. Sig. pancreatic growth after long term feeding in all I groups
Nagasawa *et al*[69] 1991	Mouse (obese)	Tree peony root (Paenia suffruticosa)	0/5% 30 wk	C: Control I: Extract	Sig. decrease in food intake and Lee index	Improvement in glucose tolerance. No sig. difference in serum FFA levels
Wang *et al*[70] 2008	Mouse	Parasitic loranthus from Loranthacea or Viscaceae	20 d	-	Sig. decrease in body wt. & food intake	High inhibitory ability on FAS-Loran thacea was nearly 400 fold stronger than that from the viscaceae
Hu *et al*[71] 2008	Mouse (female)	Escins extracted from Aesculus turbinata Blume (Hippocastanaceae)	2%/11 wk	I: HFD C: HFD + extract	Suppressed the increase in body & parametrial adipose tissue wt.	Suppressed the increase of liver TG content; increased TG in feces after fat ingestion
Ohkoshi *et al*[72] 2007	Mouse	Nelumbo nucifera Gaertn leaves (Nymphaceae)	50%	C: STD/HFD I: Same groups + extract	Sig. suppression of body wt. gain	Exhibition of lipolytic activity especially in visceral adipose tissue; β adrenergic receptor pathway was partly involved
Kang *et al*[73] 2004	Rat	PM-F2-OB composed of *Lycii Fructus*, *Rehmanniae Radix*, *Coicis Semen*, *Carthami Flos*, *Hoelen*, *Angelicae Radix*, *Nelumbinis Semen*, *Radix Dioscorea* and *Aurantii Fructus*	6 wk	C: STD/HFD I: Same groups + plant	No sig. difference in wt. change if STD was used but in HFD group of I resulted in sig. decrease in body wt. gain but showed no sig. difference in amount of food intake	Sig. decrease in serum Chol/LDL and total lipids; reduction of kidney fat wt./FFA/PL & TG to levels equal or below the normal diet
Mary *et al*[74] 2003	Rabbit	Caps HT2 A herbal formulation	5 mg/kg (iv) 30 d 100/200/300/400/mg per kilogram orally	-	Sig. decrease in body wt.	Sig. increase in HDL after oral administration and decrease in atherogenic index in oral administration; Sig. increase of the release of LPL enzyme and sig. hypolipidemic effect in Ⅳ groups
Wu *et al*[75] 2005	Rat (diabetic by STZ)	Astragalus polysaccharide (APS) a component of Astragalus membranaceus roots	400 mg/kg (APS) 5 wk	-	Sig. decrease in body wt.	Sig. decrease in plasma glucose; improved insulin sensitivity
Xie *et al*[76] 2005	Mouse (Genetically obese)	Total, Ginsenosides in Chinese ginseng (TG CG), from leaves and the stem of Panax ginseng	100, 200 mg/kg (ip) 12 wk & 150, 300 mg/kg (oral)/12 wk	C: Placebo I: Extract	Sig. decrease in body wt.	Sig. decrease in FBG in 200 mg/kg dose after injection Sig. decrease in FBG in 300 mg/kg dose
Palit *et al*[77] 1999	Mouse	Galega officinalis	10% (w/w) of the diet 28 d	C: Diabetic/NL I: Same groups + plant	Sig. decrease in body wt. in both I groups, sig. wt. loss in normal mice independent of a reduction in food intake but in diabetic mice wt. loss was with reduced food intake	Striking loss of body fat in both groups; Sig. decrease in serum glucose in both groups but Sig. decrease in serum insulin in diabetic mice
Oi *et al*[78] 1999	Rat	Garlic	8 g/kg of diet 28 d	C: HFD I: HFD + extract	Sig. decrease in body wt. & perirenal adipose tissue wt. & epididymal fat pad	Sig. decrease in plasma TG levels; sig. decrease in mitochondrial protein and (UCP) in brown adipose tissue, and in urinary noradrenaline and adrenaline excretion
Yoshida *et al*[79] 1995	Mouse (obese and lean)	Bofu-tsusho-san	1/4%, 4/7% of wt. of food 8 wk	-	Sig. decrease in body wt. & retroperitoneal white adipose tissue wt. and no change in food intake	Sig. increase in GDP binding dose dependently
He *et al*[80] 2008	Rat (obese by STZ & HFD	Yi-Qi-Yang-Yin-Ye	2, 4, 8 g/kg 4 wk	-	Body wt. decreased	Decrease in TG/Chol/LDL/FFA/FBG/insulin; improvement of glucose tolerance

Jeong *et al*[81] 2008	Rat (fatty)	Gyeongshang angjeehwan: Liriope platyphylla F.T./Wang & T. Tang (Liliaceae), Platycodongrandiflorum A. DC. (Campanulaceae). Schisandrachinensis K. Koch (Magnoliaceae). Ephedra sinica Stapf (Ephedraceae)	8 wk	C: Placebo I: Compound	Sig. decrease in food intake & body wt. gain & abdominal fat	Sig. decrease in plasma leptin levels; decrease in circulating TG and inhibition of lipid accumulation in liver; increase of mRNA of genes responsible for FABO
Park *et al*[82] 2005	Rat (obese by diet)	Platycodon grandiflorum	150 mg/kg 7 wk	C: Convert to NLD/HFD I: Same groups + extract	Sig. decrease in wt. of body & adipose tissues in rats converted to NLD as compared to those remained on HFD	Sig. decrease in fat cell number & size in both I groups as compared to their state before intervention; decrease of FABP expression in HFD group of I
Akagiri *et al*[83] 2008	Mouse (obese by HFD)	Bofutsushosan (BOF)	1%/4 wk	C: Placebo I: Compound	The wt. of WAT and increase in size of adipocytes inhibited	Expression of UCP1 mRNA in WAT was found but not sig.
Kim *et al*[84] 2005	Mouse (diabetic)	Pine extract (bark and needle)	21 d	C: Control I: Extract	Sig. decrease in body wt.	Effectively suppressed the increase of postprandial blood glucose level by delaying absorption of diet
Attele *et al*[85] 2002	Mouse (obese diabetic)	Panax ginseng berry	150 mg/kg (ip) 12 d	C: Control I: Extract	Sig. loss of wt. with a sig. reduction in food intake & a very sig. increase in energy expenditure & body temperature	Sig. improvement in glucose tolerance & sig. reduction in serum insulin levels & plasma chol levels

MSG: Monosodium glutamate; FABO: Fatty acid β oxidation; STD: Standard diet; LFD: Low fat diet; NLD: Normal diet; HFD: High fat diet; FABP: Fatty acid binding protein; FFM: Fat free mass; sig.: Significant; AST: Aspartate transaminase; ALT: Alanine transaminase; C: Control; I: Intervention; FAS: Fatty acid synthetase; UCP: Uncoupling protein; GDP: Guanosine 5' diphosphate; FAS: Fatty acid synthetase; TG: Triglyceride; HDL: High density lipoprotein; LDL: Low density lipoprotein; FBG: Fasting blood glucose; ip: Intraperitoneal; iv: Intravenous. Caps HT2 is a herbal formulation containing methanolic extract of selected parts of plants: commiphora mukul; Allium Sativum; Plumbago indica/some carpus anacardium/Hemidesmus indicus/Terminalia arjuna/Tinospora cordifolia/Withania somnifera ocimum sanctum.

caffeine[9,20] which caused minor adverse effects such as dry mouth, insomnia, nervousness, palpitation and headache. Bofutsushosan[14] caused loose bowel movements.

ANIMAL STUDIES

Change in body weight and body fat

The majority of animal studies (41 out of 58) showed significant weight loss or inhibition of weight gain when supplemented with high fat diets containing extracts of plants, with or without an efficient decrease in fat mass[27-85] (Table 2).

Food intake

Clinical trials with *Agave tequilana* (TEQ) and Dasylirion spp (DAS)[30], Pomegranate leaf[43], Korean red ginseng[58], Tree peony[69], Gyeongshang angjeehwan containing a variety of plants including platycodongrandiflorum, Magnoliaceae and Ephedra[81], Parasitic loranthus[70], and Panax ginseng berry[85] showed significant reductions in food intake or appetite. In studies with *Cucurbita moschata*[40], *Cyperus rotundus*[42], Nomame Herba[66], *Acanthopanax senticosus*[57] PM-F2-OB (a traditional herbal medicine used for the treatment of obesity in Korea composed of Lycii Fructus), and several other plants[73], bofu-tsusho-san[79], *Galega officinalis*[77], and Oolong tea[67], no change in the amount of food intake or appetite was observed.

DISCUSSION

In many studies[8-10,12-16,20-23,27,39,73,74,79-81,83], a combination of plants or compounds containing minerals and or chemical extracts of plants were investigated and the scientific names are summarized in Tables 1 and 2. Most of these studies showed anti-obesity effects such as decreasing body weight in humans or body weight gain in animals with or without changes in body fat.

Currently available anti-obesity medications attack the body fat dilemma in three different ways. They can stimulate metabolism, suppress appetite, affect serotonin, or they can impede digestion of fat. In this review, we can categorize the target effects of herbal medicines in the same way.

Arachis hypogaea[50] decreased body weight gain, liver triglyceride content and liver size in association with increased fecal lipid excretion, suggesting an inhibitory mechanism on lipid absorption. Phillyrin[52], *Allium victorialis*[32], Pomegranate leaf[43], *Kochia scoparia*[46], *Panax japonicus*[55], Oolong tea[67], and *Aesculus turbinata* Blume[71] also had the same effect.

A decrease in food intake as a result of a decrease in appetite and an influence on hormonal status was observed with TEQ and DAS[30], Pomegranate leaf[43], Korean red ginseng[58], Tree peony[69], Gyeongshang angjeehwan containing a variety of plants including platycodon grandiflorum and Magnoliaceae and

ephedra[81], and Parasitic loranthus[70], refined Rhubarb[34], *Caralluma fimbriata*[19] and Panax ginseng berry[85]. Possible stimulation of metabolism has been reported as a mechanism of action for compounds such as Slimax[8], supplements containing ephedra[9,13,14,20] and *Terminalia arjuna* Roxb[11] which showed modification of lipid metabolism and a reduction in serum lipid levels.

Ephedra known as *Ma Huang* is a well known natural product with amphetamine-like stimulation effects. Although it's efficacy in weight loss need more investigations, its adverse effects are well established in the literature. In this review, nine studies investigated the effects of ephedra as one of the major components in the combinations with caffeine[9,13,22] or with several other plants[14,20,79,81,83] 5 of which were human studies[9,13,14,20,22].

In one study[13], efficient decreases in body weight and fat were observed with the administration of 210 mg caffeine and 72 mg ephedra per day for 12 wk with an improvement in lipid metabolism and blood pressure without serious adverse effects. In this study, the weight loss at 12-wk was -3.5 ± 0.6 kg with the test compound which was significantly ($P < 0.02$) higher than that of the placebo. The percentage fat loss shown by DXA was -7.9% ± 2.9% and -1.9% ± 1.1%, respectively ($P < 0.05$). In another study[20], ephedra at a dose of 40 mg/d and caffeine at a dose of 100 mg/d for a longer time (9 mo) was found to be more efficient than the previous study in lowering body fat and weight, improving lipid metabolism and blood pressure and had no serious adverse effects. The treatment group lost significantly more body weight (-7.18 kg) and body fat (-5.33 kg) than the control group (-2.25 and -0.99 kg, respectively). The difference in data from these two studies possibly resulted from the different dosages and duration of interventions.

In a human study[9], a significantly greater weight loss was observed (-4.0 ± 3.4 kg or 3.5% of baseline) in the test group *vs* (-0.8 ± 2.4 kg or 0.09% of baseline) in the placebo group. Changes were significantly greater for body fat and percentage of body fat in the active group (-3.5 ± 3.3 kg and -2.1% ± 3.0%) in comparison to the placebo group (-0.7 ± 2.9 kg and -0.2% ± 2.3%). The tested product also produced several untoward side effects, leading to some actively treated subjects withdrawing from the study. Additional long-term studies are needed to elucidate the effects of chronic treatment. Thus further examinations in healthy individuals using scientific combinations and dose/duration adjustments are required.

Four studies[58,59,65,76] investigated different doses and types of ginseng which is a very popular Chinese herbal medicine. Ginseng significantly decreased weight gain and efficiently improved glucose tolerance[59,76].

It has been reported[58] that hormonal influences can reduce food intake and decrease serum leptin and neuropeptide Y in the brain hypothalamus although not significantly. Thus the anti-obesity effect of this plant requires further investigation.

CQ, a succulent vine native to West Africa and Southeast Asia, has been used in traditional African and Ayurvedic medicine for more than a century. Although some studies have examined other uses for CQ, its role in fighting against obesity and for symptoms of metabolic syndrome has recently attracted interest in other parts of the world, because of its milder adverse effects comparing to ephedra. In this review, two studies focused on this herb[15,26]. CQ in combination with IG[15] induced marked reductions in body weight and fat. In addition, a reduction in waist size of 1.0 cm in the placebo group *vs* 21.9 cm in the CQ-IG group was observed.

As we focused on herbal medicines, all dietary interventions such as the consumption of fruits and vegetables, whole grains, different types of fibers, functional food components including omega three fatty acids or phytochemicals such as flavonoids were omitted. Lifestyle modification is still the safest and efficacious method of inducing a persistent weight loss. In this review, some of the studies were carried out on subjects who simultaneously received diet and exercise programs (mentioned as weight loss programs in Tables). These results demonstrated that specific phytochemical supplements increase the effectiveness of weight loss programs and additional significant anti-obesity effects are observed.

Although few studies mentioned adverse effects, it should be noted that many serious adverse events which would have stopped a trial of a pharmaceutical agent would likely not have been identified by the authors' search methods. Moreover, important safety issues including significant adverse events or supplement-drug interactions relevant to many clinical populations may not be fully addressed by the trials available for review.

CONCLUSION

Compliance with conventional weight-management programs, which often include increasing energy expenditure *via* physical activity, is low. It is not surprising to see the marketing of many new dietary slimming aids aimed at satisfying the need for palatable (as well as safe, effective, and therapeutic) options. In accord with this approach there are numerous investigations on the effectiveness of medicinal plants as natural supplements to reduce body weight. In this paper a variety of herbal supplements had beneficial effects on obesity especially compounds containing ephedra, CQ, ginseng, bitter melon (*Momordica charantia*), and zingiber. Most of the introduced herbals (Tables 1 and 2) have also been shown to have antioxidant effects, and with regard to the role of oxidative stress in the pathophysiology of some diseases and conditions, their further positive effects may be very promising[86-95]. Attention to these natural compounds and further work on the isolation and characterization of their constituents is highly recommended.

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REVIEW

An overview of animal models for investigating the pathogenesis and therapeutic strategies in acute hepatic failure

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Abstract

Acute hepatic failure (AHF) is a severe liver injury accompanied by hepatic encephalopathy which causes multiorgan failure with an extremely high mortality rate, even if intensive care is provided. Management of severe AHF continues to be one of the most challenging problems in clinical medicine. Liver transplantation has been shown to be the most effective therapy, but the procedure is limited by shortage of donor organs. Although a number of clinical trials testing different liver assist devices are under way, these systems alone have no significant effect on patient survival and are only regarded as a useful approach to bridge patients with AHF to liver transplantation. As a result, reproducible experimental animal models resembling the clinical conditions are still needed. The three main approaches used to create an animal model for AHF are: surgical procedures, toxic liver injury and infective procedures. Most common models are based on surgical techniques (total/partial hepatectomy, complete/transient devascularization) or the use of hepatotoxic drugs (acetaminophen, galactosamine, thioacetamide, and others), and very few satisfactory viral models are available. We have recently developed a viral model of AHF by means of the inoculation of rabbits with the virus of rabbit hemorrhagic disease. This model displays biochemical and histological characteristics, and clinical features that resemble those in human AHF. In the present article an overview is given of the most widely used animal models of AHF, and their main advantages and disadvantages are reviewed.

Key words: Acute hepatic failure; Surgical models; Chemical models; Viral models

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INTRODUCTION

Acute or fulminant hepatic failure (AHF) is a severe liver injury accompanied by hepatic encephalopathy which causes multiorgan failure with an extremely high mortality rate, even if intensive care is provided. Management of severe AHF continues to be one of the most challenging problems in clinical medicine[1]. Liver transplantation has been shown to be the most effective therapy, but the procedure is limited by shortage of donor organs combined with the disadvantage of needing immunosuppressant treatment[2,3]. Survival rates are substantially improved today compared with the mortality rate that approximated 100% when the

syndrome was first described nearly five decades ago. Nonetheless, survival has plateaued in recent years, prompting us to consider whether major new advances in disease understanding are needed to further improve the overall outcome[4].

Since 1970, when Trey and Davidson[5] introduced the term fulminant hepatic failure, various authors have suggested different classifications aimed to establish prognosis and adequate therapeutic strategies. These classifications are fundamentally based on time elapsed from onset of clinical symptoms or jaundice to the development of encephalopathy[6-9]. The causes of AHF are varied and in many patients remain unknown. A 6-year study (1992-1998) carried out in Spain into the causes of AHF indicated that the basic etiopathogenic agents of AHF were viral hepatitis (39%), unknown cause (30%), toxins or drugs (21%) and others (10%)[10]. Acute viral hepatitis constitutes a frequent cause of AHF[7,11,12]. The viruses causing hepatitis A, B and E are capable of producing AHF, which is rarely seen with hepatitis C virus. The hepatitis B virus is the main causal agent worldwide, responsible for 70% of all cases of viral origin[13]. The hepatitis E virus causes AHF principally in women in their third term of pregnancy. Other viruses involved comprise the herpes virus, varicela-zoster, cytomegalovirus, Epstein-Barr virus, human herpes type 6, adenovirus and paramyxovirus, mainly in the setting of immunosuppression[14]. Non-steroidal anti-inflammatory analgesic and anti-bacterial drugs are among the pharmaceuticals which most frequently trigger AHF[7,15-17]. Other less common causes of AHF include pregnancy, veno-occlusive disease, Budd-Chiari syndrome, Wilson's disease, hemochromatosis, tumoral metastases, sepsis, ischemia and hepatic transplant failure[18,19].

Etiologies also vary worldwide with considerable differences apparent between Western countries and the developing world. In Europe and North America a large proportion of cases are due to acetaminophen and to idiosyncratic drug reactions[20], whereas reports from emerging countries in Asia and Africa feature viral illnesses, particularly hepatitis B and E[17]. The resulting clinical picture is remarkably similar across the different etiologies, reflecting common patterns of response of the innate immune system and the resulting inflammatory response[21]. Determining etiology is important for two reasons: specific antidotes or therapies may be indicated once the diagnosis is known, and knowing the cause provides a reasonably valid guide to predicting outcome.

The AHF syndrome occurs as a result of the functional failure of a large part of the hepatic parenchyma, and severity is proportional to the level of hepatic damage. AHF provokes profound physiological alterations characterized by encephalopathy, hemodynamic changes and coagulopathy, with frequent development of cerebral edema and renal failure[14,22,23]. Diagnosis is based on biochemical and hematological data indicating hepatic cell hypofunction. Although prolonged prothrombin time not corrected by vitamin K and impairment of factor V are widely used, research into prognostic indexes remains an open field of investigation[24].

The pathophysiology of AHF is an area of great interest. It is evident that a relationship must exist between different pathogenic factors, such as bacteria toxins, cytokines, free radicals and other components of the inflammatory system which cause local lesions[25]. It seems that the endothelium is the first to release vasoactive agents which affect local and distal blood flow in the critical phase of the disease, with nitric oxide, prostacyclins and endothelins being essential components of the response[26]. Hyperbilirubinemia is generally conjugated and jaundice is an early indicator which progresses rapidly. Severe coagulation problems arise as a result of a variety of mechanisms. Consumption of factor V indicates hepatic damage regardless of vitamin K levels. Renal failure occurs in 30%-75% of cases, and is associated with a poor prognosis. Thrombocytopenia is also common[27]. An increase in the plasma concentration of aromatic amino acids (AAA) and normal or slightly elevated values for branched amino acids (ACR) are typical findings in patients with AHF. In fact, a fundamental clinical parameter for AHF is the Fischer index, that is, the ACR/AAA molar ratio, which decreases as the severity of hepatic symptoms develops[28]. An increase in the amino acids phenylalanine and tyrosine, and a decrease in the Fischer index have been reported in both surgical models[29] and models using galactosamine[30].

Intracranial hypertension is a major cause of morbidity and mortality of patients suffering from fulminant hepatic failure. The etiology of this intracranial hypertension is not fully determined, and is probably multifactorial, combining a cytotoxic brain edema due to the astrocytic accumulation of glutamine, and an increase in cerebral blood volume and cerebral blood flow; in part due to inflammation, to glutamine and to toxic products of the diseased liver[31]. Cerebral edema is a potential life-threatening complication in patients with AHF who progress to grade Ⅲ/Ⅳ encephalopathy[26]. The current view on the pathogenesis of cerebral edema is that hyperammonemia plays a main role. High arterial ammonia concentrations have been proposed as a predictor of brain herniation and mortality in patients with AFL[32-34]. Moreover, arterial ammonia concentration, ammonia delivery to the brain, and its metabolic rate are higher in patients with high intracranial pressure, and increased arterial ammonia correlates with increased cerebral flow[35]. Recent work has also suggested free radical formation occurring at a mitochondrial level as being the potential mediator of cellular dysfunction as opposed to ammonia *per se*[36].

Research into the molecular mechanisms of hepatic regeneration has aroused wide-spread interest[37,38]. Although little is still known about the hepatic regenerative process, it is clear that cellular loss and damage in the liver are accompanied by a lack of regenerative activity[39]. Plasma levels of hepatocyte growth factor (HGF) and transforming growth factor (TGF)-β rise[40]. An increase in the activity of the

fibrinolytic system, responsible for the activation of both HGF and TGF-β, is also observed[41]. It has been reported that the serum of people affected by AHF has a negative effect upon culture cell growth when compared with a control serum, due to cell proliferation inhibition rather than to an increase of apoptosis[42]. Recent studies using various animal models have shown that the over-expression of calpastatin, an endogenous inhibitor of calpain, helps prevent further liver damage when hepatic regeneration is compromised[43].

Knowledge concerning the pathophysiological basis of the AHF hemodynamic alterations, immunological dysfunction, and multiorgan failure is still very rudimentary. It is therefore crucial to investigate the molecular basis of AHF in more depth[44]. Furthermore, although many AHF treatment options have been proposed and applied in recent years, only hepatic transplantation is widely accepted among clinical specialists. However, the lack of donors combined with the high costs, technical difficulties, viability issues and the disadvantage of needing life-long pharmacological immunosuppressant treatment following surgical intervention (with the added complication that the immunosuppressant agents used themselves produce side effects in the kidneys, liver and other organs), mean that liver transplantation is not always an option. For these reasons, other therapeutic options to bridge patients to recovery or stabilization have to be considered. Artificial liver support, intended to remove protein-bound toxins and water-soluble toxins without providing synthetic function, and bioartificial liver support systems, using hepatocytes in an extracorporeal device connected to the patient's circulation, are being tested, and molecular adsorbent recirculating systems (MARS)[45] or cell-based therapies are increasingly the focus of attention[46,47]. These systems improve clinical and biochemical parameters and can be applied safely to patients[48], but their effectiveness and viability have not yet been conclusively demonstrated[38]. In terms of clinical applications, functional studies using animal models are absolutely crucial.

ANIMAL MODELS OF AHF

Knowledge of the pathophysiology and treatment of AHF are limited by the lack of satisfactory animal models. Many attempts have been made to develop a suitable model which can be replicated, using a wide variety of species and approaches, from surgical models to the use of hepatoxic drugs (Table 1). However, to date a simple model which accurately reproduces the pattern of human AHF has not been reported, and the models currently in use present significant limitations[49,50].

An ideal model would present well-defined clinical and biochemical criteria, and, as in the case of the King's College AHF prognostic criteria[11], be capable of providing an accurate prognosis. However, none of the models which have been developed until now meet these requirements. Furthermore, the clinical and biochemical criteria used to indicate the existence of AHF in animal models often have very little in common with those used in clinical practice. However, given the current state of knowledge concerning AHF and the difficulties involved in carrying out research on patients, animal models have a fundamental role to play in future studies despite their limitations. Therefore, although progress is being made, research in this field must continue, with the aim of developing a reliable and suitable animal model, capable of accurately reflecting the human clinical syndrome and presenting a minimum of disadvantages[51].

Ideal AHF models, according to criteria widely accepted (Table 2)[49-54], would benefit from complying with a series of requirements including that the model should be reversible, ie that some animals would survive the process if a suitable treatment were administered, and that the results obtained can be replicated, i.e. that death occurs at recognised intervals and that the extent of hepatic damage can be measured and standardised. Furthermore, death would need to be a result of hepatic damage, i.e. the complications produced following damage would need to accurately reflect the typical human clinical picture and death should be the direct result of the liver damage produced. Therefore, the untreated animals should die with signs of progressive hepatic failure within a recognised period of time. In addition, the animal used would need to be of a size permitting sufficient samples of blood and tissue to be taken during treatment. Finally, all the methods used should represent the lowest possible health risk for personnel participating in the research. An additional criterion could be the use of a conscious animal model to evaluate the development of hepatic encephalopathy, since this is an essential part of the pathology of AHF[55].

Numerous studies have been carried out in an attempt to develop a suitable AHF model. The majority of animal models are based on surgical techniques or hepatoxic drugs. Surgical models include the use of hepatic ischemia and partial/total hepatectomy, whilst chemical models are based on the use of drugs and toxins such as acetaminophen, azoxymethane, concanavalin A, galactosamine, halothane, thioacetamide, amatoxin-endotoxin, *etc.* Nevertheless, to date, no model accurately reflects human AHF, and most demonstrate significant limitations.

Surgical models

Surgical models of AHF can be divided into three categories: hepatectomy (total or partial), devascularization (total or partial) and models which are a combination of the previous two.

Total and partial hepatectomy: Surgical models employing total or partial hepatectomy have been successfully developed in various animal species following the first attempt carried out by Mann on dogs in 1921[56]. It has been demonstrated that 95% liver resection in rats provides a good AHF model[57], whilst a less than 90% hepatectomy is the upper limit for a liver regeneration research model in mice, as higher values produce mortal hepatic failure[58]. A potentially reversible model using pigs has been described which combines partial hepatectomy (70%) with porta-caval derivation and produces death from AHF after an interval which is sufficiently prolonged

Table 1 Main AHF animal models in different species

Animal model	Species	Advantages/disadvantages
Surgical		
Total/partial hepatectomy	Pig, dog, rabbit, rat, mouse	Hepatic encephalopathy; reproducible/no reversibility; no long-term survival
Total/partial devascularization	Pig, dog, rabbit, rat	Hepatic encephalopathy; reproducible/no reversibility; no long-term survival
Chemical		
Acetaminophen	Pig, dog, rabbit, rat, mouse	Hepatic encephalopathy; no hazard/non-reproducible; variable interval between damage and death; species and age variability
Amanitin	Pig	Hepatic encephalopathy; specific toxic effects; large animal
Azoxymethane	Mouse	Hepatic encephalopathy; reproducible/small size; hazard
Carbon tetrachloride	Pig, rabbit, rat, mouse	Hepatic encephalopathy/non reproducible; extrahepatic toxicity; small time window before death
Concanavalin A	Rat, mouse	Hepatic encephalopathy/small size
Galactosamine	Pig, dog, rabbit, rat, mouse	Hepatic encephalopathy; biochemical markers/non-reproducible; hazard; variable interval between damage and death; species differences
Lipopolysaccharide	Rat, mouse	Hepatic encephalopathy/non-reproducible; small size; hazard; small time window before death
Thioacetamide	Rabbit, rat, mouse	Hepatic encephalopathy; reproducible; large time window before death/hazard
Viral		
Rabbit hemorrhagic disease	Rabbit	Hepatic encephalopathy; reproducible; no hazard

Table 2 Main criteria for an AHF animal model (according to Terblanche and Hickman (1991)

Reversibility	Suitable treatment may reverse and improve survival
Reproducibility	Reproducible end-points are required to standardize the model
Death from liver failure	Should reflect biochemical, histological and clinical changes including death from AHF
Therapeutic window	Time for treatment should be available between insult and death
Adequate animal size	Size large enough to allow blood and tissue analysis to take place serially
Minimal hazard to personnel	Minimum risk for operators and associated staff

to enable studies of hepatic support measures to be carried out. In addition, the animal is of an appropriate size, and the technique does not represent a health hazard[59].

The partial hepatectomy models are equivalent to patients who have undergone large liver resections for liver tumors. It has been demonstrated by DNA analyses of rats subjected to various levels of partial hepatectomy that induced AHF is a consequence of both an increased rate of apoptosis and a reduction in liver regeneration[60]. Moreover, models of partial hepatectomy have been used to test the usefulness of different support systems. Thus, intraperitoneal transplant of syngeneic-bioencapsulated bone marrow cells, which can transdifferentiate into hepatocyte-like cells in the peritoneal cavity of 90% hepatectomized rats, increases the survival rate of these animals[61]. Examination of the effects of a series of allogenic hepatocyte transplantations in rats with subtotal hepatectomy indicates that intrasplenic hepatocyte transplantation 1 d before liver surgery shows the best results in terms of survival[62]. The usefulness of an artificial liver module having a liver lobule-like structure has been recently tested in rats with combined partial hepatectomy and hepatic ischemia, demonstrating that in treated rats the increase in blood ammonia was completely suppressed and all animals recovered[63].

The clinical equivalent of liver total hepatectomy is the massive liver damage due to liver trauma or a primary graft failure[51]. Main disadvantages are the absence in circulation of the toxic substances and inflammatory factors which play a role in the pathogenic mechanisms of AHF. Advantages are related to replicability and its usefulness in the *in vivo* study of artifical support devices in the absence of toxic products eliminated or produced by the damaged liver. Despite the disadvantages indicated, total hepatectomy has been used on rats to study hepatic regeneration[64], and with pigs as a replicable model for testing the effectiveness and function of various temporary support device systems[65,66]. A new surgical model for hepatectomy in pigs, requiring prior to *en bloc* hepatectomy a Y-shaped bypass starting with end-to-side anastomosis between the vena cava and the portal vein, followed by anastomosis to the intrathoracic vena cava has been recently described. This model permits total hepatectomy with minimal blood loss under stable circulation without requiring an extracorporeal bypass[67].

Devascularization: Complete devascularization of the liver may be achieved by portacaval shunt followed by occlusion of the hepatic artery, and in most cases also occlusion of the common bile duct and accessory hepatic vessels[68,69]. Depending on the time of temporary occlusion of the hepatic artery the model is more or less reversible. These techniques have been successfully used to induce a reproducible hepatic failure in pigs, which could be useful in the study of different artificial and/or bioartificial hepatic support devices[70,71] or to test the effects of antioxidant molecules such as N-acetylcysteine[72]. For example, a reproducible model has been developed using dwarf pigs for the study of reversible devascularization through hepatic artery ligation and porto-caval anastomosis, where intracranial pressure was monitored in addition to other classic parameters

indicative of AHF. This model provides an 8-h therapeutic window, enabling tests on different bioartifical support systems to be carried out[73]. In fact, with the use of a similar model in pigs, albumin dialysis using the molecular adsorbents recirculating system (MARS) has been reported to attenuate extracellular brain ammonia and lactate levels[74]. Hepatic devascularization in pigs has also allowed the demonstration that endothelium-dependent hyperpolarization of vascular smooth muscle contributes to the development of hyperdynamic circulation in AHF[75].

A model using total clamping of the portal triad in dogs demonstrated that the damage caused by ischemia-reperfusion as a consequence of the surgical procedure was reduced following administration of a bradykinin β2 receptor antagonist[76]. Dogs were also used in another AHF model employing porto-caval derivation combined with bile duct ligation, in order to test a new system of bioartificial liver by inoculation of hepatocytes. This model was configured by inoculating porcine hepatocyte spheroids into the cell circuit of a hollow fiber bioreactor[77]. Recently a new pig model has been developed in which a 75%-80% liver resection is combined with an ischemia period[78].

Studies carried out on survival time, technical ease, safety and reproducibility of AHF surgical models have reported that devascularization was more useful for studying the development and treatment of AHF caused by ischemia and related side effects, whilst partial hepatectomy was the most suitable technique for studying liver deficiency status and AHF treatment *via* bioartifical support devices[79].

Chemical models

The use of chemical agents such as acetaminophen, thioacetamide or galactosamine may reproduce a number of important AHF clinical characteristics, such as hypoglycemia, encephalopathy, and increased blood levels of hepatic enzymes, and hepatotoxic chemical agents are still frequently used as a model for AHF. However, repeated administration or a support therapy may be required in some models. In addition, intracranial hypertension, one of the main characteristics of human AHF, is absent in some chemical models whilst in other cases, an increase in toxins involved in hepatic encephalopathy and cerebral edema in human AHF cannot always be demonstrated[49].

Acetaminophen: Acetaminophen (paracetamol) is a commonly used drug which can produce hepatic damage. In fact, it is the drug most frequently used to commit suicide in the United Kingdom despite the existence of the antidote acetylcysteine. Acetaminophen overdoses are the number one causes of AHF in USA, United Kingdom, and most of Europe, accounting for nearly 50% of USA cases[13]. Acetaminophen toxicity is dose-dependent, but its effects can be exacerbated by fasting, cytochrome P-450 inducer drugs and especially by alcohol. Studies on both hepatocyte cultures and mice have shown that c-jun kinases (JNK) play a major role in the toxic effect of the drug[80]. More recently, it has been shown that apoptosis signal-regulating kinase 1 (ASK1), a member of the mitogen-activated protein kinase kinase kinase family, is activated by acetaminophen overdose in mice, most likely *via* a mechanism involving thioredoxin-ASK1 dissociation, and that it plays a role in acetaminophen-induced liver injury through JNK activation[81]. The fact that JNK inhibition is not protective in acute carbon tetrachloride-mediated or anti-Fas antibody-mediated hepatic injury, suggests specificity for the role of JNK in the pathogenesis of acetaminophen-induced liver failure, thereby identifying JNK as an important therapeutic target in the treatment of acetaminophen hepatotoxicity[82].

The results of numerous studies with animal models using acetaminophen to induce AHF have produced heterogeneous results due to the existence of significant variations in the hepatic detoxifying metabolism of the drug related to species and age[83,84]. Under normal conditions, acetaminophen hepatic metabolism is produced by glucuronidation and sulfation reactions, with formation of metabolites which are later excreted through the kidney. When an excess of the drug is present, normal detoxifying pathways are saturated and the drug is metabolized through cytochrome P-450 to N-acetyl-p-benzoquinoneimine which, unless conjugated with glutathione, is thought to interrupt mitochondrial calcium flux and to induce cell damage by the formation of hydroxyl radicals, nitrites, and nitrates, leading to apoptosis and cell necrosis[85]. Therefore, in order to potentiate acetaminophen toxicity, inducers of the cytochrome P-450 systems such as phenobarbitone and 3-methylcholanthrene, glutathione depletion induced by the glutathione synthetase inhibitor buthionine sulfoximine or a combination of both systems are used[84,86].

Other important aspects which have not been standardized in acetaminophen models, and which produce variable results, include the optimal drug dose, the most suitable method of administration and the necessity or not of induction of the cytochrome P-450 system[86,87]. Lack of standardization is the origin of some of the major disadvantages of these models, specifically, their lack of reproducibility and the variable interval between inducing damage and the death of the research animals[49,52]. Furthermore, in some rodents significant differences have been found in concentrations of the main coagulation factors compared to those found in human AHF[88].

Acetaminophen-induced animal models of AHF are widely used to improve our insight into the metabolic and physiological derangements of AHF and to facilitate the development of new therapeutic modalities. Thus, implantation of encapsulated lentivirally immortalized human hepatocytes rescue mice from lethal doses of acetaminophen, confirming that lentiviral vectors represent tools of choice for immortalization of non-dividing primary cells and that immortalized human hepatocytes are promising reagents for cell-based therapy of acute liver failure[89]. More recently, it has

been found that adult-derived mononuclear bone marrow fraction is capable of significantly increasing the survival rate of rats with acetaminophen-induced AHF[90]. Research has also shown that acetaminophen-induced hepatocellular damage is associated with increased circulating catecholamines, which may contribute to the pathophysiology of acetaminophen-induced hepatotoxicity by compromising hepatic perfusion, and that toxicity may be abolished by the use of α(1) antagonists[91].

Galactosamine: D-galactosamine is a molecule which, when metabolized *via* the galactose pathway in the liver, causes serious metabolic alterations and hepatic necrosis through depletion of different uridine intracellular mediators[49], and has therefore been used to develop AHF models. In one of the first models using rabbits[92], death occurred between 21 and 44 h, following a coma lasting on average 2.6 h, with histologic and biochemical findings compatible with AHF. Furthermore, it was possible to show that in this same species, hepatotoxin did not cross the hematoencephalic barrier[93]. More recently, galactosamine has been used on anesthetized dogs. This model also displays the characteristic effects of human AHF, such as an increase in blood levels of liver enzymes, bilirubin, ammonium or lactate and the associated coagulopathy, hypoglycemia, coma and increase in intracranial pressure[94]. However, the effects were not the same in dogs without anesthesia, probably due to the added effect of the anesthetic. A reproducible model has been developed with pigs which, because of their size, are suitable for the assessment of different support systems designed for treating AHF in humans[95]. Significant differences in galactosamine sensitivity across different species exist. Furthermore, the interval between damage caused and death is not uniform, the agent is expensive to use in large-scale models, and lastly, it carries health risks[50].

Galactosamine models have been used to investigate the renal damage which accompanies AHF[96] and the liver metabolic pathways involved[97]. In addition, the potential protective effects of substances such as the chimeric protein hyper-IL-6[98] or 1,6 diphosphate fructose have been investigated in rats[99]. Cardiotrophin 1 may improve the outcome of D-galactosamine-induced AHF through its effects on anti-apoptosis and cell repair[100]. Blocking of N-methyl-D-aspartate receptors prevents ammonia-induced death[101] and also prevents or delays death of rats by galactosamine-induced AHF[102]. Moreover, this model has been used to identify the contribution of cytosolic polypyrimidine tract-binding protein to the mechanisms of hyperinsulinemia by stabilization of mRNA encoding insulin and its secretory granule proteins[103]. D-galactosamine models have also allowed testing of different extracorporeal hepatic support devices[104] and bioartificial systems, including hepatocytes transfected with the human gene interleukin-1 receptor antagonist in rats[105], the use of a nonwoven fabric bioreactor containing porcine hepatocytes[47], or the study of the potential effects of cerebrospinal fluid drainage and cranial decompression in rats[106].

A combination of D-galactosamine and lipopolysaccharide has also been widely used to induce AHF in rats. This model has allowed the demonstration of the potential therapeutical role of vascular endothelial growth factor[107]. Using this approach, evidence for a direct link between tumour necrosis factor (TNF)-α and Fas/FasL in mediating hepatocyte apoptosis has been provided[108], it has been reported that type I inositol 1, 4, 5-triphosphate receptors increase in the kidney[109], and it has been demonstrated that transcription factor early growth response (Egr)-1 plays an important role in acceleration of hepatic inflammation, apoptosis, and subsequent mortality in acute liver injury[110]. Research with this model has also found that the expression and activity of both leukotriene C4 synthase and microsomal glutathione-S-transferase are up-regulated, being partly responsible for cysteinyl leukotriene hepatic accumulation[111], and that a combination of 5-hydroxyindole acetic acid, glucose, β-hydroxybutyrate, and phosphate concentrations in the plasma is a potential marker for AHF, as well as for the early prognosis of AHF[112]. Studies using SP600125, a small molecule JNK-specific inhibitor have confirmed the role of JNK as a critical apoptotic mediator in galactosamine/lipopolysaccharide-induced AHF[113]. Very recently it has been demonstrated that in mice challenged with D-galactosamine and lipopolysaccharide, deficiency of uncoupling protein-2, which plays a role in liver cell death through its involvement in the production of reactive oxygen species and adenosine, provides protection under endotoxemic stress conditions, underlining the significant role of the bioenergetic status in critical illness[114].

Carbon tetrachloride: Carbon tetrachloride has been widely used to induce chronic liver damage, especially as a model of primary hepatic cirrhosis. Nevertheless, its use to induce AHF has been very limited due to low reproducibility and wide variation between species[50,115]. The mechanism of action is produced in the endoplasmic reticulum by formation of reactive intermediates through isoenzymes of cytochrome P-450[116]. This mechanism also involves significant alterations to mitochondrial calcium homeostasis and is dose-dependent[117].

A relatively uniform model was developed using pigs which induced coma and death between 12 and 52 h through a combination of pretreatment with phenobarbital and a 2-h interruption of arterial blood flow followed by intragastric administration of the toxin[118]. The administration of carbon tetrachloride in rats has been shown to simultaneously induce both severe damage processes and hepatic regeneration[119]. Depending on the dose administered, exposure time, the presence of exacerbating agents, or the age of the organism affected, regeneration can occur and lead to the total recovery of the damaged liver[120,121].

Rats have been used for the study of intrasplenic transplant of hepatocytes[122], and to investigate the mechanisms involved in compensatory liver regeneration which avoids progressive toxic damage[43]. Carbon

tetrachloride-induced AHF has also allowed the demonstration in rats of the therapeutical efficacy of Gabexate mesilate, a synthetic protease inhibitor[123], the sulfated polysaccharide extracted from brown algae fucoidan[124], or naringenin-loaded nanoparticles[125], but not of granulocyte colony stimulating-factor[126].

Criticisms of these models include the fact that carbon tetrachloride mainly affects the central zone of the hepatic acinus, and the characteristic massive necrosis of human AHF is not present. Furthermore, carbon tetrachloride is not completely metabolized in the liver and some of the non-metabolized toxin affects and damages other organs, especially the lungs and kidneys[52]. Finally, there is a wide variation in species and age sensitivity, basically due to different levels of development and effectiveness of the cytochrome P-450 detoxifying system[49].

Thioacetamide: Thioacetamide causes hepatocellular necrosis following biotransformation by mono-oxygenases[127], and has been used to explore the role of reactive oxygen species[128], and the protective effect of antioxidants such as curcumin[129], pro-regenerative substances[130], or the worsening of encephalopathy following long-term treatment with substances such as indometazine[131]. Using the thioacetamide model of AHF, it has been recently shown that cannabinoids and capsaicin improve liver function[132] and that *Gingko biloba* ameliorates hepatic damage most probably due to its free radical-scavenging effects[133]. Simvastatin improves encephalopathy and survival in thioacetamide-treated rats, an effect that is offset by N(G)-nitro-L-arginine methyl ester (L-NAME), a non-selective inhibitor of nitric oxide synthase (NOS), which supports the role of nitric oxide in liver damage and encephalopathy[134]. Moreover, the fact that L-NAME administration, but not L-canavanine (specific inhibitor of inducible NOS), had detrimental effects on the severity of hepatic damage and motor activities in thioacetamide-treated rats, suggests that constitutive NOS activities play a major protective role[135].

Azoxymethane: Azoxymethane administration induces in mice alterations similar to those encountered in human AHF[136]. In fact, it has been shown that mice present decreased locomotor activity followed by loss of righting and corneal reflexes, are hyperammonemic, and develop spontaneous hypothermia and brain amino acid profiles typical of AHF in other species including humans. These findings demonstrate that azoxymethane treatment affords a reproducible model which may be suitable for the study of the cerebral complications of AHF[137]. Induction of AHF in C57BL/6J mice by using azoxymethane has recently allowed the observation that altered expression of zonula occludens-2 precedes increased blood-brain barrier permeability, suggesting that zonula occludens-2 may play an important role in the pathogenesis of brain edema in AHF[138].

Concanavalin A: A single injection of concanavalin A has also been proposed as a model of AHF as it induces hepatocellular destruction[139] through mechanisms which appear to involve participation of immune cells, including macrophages and activated CD4+ T cells[140]. The use of this animal model has demonstrated that suppressor of cytokine signaling-1 (SOCS1) plays an important negative role in fulminant hepatitis and that forced expression of SOCS1 is therapeutic in preventing the disease[141]. In concanavalin A-treated mice it has been reported that TNF-α levels are not affected by adiponectin, whereas IL-10 production is increased. Therefore, adiponectin might play a role in the control and limitation of inflammation in the liver, and a contribution has been suggested for IL-10 in adiponectin-mediated hepatoprotection[142]. siRNA delivery for osteopontin, which has been implicated in various helper T cell type 1 immunity-mediated diseases, has therapeutic potential in concanavalin A-mediated AHF[143].

Other models: AHF has also been induced through the use of poisons such as the derivative of *Amanita phalloides* which, although not a frequent cause of poisoning, has well-known effects on humans. In fact, the effect of amatoxins is due to ARN polymerase induction, producing cell toxicity in hepatocytes, intestinal mucosal cells and kidney tubular cells and they have been used in combination with lipopolysaccharide to develop an animal model of AHF using pigs[144]. Models with pigs have also been reported which combine amanitine with lipopolysaccharide, with the aim of studying survival following orthotopic liver transplant and tacrolimus administration[145].

Other models employ parenteral administration of *Propionebacterium acnes* and lipopolysaccharide in mice to study the inhibition of the acquired immune response[146], and intraportal administration of α-amanitine and lipopolysaccharide in pigs to study bioartifical liver support devices[147].

Intrahepatic upregulation of the immunoactivating molecules CD40 and CD40 ligand (CD40L) are early mechanisms for liver cell damage in human and murine AHF. The use of a model based on intrahepatic overexpression of CD40L by adenoviral-mediated gene transfer (AdCD40L) in mice, has led to the demonstration that CD40-CD40L interaction can induce liver damage, and that CD40L-induced AHF depends on competent lymphocytes[148].

Although their use has been limited, various models combining drug administration and surgical procedures have been described, such as a combination of 70% liver resection and endotoxin administration in rats[149], or resection of all of three hepatic arteries combined with intraportal injection of carbon tetrachloride[150].

Viral models

Despite the fact that viral hepatitis is a main cause of AHF in many countries, the use of infective agents to develop animal models of AHF has in general been unsuccessful and only the use of transgenic mice over-expressing virus B hepatitis proteins (HBV) or BALB/cj mice infected with MHV-3[151,152] has shed some light

on virus-induced AHF mechanisms. However, these murine models display significant limitations as regards the absence of intracranial pressure measurements, or the lack of data concerning toxins involved in hepatic encephalopathy and cerebral edema, as well as the small size of the models used which renders testing of new liver support systems impossible[49,50].

More recently, our research group has described a new animal model of AHF using experimental infection of rabbits with 104 hemagglutination units of an isolate of the rabbit hemorrhagic disease virus (RHDV)[153]. First reported two decades ago, RHDV is a member of the *Caliciviridae* family which causes an acute and highly fatal disease in wild and domestic rabbits[154]. Rabbit hemorrhagic disease (RHD) is a viral hepatitis which displays surprising clinical, anatomopathological and transmission mode similarities to fulminant human viral hepatitis B, C, and E[155]. The virus does not replicate in any other vertebrate[156] and to date there is no indication that it can be transmitted to humans, even among those populations most exposed to the virus.

It has been shown that the viral antigen can already be found in hepatocytes at 12 h postinfection (p.i.) and that at 36 h and 48 h p.i., it is localised in 60%-80% of hepatocytes[157]. RHD is characterized by a high morbidity and a mortality rate that approaches 90%[153,158]. Rabbits die within 36 to 54 h p.i. with clinical signs characteristic of progressive AHF and coma. In addition, the interval between infection and death, in the majority of animals, provides a wide therapeutic window which indicates that our model complies with another of the essential prerequisites of a good AHF animal model, that is, the existence of a sufficiently prolonged interval between intervention and death to enable research into various treatment methods or liver support technologies. In addition, the use of a medium-sized animal facilitates serial collection of blood samples, and makes easier monitoring of intracranial pressure and biochemical alterations produced during the course of the infection[153].

This model reproduces representative biochemical and histological parameters and clinical signs of human AHF. Thus, significant increases in blood transaminase and lactate dehydrogenase activities, and in blood bilirubin concentrations, are detected. Moreover, blood concentration of aromatic amino acids increases significantly, with a decrease in the Fischer index and hypoglycemia. Prolonged prothrombin time, a prognostic element in AHF, and exhaustion of factor V and Ⅶ are systematic findings. These effects could occur as a consequence of diminished synthesis of clotting factors and the development of disseminated intravascular coagulation[153]. In addition to biochemical and histological abnormalities, infected rabbits demonstrate a clinical picture consistent with AHF. Prostration and side recumbency are present at later stages and neurologic symptoms (convulsions, ataxia, and posterior paralysis) rapidly progress to coma and brain death in the terminal phases. In our model, intracranial pressure rises progressively in the terminal phases, suggesting a loss of intracranial compliance, and short episodic spikes are also observed. The rise in intracranial pressure in RHDV-infected animals is accompanied by an increase in plasma ammonia levels[153].

Histological and immunohistochemical examination reveals necrotic areas associated with hemorrhages and neutrophil infiltration, and large apoptotic areas with a high caspase 3 expression, mainly in the periportal areas of hepatic acini[159]. A significant increase in inducible nitric oxide synthase expression and TNF-α activity, similar to those reported in AHF[160], are also observed in infected rabbits[161]. TNF-α may lead to cell proliferation or to apoptosis, and its over-expression correlates with both apoptosis and hepatic regeneration in AHF[162]. Balance between proliferation and apoptosis may be influenced by an excess of reactive oxygen species that, if not neutralized by glutathione and antioxidant enzymes, may cause mitochondrial damage and cytosol release of cytochrome c, causing caspase activation and cell death[163]. This also happens in RHDV-infected rabbits, which show impaired glutathione levels and antioxidant enzyme activities[161], with a marked activation of the apoptotic intrinsic pathway[159].

Therefore, RHDV experimental infection induces an AHF in rabbits which has a number of physiological and biochemical features seen clinically in humans, is highly reproducible, has a long therapeutic window and generates intracranial hypertension and an associated-encephalopathy. Thus, it is the first successful model using infective agents and satisfies the criteria applicable to an animal model of AHF. This model could provide a useful tool for the study of AHF and the evaluation of new liver support technologies in humans.

CONCLUSION

AHF is a potentially devastating sindrome whose treatment has been limited by the lack of satisfactory animal models. The potential disadvantages of surgical models are that they do not offer reversibility or recovery, they are difficult to replicate, they depend on surgical skill, many of the clinical and biochemical parameters typical of human AHF are not present, and that they do not reproduce an environment complicated by the release of inflammatory mediators and products of cell necrosis. Thus, their usefulness is limited to the evaluation of various liver support systems. Models using hepatoxins do not suffer from the above limitations, but nevertheless they may present disadvantages, such as the necessity for adjusting dosage and the potential health hazard which in most cases such chemical agents represent. As for the only viral model developed to date which has proved to be viable, induced RHDV infection in rabbits, it is reproducible and presents characteristics similar to human AHF. The only limitation is that the only susceptible species is the rabbit, although this could also be considered an advantage as it does not represent a health hazard to researchers.

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REVIEW

Molecular characteristics and stages of chronic hepatitis B virus infection

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Abstract

Hepatitis B virus (HBV) is a common viral pathogen that causes a substantial health burden worldwide. Remarkable progress has been made in our understanding of the natural stages of chronic HBV infection. A dynamic balance between viral replication and host immune response is pivotal to the pathogenesis of liver disease. Knowledge of the HBV genome organization and replication cycle can unravel HBV genotypes and molecular variants, which contribute to the heterogeneity in outcome of chronic HBV infection. Most HBV infections are spontaneously resolved in immunocompetent adults, whereas they become chronic in most neonates and infants at a great risk of developing complications such as cirrhosis and hepatocellular carcinoma (HCC). Those with chronic HBV infection may present in one of the four phases of infection: immune tolerance, immune clearance [hepatitis B e antigen (HBeAg)-positive chronic hepatitis B (CHB)], inactive carrier state, and reactivation (HBeAg-negative CHB). Understanding the dynamic nature of chronic HBV infection is crucial in the management of HBV carriers. Long-term monitoring and optimal timing of antiviral therapy for chronic HBV infection help to prevent progression of HBV-related liver disease to its later stage, particularly in patients with higher risk markers of HCC, such as serum DNA concentration, HBeAg status, serum aminotransferase, HBV genotypes, and pre-core or core mutants.

Key words: Hepatitis B virus; Pathology; Immune tolerance; Immune clearance; Inactive hepatitis B surface antigen carriers; Reactivation; T-cell response; Cytokines

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem worldwide. Some individuals can develop acute HBV infection and achieve complete immune clearance of virus, yielding a life-long immunity, while others can develop chronic HBV infection depending on the host immune response. Chronic HBV infection is associated with a wide range of clinical manifestations, from an asymptomatic carrier state with a normal liver histology to severe and chronic liver diseases, including cirrhosis and hepatocellular carcinoma (HCC)[1,2].

There is a particular concern in the Asia-Pacific region, where chronic HBV infection is prevalent, with a carrier rate of approximately 10% of chronic HBV carriers. About 25%-40% of them will eventually die of liver disease (cirrhosis with and without HCC) with a death rate of 50% for male carriers and 15% for female carriers, respectively. Chronic HBV infection is a dynamic process with a replicative or a non-replicative (or low replicative) phase based on virus-host interaction which is pivotal to the pathogenesis of liver disease. Understanding the dynamic nature of chronic HBV infection is crucial in the management of HBV carriers. Long-term monitoring and optimal timing of antiviral therapy for chronic HBV infection patients can help to prevent progression of HBV-related liver disease to its later stage[3,4].

PATHOLOGY OF HBV INFLAMMATORY REACTION

Viral hepatitis, characterized by diffused inflammatory

reaction, is associated with cell damage and death. It has been recently reported that HBV replication is associated with cell death, which is different from the widely accepted non-cytopathic characteristics of HBV[5]. The mechanism of cell damage is generally defined as the result of cytotoxic T-lymphocyte (CTL)-mediated immune responses to viral infection[6]. Another typical process causing cell death is apoptosis. It has been shown that HBV proteins, such as HBx and HBsP, can induce apoptosis[7]. A careful light microscopic examination of HBV genotypes A-C transfected cells can reveal rounded up and death cells which are apoptotic signs. To identify the observed cell death, FAC_s is used because apoptotic cells can show phoplaridylserine on cell membrane. $HePG_2$ cells can be transfected with HBV genotypes A-C. Cells observed under a phase contrast microscope, can be stained with apoptosis markers and analyzed by flow cytometry. HBsP expression can be detected by Western blotting assay. BH3 sequences can be aligned and analyzed with the vector NT1. HBV genotypes A-C transfected cells display cell death which has been further proved as apoptosis. HBsP, a pro-apoptotic protein, is detectable during transfection of virus genomes. Different apoptotic effects are correlated with the expression of different genomes. Alignment and analysis of the HB3 domains of three virus genomes can reveal a slight variance. It has been reported that variant HBsP expression and BH3 sequence of HBV genotype may be involved in differential apoptotic effects on transfected cells[8]. However, HBV can also directly cause death of hepatocytes[6].

HBV TRANSMISSION AND INFECTION

In high endemic regions, such as Asia, Africa, Pacific Islands and the Arctic, early perinatal and horizontal infection in childhood is the main route of HBV transmission with a hepatitis B surface antigen (HBsAg) positive rate of 8%-15%, while in low endemic areas, such as Western countries, HBV is a predominant disease in adolescents and adults due to high risk sexual behaviors or drug injections, with a HBsAg positive rate of less than 2%[9].

The vast majority of early perinatal or horizontal infections in childhood are the main route of HBV transmission in untreated infants whose mothers are hepatitis B e antigen (HBeAg) positive, and over 90% of them will become chronic HBV carriers. In contrast, about 90% of HBV infections may occur as acute infection and only 5%-10% may occur as chronic infection in adults. This dramatic difference in chronic rates is believed to reflect the host immunologic status and the time of infection.

Although infants whose mothers are HBeAg-positive HBV carriers are at a high risk of developing infection and subsequently become viral persistence, the age of infants at the time of HBV infection is inversely correlated with the chronic rate[10,11]. A HBc/HBe-specific Th-cell tolerance model can show the reversibility of T-cell tolerance. It has been shown that a single prenatal dose of HBeAg can result in apparent T-cell tolerance in mice at the age of 8-12 wk, but the tolerance may disappear at the age of 16 wk[12], suggesting that T-cell tolerance can be maintained and HBcAg/HBeAg will continuously present. HBc/HBe-specific thymocytes are absent in thymus and this "repertoire renewal process" requires about 16 wk. Similarly, human fetus may be exposed to tolerogenic HBeAg in uterus not infected at birth. The longer the elapsed time before HBV infection is, the greater the probability of renewing HBc/HBe-specific T-cell repertoire is, because the neonate would no longer be exposed to HBeAg[13].

There is an obvious difference between patients infected with HBV in adolescence or adulthood immediately entering immune clearance phase, and short duration and tendency quiescent after seroconversion from HBeAg to antibody against HBeAg (anti-HBe). Such patients are termed "healthy" carriers. In contrast, patients with early HBV infection have a prolonged immune tolerance phase and a prolonged immune clearance phase, indicating that their diseases tend to progress after HBeAg seroconversion.

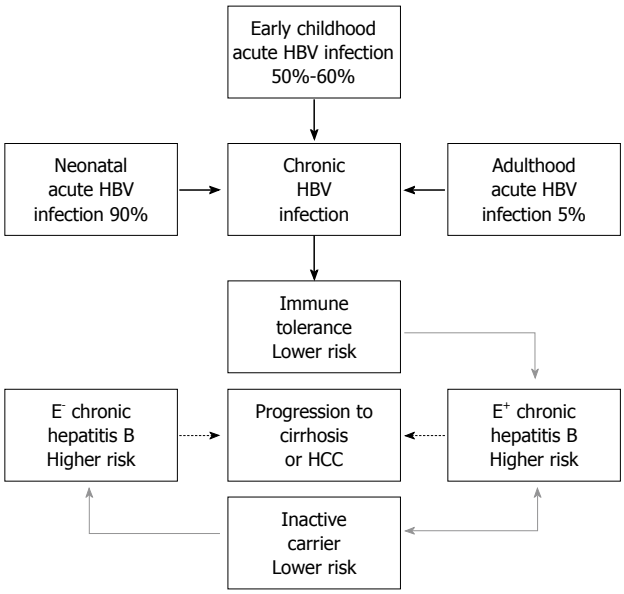

Figure 1 Phases of chronic hepatitis B virus (HBV) infection. Thick arrows indicate HBV infection rates in different age groups, dotted arrows indicate changes in histology, grey arrows indicate changes at risk of progressing to cirrhosis or HCC (E^+: HBeAg positive; E^-: HBeAg negative).

STAGES OF HBV INFECTION

Remarkable progress has been made in our understanding of the four natural stages of chronic hepatitis B (CHB): immune tolerance stage, immune clearance stage, inactive HBsAg carrier stage, and reactivation stage. However, not all CHB infection patients go through all the four stages (Figure 1).

Immune tolerance stage

Patients with perinatal or early childhood-acquired HBV infection have an initial tolerance stage characterized by the presence of HBeAg, high serum DNA level, normal serum aminotransferase level, and minimal or

no inflammation on liver biopsy[14]. Such manifestations can rarely be seen in those who are infected with HBV in later childhood or adulthood and whose infection subsequently develop into chronic HBV infection[14].

Although a high serum DNA level in liver disease patients with minimal or no inflammation is considered as a sequela of immune tolerance to HBeAg, it has been shown that HBeAg may promote HBV chronicity by functioning as an immunoregulatory protein[15]. For example, in transgenic mice, transplacental transfer of maternal HBeAg may preferentially behave as a tolerogen and inactive HBe/HBc-specific Th cells through at least central deletion of high-affinity HBe/HBcAg-specific CD4 T cells or clonal ignorance and anergy in periphery blood, resulting in ineffective cytotoxic T cell lysis of infected hepatocytes. Such a mechanism may be responsible for the high chronic HBV infection rate (-90%) observed in babies infected by their HBeAg positive mothers accounting for the inability of infants to clear perinatal HBV infection. After neonatal or prenatal HBV infection (absent in uterus tolerance), secretion of monomeric HBeAg in the relatively Th_2-bised neonatal immune system may also have an anti-inflammatory influence on nucleoprotein-specific immune response by eliciting Th_2-like cytokines. Secreted HBeAg can also enter thymus. It has been subsequently reported that HBeAg specific Th_2-like cells can preferentially survive tolerance production to a greater extent than HBeAg-specific Th_1-like cells[16]. Therefore, chronicity resulting from vertical transmission of HBV characterized by the predominance of HBeAg-specific Th_2-like cells and secretion of anti-inflammatory cytokines, such as IL-4, IL-5, and IL-10, can enhance antibody production, and viral persistence would characterize the HBeAg-specific T-cell response. If the *status quo* of various clonal tolerance phenotypes could be maintained as long as the HBeAg concentration and/or the non-inflammatory hepatic environment remain unchanged, the immune tolerance would last 1-4 decades. During this phase, the rate of spontaneous or treatment-induced HBeAg seroconversion is less than 5%[17,18]. Patients in the immune tolerance phase are considered at a low risk of progressing to cirrhosis or HCC based on serial monitoring of virological, clinical and ultrasonographic assessments.

Although antiviral therapy is not recommended for immune tolerance patients, they should be closely monitored for progression to the immune clearance phase. Once this occurs, antiviral therapy should be considered more diligently after 6-12 mo if HBeAg seroconversion does not occur because disease progression can occur in the immune clearance phase.

Immune clearance stage

As the host immune system matures, a nonspecific increase in hepatic inflammation or decrease in HBeAg serum concentration, perhaps due to the emergence of core promoter region or pre-core region mutants, may allow activation of intermediate-or low-avidity HBeAg-specific T cell clones that are not physically deleted and/or reverse the anergic state of others[17]. Therefore, treatment modalities for chronic HBV infection should be directed at activating the relatively low-avidity HBeAg-specific T cells. Such a shift from HBeAg-specific Th_2 cell tolerance to Th_1 cell activation may recognize HBV-related epitopes on hepatocytes, and immune-mediated hepatocellular injury ensues, the so called clearance phase of CHB infection. Then, IL-2, INF and tumor necrosis factor are secreted following inflammation. The HBcAg-specific T-cell response is characterized by CTL induction, liver injury and inhibition of viral replication[6]. In patients with prenatally acquired HBV infection, transition from immune tolerance phase to immune clearance phase occurs during the second or third decade of life. Although HBV replication and viremia continue in the liver, the serum virus level becomes lower in immune clearance phase than in immune tolerance phase when viral replication is completely unopposed. The active phase of CHB is often marked by increased levels of alanine aminotransferase (ALT), necrotic inflammatory activity, and cycling HBV-DNA and HBeAg due to liver injury. Pre-core minus mutants and mutations within the core gene begin to accumulate at the time of ALT flare up because they have a better ability to evade immune clearance[19], suggesting that nucleoprotein antigens are the major immune attacking foci during chronic HBV infection, perhaps because the nucleoprotein-specific T-cell repertoire has been eroded to a lesser extent than the envelope-specific repertoire simply due to the lower concentration of HBe/HBcAgs. $CD4^+$ HBeAg-specific T cells, identified in HBeAg-single-Tg and TCR-HBeAg-Tg mice, are not deleted or anergized and remain quiescent in the presence of serum HBeAg, but can mediate seroconversion and liver injury once they are activated. These HBeAg-specific T cells escape tolerance induction due to their low avidity and/or low TCR density[18].

This active phase is characterized by the presence of HBeAg, high serum HBV DNA and aminotransferase levels, as well as active inflammations and fibrosis in the liver. A key event in the natural history of HBeAg positive CHB patients is HBeAg seroconversion[20]. Several studies have shown that seroconversion with a marked reduction in HBV replication is associated with biochemical and histological remission of inflammatory activity in the majority of patients[2,10,20]. Most studies showed that the mean annual rate of spontaneous HBeAg seroconversion ranges 8%-15% in children or adults with an elevated ALT level[20]. Although the ALT level is normal in most Asian children, their spontaneous HBeAg seroconversion rate is less than 2% during the first 3 years of age and then increases to 4%-5%. In some cases, spontaneous flare up of hepatitis is not frequently recognized because it is usually asymptomatic. Since subsequent HBeAg seroconversion would not occur in such flare up of hepatitis, it can thus be viewed as an abortive attempt at seroconversion. However, some patients present with a symptomatic flare up of hepatitis that mimics acute hepatitis and even present with fulminant hepatic failure. Regression of fibrosis occurs several months or years after HBeAg seroconversion. These flare ups of hepatitis may precede the disappearance

of HBeAg and development of HBeAg antibody, culminating in the remission of hepatitis activity. It has been recognized that the duration of immune clearance phase and the frequency and severity of flare ups are correlated with the risk of progressing to cirrhosis and HCC[21-23].

Inactive HBsAg carrier stage

This inflammatory phase of HBV infection also leads to HBeAg seroconversion and enters into inactive HBsAg carrier status. Inactive carriers form the largest group of chronic HBV infection patients. After seroconversion, most patients remain negative for HBeAg and positive for anti-HBe antibody with an undetectable or a low HBV DNA level, while the minority have undetectable viral loads. Biopsy findings can range from mild inflammation and minimal fibrosis to inactive cirrhosis if the disease is severe during immune clearance[20].

The progress of inactive HBsAg carrier state is usually benign. A long-term follow-up (up to 18 years) of these carriers can indicate a sustained biochemical remission and a very low risk of developing cirrhosis or HCC in them[19]. Patients even with no cirrhosis may develop live cancer in their inactive HBsAg carrier state. In addition, approximately 20%-30% of inactive HBsAg carriers may undergo spontaneous reactivation of hepatitis B during the follow-up. Multiple episodes of reactivation or sustained reactivation can cause progressive liver damage and even decompensation. HBV reactivation is usually asymptomatic but can occasionally mimic acute viral hepatitis. Some carriers eventually become HBsAg negative and develop anti-HBs. The estimated incidence of delayed HBsAg clearance is 1%-2% per year in Western countries where HBV infection is acquired in adulthood, and 0.05%-0.8% per year in endemic areas where HBV infection is mostly acquired perinatally or in early childhood. Prognosis can be improved by loss of HBsAg as liver disease is inactive or non-progressive, but HBsAg clearance does not completely prevent occurrence of liver decompensation or HCC in patients with cirrhosis[23-25].

Reactivation stage

Chronic HBeAg-negative patients can be divided into chronic inactive HBsAg carriers and CHB patients with biochemical and intermittent virological activity[5]. HBeAg-negative chronic hepatitis may recur in one third of inactive HBV carriers without serum reversion of HBeAg[22,23].

It is believed that seroconversion of HBeAg to HBeAb is accompanied with cessation of HBV replication and remission of liver disease. However, HBsAg-negative CHB has been recognized as an important form of chronic hepatitis, and e-antigen negativity is due to mutations in pre-core and core promoter regions. The most frequent pre-core mutation is a G-A change in nucleotide 1896 (G1896A) which creates a stop codon[23] and the most common core promoter mutation involves a substitution of nucleotides 1762 and 1764, which can result in loss of HBeAg synthesis. Loss of circulating HBeAg can decrease the induction of HBeAg-specific Th_2 cell activity and result in a predominance of inflammatory Th_1-like cells[16]. HBeAg-negative CHB (pre-core mutant) occurs as the predominant species during typical HBV infection with wild-type virus which is selected during the immune clearance phase (HBeAg seroconversion). Several studies have shown that HBeAg may be a target antigen on HBV-infected hepatocytes[5,15,18]. Failure to produce a target antigen may be a means of evading immune clearance. The clonal heterogeneity of HBeAg-specific T-cell tolerance may explain how a primarily tolerogenic protein can exert its pressure on the immune response to the selection of HBeAg negative mutant. For example, high-avidity HBeAg-specific T-cell clones may be tolerized and low-activity T-cell clones may be activated and involved in selecting HBeAg-negative mutant in the same patient[18,24-26]. The occurrence of HBeAg-negative mutants during chronic active HBV infection, especially in the presence of a high viral load, is correlated with an exacerbation of liver injury and a worse prognosis. Serum HBeAg can act as an efficient T-cell tolerogen which reduces the frequency of liver injury and down-regulates anti-HBc production. Anergy of HBc/HBeAg and HBeAg specific T-cells depends on HBeAg concentration and is reversible in the absence of HBeAg, which may explain the correlation between pre-core and core promoter mutations and severe liver injury[19,22,27,28].

Progress to this phase occurs spontaneously or to inactive carriers during immune suppression. Some patients can progress directly from HBeAg positive to HBeAg negative CHB. Identification of pre-core/core promoter mutations and recognition of HBeAg negative CHB indicate that the disease occurs after HBeAg seroconversion[19]. Age is significantly higher in HBeAg-negative patients than in HBeAg-positive patients. ALT and HBV DNA levels are significantly lower in e-antigen negative patients than in e-antigen positive patients. However, spontaneous recovery is rarer, long-term prognosis is poorer, and histological lesions are more severe in HBeAg-negative patients than in HBeAg-positive patients. Necrotic inflammatory activity is almost identical in both HBeAg-negative and positive patients. However, fibrotic activity is higher in e-antigen negative patients than in e-antigen positive patients. The estimated annual incidence of cirrhosis is 2%-6% in HBeAg positive CHB patients and 8%-10% in HBeAg negative CHB patients. The higher incidence of cirrhosis in HBeAg-negative patients is related to age and fibrosis stage, suggesting that HBeAg-negative chronic hepatitis can progress to cirrhosis and HCC in the natural history of HBV infection rather than *de nove* infection with HBV variants that do not produce HBeAg[29-31]. HBeAg-specific T cell tolerance is reversible in the absence of tolerogen. Since antiviral treatment can reduce HBeAg and viral load possibly in combination with HBc/HBeAg-specific immunization, it can alleviate chronic HBV infection by shifting the cytokine profile from Th_2 to $Th_{0/1}$[26,32].

Occult HBV infection

Occult HBV infection is defined as the existence of HBV DNA in serum, although it is not considered as a phase of CHB[33,34].

In addition to a symptomatic and serologically

Table 1 Characteristics of chronic hepatitis B at different stages

Phase	ALT	HBsAg	HBeAg	HBeAb	HBV DNA (IU/mL)	Th cell biased	Liver histology
Immune tolerance	Usually normal	Present	Present	Absent	≥ 20000	Th_2 cell	Normal or mild inflammation
Immune clearance	Elevated	Present	Present	Absent	≥ 20000	Th_2/Th_1 cell	Active inflammation
Inactive HBsAg carrier	Usually normal; can have flares	Present	Absent	Present	> 20000	Th_1 cell	Mild inflammation or inactive cirrhosis
$HBeAg^-$ CHB	Perioid flares	Present	Absent	Present	> 20000 < 20000	Th_1 cell	Active inflammation
Occult hepatitis B	Rarely elevated	Absent	Absent	Present when HBV recovered	< 20000	Th_1 cell	From normal to cirrhosis HCC

evident infection, occult persistent HBV carriage has been identified since nucleic acid amplification assay enhances its sensitivity to hepadnaviral genomes and their replicative intermediates. There is evidence that occult HBV infection is a common and long-term consequence of acute hepatitis B resolution. This form of residual infection is termed as secondary occult infection (SOI). The data from the woodchuck model of HBV infection indicate that exposure to a small amount of hepadnavirus can also cause primary occult infection where virus genome but not serological makers of virus exposure are detectable without liver involvement. However, both forms of virus replicate at a low level in the lymphatic system. Serological testing for SOI can reveal the presence of antibodies to HBV core antigen (anti-HBc), which has been recognized not only as a valuable marker of prior HBV exposure but also as an indicator of progressing occult HBV infection[35]. It has been recently reported that up to 20% of individuals with occult HBV carriage are not reactive to anti-HB_x or any other serological indicators of HBV exposure, and detection of naturally acquired antibodies to HBsAg (anti-HBc) does not exclude the existence of occult HBV infection[33,36].

The severe consequences of occult HBV infection have not been fully recognized. There is evidence that occult HBV can be a source of virus contamination in blood and organ donations, as well as a reservoir from which full blown hepatitis can arise[37]. Case reports also indicate that immunosuppression caused by chemotherapy or immunomodulatory agents or immunodeficiency due to HIV infection or hematological malignancies can induce reactive occult infection[38,39]. Mild necrotic inflammation has been documented in liver samples obtained from acute hepatitis B patients many years after recovery[40]. Liver fibrosis and cirrhosis of unknown origin have been explained by occult HBV infection in many retrospective studies[35,41,42]. The oncogenic potency of occult HBV persistence becomes progressively evident and is further elevated in alcoholics and patients with other liver ailments like hepatitis C[41,42]. No reports are available on the treatment of occult HBV infection (Table 1).

CONSEQUENCES OF CHRONIC HBV INFECTION

Individuals with chronic HBV infection are at an increased risk of developing end-stage liver diseases including cirrhosis, hepatic failure, and HCC. It has recently been estimated that about 53% of HCC cases in the world are related to HBV infection. The lifetime risk of developing HCC is increased even in patients with cleared HBsAg or occult HBV infection. Further risk factors include chronic HCV infection, exposure to aflatoxin B_1, alcohol abuse, obesity and diabetes[4,43]. Thus, it is important to identify HBV-infected patients at a higher risk of progressing to HCC.

The reason why some CHB patients progress to HCC remains unknown. Host factors, such as immune response to HBV, genetic predisposition to HCC, high HBV replication rate, mutations within the HBV genome, are related with HCC. Many observations revealed that the major factor for the development of HBV-associated HCC is the immune system[41,43,45]. Development of hepatitis, chronic hepatitis, and HCC could be exclusively observed in mice reconstituted with bone marrow and in non-transgenic animals, but not in controls, suggesting that ineffective immune response is the principle oncogenic factor during chronic HBV infection of human beings. In other words, the same T-cell response has different effects. If T cell response is strong enough, HBV can be eliminated from the liver. If not, a pro-carcinogenic effect can be induced by triggering necrotic inflammatory disease without final eradication of HBV from the liver. It can, thus, be concluded that the immune system-mediated chronic inflammation of the liver, continuous cell death and subsequent cell proliferation may increase the frequency of genetic alteration and the risk of developing cancer. However, the molecular basis of inflammatory liver carcinogenesis caused by HBV remains largely unsolved. Cytokines modulate inflammation and the presence of inflammatory cells with the production of inflammatory cytokines activates cellular oxidant-generating pathways. Reactive oxygen species that are generated in inflammatory conditions induce oxidative DNA damage and increased oxidative stress caused by chronic inflammation can produce genetic mutations and gross chromosomal alterations[4,44,45]. Extensively oxidative DNA damage has been detected in hepatocytes of HBV-transgenic mice and humans with chronic hepatitis[46].

HBV genotype C infection is associated with a higher risk of developing HCC than HBV genotype B infection[29]. The BCP A1762T/G1764A mutant is associated with an increased risk of developing HCC

compared with the double wild type variant, whereas the pre-core G1896A mutation is associated with a decreased risk of developing HCC compared with the wild-type variant. Several mechanisms of liver carcinogen are related to the BCP A1762T/G1764A mutation which may enhance HBV virulence by increasing host immune response and viral replication, or by altering the coding region of the X antigen. Mutant BCP may augment the host immune response to HBV-infected hepatocytes by diminishing circulating HBeAg and increasing hepatocyte apoptosis and regeneration, thus leading to liver injury[47,48]. The BCP mutation appears to enhance the efficacy of viral replication either by modulating the relative levels of pre-core and core RNAs or by creating a transcription factor binding site for hepatocyte nuclear factor 1. Mutations in the BCP region over lapping the coding sequence of the X antigen of HBV may result in changes of amino acids, K130M and V131I, in the X gene. These amino acid changes may interfere with cell growth control and DNA repair, thus leading to HCC[49,50]. There is experimental evidence that HBx, a multifunctional protein with oncogenic potentials, can interact with a large number of cellular factors and modulate their normal function, thus leading to deregulation of normal cell activities and HCC[46,51]. Despite its importance in HCC development, the clinical significance of genetic variability in the *x* genetic region still remains poorly understood[52].

Several factors, including age, male gender, repeated episodes of severe acute exacerbation, and HBV reactivation after HBeAg seroconversion, are related with the risk of developing advanced liver disease in patients with CHB. Previous studies showed that HBV genotype C infection is associated with later HBeAg seroconversion and multiple episodes of acute exacerbation without HBeAg seroconversion than genotype B HBV infection[9,21,25,30,49]. The delayed HBeAg seroconversion may prolong the inflammation process and subsequently result in more severe liver damage[30]. Several nucleotide mutations in the pre-core and core promoter regions may reduce HBeAg production and are associated with advanced liver disease[47]. In Asia, genotype C and T1762 and A1764 mutants may play a role in HBV-related liver cirrhosis, and can be used in predicting the clinical outcome of patients with chronic HBV infection.

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ORIGINAL ARTICLES

Constructive thinking, rational intelligence and irritable bowel syndrome

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Abstract

AIM: To evaluate rational and experiential intelligence in irritable bowel syndrome (IBS) sufferers.

METHODS: We recruited 100 subjects with IBS as per Rome Ⅱ criteria (50 consulters and 50 non-consulters) and 100 healthy controls, matched by age, sex and educational level. Cases and controls completed a clinical questionnaire (including symptom characteristics and medical consultation) and the following tests: rational-intelligence (Wechsler Adult Intelligence Scale, 3rd edition); experiential-intelligence (Constructive Thinking Inventory); personality (NEO personality inventory); psychopathology (MMPI-2), anxiety (state-trait anxiety inventory) and life events (social readjustment rating scale). Analysis of variance was used to compare the test results of IBS-sufferers and controls, and a logistic regression model was then constructed and adjusted for age, sex and educational level to evaluate any possible association with IBS.

RESULTS: No differences were found between IBS cases and controls in terms of IQ (102.0 ± 10.8 *vs* 102.8 ± 12.6), but IBS sufferers scored significantly lower in global constructive thinking (43.7 ± 9.4 *vs* 49.6 ± 9.7). In the logistic regression model, global constructive thinking score was independently linked to suffering from IBS [OR 0.92 (0.87-0.97)], without significant OR for total IQ.

CONCLUSION: IBS subjects do not show lower rational intelligence than controls, but lower experiential intelligence is nevertheless associated with IBS.

Key words: Constructive thinking; Intelligence tests; Intelligence; Irritable bowel syndrome

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common disease worldwide, with a prevalence in the general population ranging from 2% to 15%, depending on the definition criteria used[1,2]. Its pathophysiology is unknown, yet several biological factors have been implicated[3]; and aside from these biological factors, psychosocial factors have long been known to be involved in IBS. While anxiety has consistently been associated with IBS[4], to what precise degree it is a cause or consequence remains unresolved[5]. Personality traits, and neuroticism in particular, have been linked to IBS, although it is a matter of debate whether they are associated with consultation behavior[6] or with the disease itself[7].

Cognition (beliefs, interpretation and expectations) plays a pivotal role in the interaction of subjects with

the environment, and cognitive abilities should drive this interaction on a daily basis and for long-term success. Several items of information suggest that cognitive processes may be important in IBS. Abnormal illness behavior which has been associated with many chronic diseases, including non-consulters' IBS[8], is to a great extent the result of a cognitive process[9], involving appraisal and interpretation, and is likely associated with worry[10]. Hypervigilance as to abdominal perceptions may be viewed as a cognitive process[11,12]. IBS patients use less effective coping styles[6,13], which are indeed modulated (appraisal of threat, adapting the response to the situation) by the use of cognitive abilities.

Although intelligence is not easy to define[14], it may be construed as the ability to solve problems[15]. Problems arising from life may be categorized as abstract problems, which call for an analytical approach and a slow response, and daily life problems, which call for automatic analysis and a quick response. Solving abstract problems is a task performed by rational intelligence and can be measured by IQ[14]. IQ has been found to be associated with health[16] and longevity[17], likely through an influence on health knowledge and health behavior[18]. Attree *et al*[19] recently found a lower IQ in IBS subjects *vs* controls. Rational intelligence might enhance one's ability to identify environmental factors precipitating bowel symptoms and change one's lifestyle accordingly.

Solving daily problems is not a rational task but rather relies on cognitive abilities to interpret events efficiently. Epstein proposed the concept of constructive thinking (experiential intelligence), defined as automatic thoughts in daily life to survive at a minimum cost of stress[20]. According to his cognitive-experiential theory, constructive thinking operates passively and automatically at a preconscious level, thinking in terms of associations and broad categories, is closely connected with emotions, interpreting experience, and guiding conscious thoughts and behaviour. It can be measured by the Constructive Thinking Inventory (CTI), which is independent of IQ and moderately related to success in life and physical well-being[21]. Under the hypothesis of stress-driven changes in brain-gut interaction (IBS as emotional motor system output)[22], it might be hypothesized that the lower a person's constructive thinking, the higher the stress generated by daily life and, by extension, the higher the possibility of suffering from IBS symptoms.

Accordingly, this study sought primarily to assess whether IBS sufferers might be different to healthy non-IBS sufferers in terms of intelligence (rational and experiential), and, secondarily, whether there might be a link between intelligence and IBS-related medical consultation.

MATERIALS AND METHODS

Study design

Case-control study including IBS sufferers and matched healthy subjects.

Study population

Cases were defined as subjects, both consulters and non-consulters, who met the Rome Ⅱ IBS criteria. An IBS consulter was defined as any currently symptomatic subject who had consulted a physician due to bowel symptoms and had been diagnosed with IBS after an appropriate work-up. An IBS non-consulter was defined as any subject from the general population with symptoms of IBS who had not consulted a physician in this connection.

Controls were defined as subjects from the general population without recurrent abdominal pain or bowel symptoms, who suffered no relevant chronic diseases and had undergone no relevant abdominal surgery.

Recruitment, selection and inclusion

IBS consulters were recruited from primary care and secondary-level gastroenterologist offices at the 7th Health Area of Madrid (Spain), which provides medical attention to approximately 515 000 inhabitants. Patients diagnosed with IBS in accordance with the above definition, were invited to participate. Patients were not enrolled at a tertiary-care facility so as to better represent the population of patients with IBS.

IBS non-consulters and healthy controls were recruited from the general population residing in the same geographical area as patients. Members of the public were directly approached at corporate offices, leisure centers or department stores and invited to participate in the study. Relatives of recruited IBS patients and patients or patients' relatives attending medical facilities were excluded. After initial agreement, all subjects were briefly interviewed about any medical conditions (to exclude relevant chronic diseases), recurrent abdominal pain, bowel-related symptoms (to classify them as potential IBS subjects as per Rome Ⅱ criteria or as subjects free of bowel-related symptoms), and prior medical consultation on account of such symptoms (to classify them as non-consulters): subjects with bowel-related symptoms who failed to meet the Rome Ⅱ criteria as well as subjects who met the Rome Ⅱ criteria but reported consulting a physician in this regard were not selected.

All subjects selected-IBS consulters, non-consulters, and controls-completed a clinical questionnaire, which included questions on sociodemographic data, symptoms and medical resource utilization, including number of physician visits due to bowels symptoms in the prior year. This questionnaire was successfully used in a population-based IBS study[2], and enables Rome Ⅱ criteria as well as consultation behavior in the preceding year to be verified. Inclusion criteria for IBS subjects (patients and non-patients) were age 18-65 years and Rome Ⅱ criteria checked by the clinical questionnaire. Healthy controls were matched to IBS patients by age (± 5 years), sex, and educational level (junior school, high school, and university). Exclusion criteria were history of psychiatric disease requiring treatment; significant visual or hearing deficit; and inability to complete the set of instruments used in the study.

Instruments

In addition to the clinical questionnaire, the study design included instruments that measured IBS severity, rational intelligence, non-intellectual intelligence, personality traits, psychopathology, and life events. These instruments were respectively.

Functional bowel disease severity index (FBDSI)

The FBDSI was developed by Drossman *et al*[23] and has been shown to correlate with symptoms interference with daily functioning and health related quality of life[24,25]. It comprises three variables, namely: current pain [evaluated by a visual analog scale (VAS)]; diagnosis of functional chronic abdominal pain (chronic pain without bowel dysfunction); and the number of medical visits in the preceding 6 mo. Severity was classified as mild (< 37), moderate (37-110) or severe (> 110). Since severity in IBS subjects as rated by the complete index included medical consultation, both the complete FBDSI and current pain assessed by VAS were analyzed.

Wechsler Adult Intelligence Scale, 3rd edition (WAIS-Ⅲ)

The WAIS-Ⅲ was designed as a comprehensive test of cognitive ability for adults. It contains 11 subtests and three additional subtests. Variables obtained are three IQ scores (total IQ, verbal IQ, and performance IQ). It has been adapted and validated for use in the Spanish population, and results were scored using normative values[26]. The test was taken in quiet surroundings and all subjects underwent a brief psychological interview beforehand; special care was taken to minimize potential anxiety regarding test performance. Moreover, it was clearly stated at the time of inclusion that test results would in no case be linked to any care change in IBS subjects or to any specific psychological intervention. Subjective scoring of the WAIS-Ⅲ was done blinded to subjects' study group.

CTI

The CTI is a self-administered test developed by Epstein under the conceptual framework of Cognitive-Experiential Theory[20]. Constructive thinking is a set of automatic habitual thoughts used in daily life, and is regarded as a measure of experiential intelligence[21]. CTI provides a global measure of constructive thinking, six subscales (Emotional coping, Behavioral coping, Personal superstitious thinking, Categorical thinking, Esoteric thinking, and Naive optimism), and two validity subscales (Defensiveness scale and Validity scale). This test has been adapted and validated for use in the Spanish population, normative values for which are available[27]. T-scores were obtained using a computerized scoring software program. Under the rules of the Spanish Manual, tests were deemed invalid when the T-score was above 70 on the defensiveness scale or below 30 on the validity scale.

NEO personality inventory (NEO-PI)

The NEO-PI is a widely used personality inventory designed to measure personality based on the "big five" theory. The Spanish version is a validated instrument and normative data are available. T-scores for the five traits (neuroticism, extroversion, openness, agreeableness, and consciousness) were obtained using a computerized scoring software program.

State-trait anxiety inventory (STAI)

A widely used questionnaire with a validated Spanish version, the STAI is a 40-item self-report measure designed to assess anxiety. Subjects indicate how they generally or right now feel, by rating the frequency of their feelings of anxiety on a 4-point scale ranging from 1 (almost never) to 4 (almost always).

Minnesota multiphasic personality inventory 2 (MMPI-2)

The MMPI-2 is a widely use inventory for the assessment of psychopathological personality. Normative values for the Spanish population are available. Clinical scales were included (hypochondriasis, depression, hysteria, psychopathic deviate, paranoia, psychasthenia, schizophrenia, hypomania, and social introversion).

Social readjustment rating scale (SRRS)

The SRRS is a measure of stressful life events developed by Holmes and Rahe in 1967[28], consisting of a 61-item list of significant life events. A validated Spanish version exists, which includes a scale of each item's emotional impact scored from 0 to 100[29]. On the 61-item checklist, the participants marked events that they had experienced in the previous year. The number of stressful life events and their total emotional impact in the preceding year were obtained for each subject.

Statistical analysis

Primary analysis was intended to disclose differences in intelligence (rational and non-intellectual) between IBS subjects (both patients and non-patients) and healthy controls. For this purpose, analysis of variance was used to perform univariate comparisons of WAIS-Ⅲ and CTI measures, and sociodemographic data, personality traits and life events were also compared between the respective groups. To evaluate to what extent global constructive thinking and IQ variables might be associated with suffering from IBS, a logistic regression model was constructed (model 1), adjusting for age, sex and educational level; a full model was constructed to adjust for variables showing significant differences between IBS and control subjects, excluding those with correlation coefficients of 0.7 or higher with Global CTI score or IQ.

A secondary analysis was performed to evaluate the possible implication of measured factors in medical consultation sought because of IBS symptoms. To this end, IBS consulters and non-consulters were compared, using the same statistical model. To evaluate illness behavior, a multiple regression model was constructed (stepwise forward method), with the number of physician visits in the last year as a dependent variable and global constructive thinking, total IQ, neuroticism, number of life stressful events, clinical scales of MMPI-2

and state and trait anxiety as independent variables. Data are expressed as mean ± SD, unless otherwise stated. Statistical analysis was performed using the SPSS version 13 computer software package.

Composition of case group and calculation of sample size

We chose a ratio of 1:1 for IBS consulters and non-consulters in the case group, which may well represent the entire IBS population in this country (49% of IBS subjects as defined by the Rome Ⅱ criteria had visited a physician in the previous year)[30].

As there were no experimental data at the time of protocol design (January 2003) for making assumptions in similar settings, a five-point difference in the overall CTI scale was taken as relevant. This five-point estimate was based on the Spanish correction manual, which suggests that T-scores of 45 to 55 be regarded as normal, and 35 to 44 as moderately low. Since the mean T score for the general population could be expected to be 50, the minimum value for inferring a relevant lower score would be 44 (a five-point margin being sufficient to detect relevant differences). Hence, assuming a standard deviation of 10 (since this is a T-score), and with α and β risks set at 0.05 and 0.1 respectively, 85 subjects would be needed in each group.

In so far as IQ was concerned, a difference of 10 points was estimated as relevant. Assuming a standard deviation of 15, then, with the same α and β risk, the sample size would be 56 subjects in each group. Accordingly, a sample of 85 subjects in each group would be enough to enable a mean difference of eight points or higher to be detected. Allowing for the possibility of a 15% data loss, the final sample size was set at 100 subjects per group.

For the secondary analysis, assuming the same relevant differences in mean scores and the same standard deviations, a sample size of 50 per group (consulters and non-consulters) would provide a power of 80% with an α risk of 0.1.

Ethics

The study was formally approved by the Institutional Ethics Committee, and prior informed consent was obtained in writing from all participants.

RESULTS

A total of 73 IBS consulters and 81 IBS non-consulters were recruited. Twenty-three IBS consulters were not included because of psychiatric diagnosis requiring treatment (four patients), not accomplishing Rome Ⅱ criteria (two patients), incomplete information (one patient) or lack of adequate matching control (16 patients). Thirty-one IBS non-consulters were not included due to not accomplishing Rome Ⅱ criteria (10 subjects), incomplete information (one subject) or lack of adequate matching control (20 subjects).

The study covered 100 subjects with IBS (50 consulters and 50 non-consulters) and 100 matching healthy controls. Of these, 70 were female in each case. The mean age of IBS subjects was 37.2 ± 12.6 years, and there were no differences vis-à-vis healthy controls (37.2 ± 13.1). Both groups reported 18 subjects with junior school, 23 with high school, and 59 with university education. Controls and IBS subjects were similar in terms of: marital status (single 50 and 53, and married 43 and 40, respectively); work status (student 10 and 9, gainfully employed 85 and 84, retired or unemployed 7 and 7, respectively); and family monthly income (€ 2684 ± 1469 and € 2354 ± 2272, respectively).

Table 1 Comparison of intelligence, personality, and life events between controls and IBS subjects

	Healthy subjects (n = 94)	IBS subjects (n = 94)
Rational intelligence (WAIS-Ⅲ)		
Total IQ	102.8 ± 12.6	102.0 ± 10.8
Verbal IQ	103.4 ± 11.6	102.6 ± 10.7
Performance IQ	102.4 ± 14.2	101.6 ± 11.6
Experiential intelligence (CTI)		
Global CTI score	49.6 ± 9.7	43.7 ± 9.4[b]
Personality traits (NEO-PI)		
Neuroticism	55.0 ± 10.6	60.9 ± 8.7[b]
Extroversion	44.6 ± 11.0	45.4 ± 10.9
Openness	49.6 ± 12.6	50.6 ± 12.2
Agreeableness	45.3 ± 7.9	44.1 ± 9.0
Consciousness	37.8 ± 9.0	35.9 ± 8.2
Psychopathology (MMPI-2)		
1-Hypochondriasis	49.6 ± 9.2	62.2 ± 12.1[b]
2-Depression	48.7 ± 9.6	52.9 ± 10.9[a]
3-Hysteria	52.2 ± 9.6	60.5 ± 11.5[b]
4-Psychopathic deviate	49.8 ± 9.2	54.8 ± 11.0[b]
5-Masculinity-femininity	52.2 ± 10.6	50.3 ± 10.0
6-Paranoia	48.2 ± 8.2	52.3 ± 9.2[a]
7-Psychasthenia	46.8 ± 8.7	51.7 ± 8.3[b]
8-Schizophrenia	47.0 ± 8.1	51.5 ± 10.0[b]
9-Hypomania	49.1 ± 9.7	52.3 ± 10.3[a]
0-Social introversion	47.4 ± 8.9	48.6 ± 8.3
Anxiety (STAI)		
State anxiety	33.4 ± 8.8	40.5 ± 11.4[b]
Trait anxiety	37.1 ± 9.2	43.7 ± 10.8[b]
Stressful life events (SRRS)		
Number in prior 12 mo	8.5 ± 5.1	9.9 ± 4.8[a]
Total emotional impact	445.1 ± 280.4	521.9 ± 252.5[a]

IBS: Irritable bowel syndrome. [a]P < 0.05 (ANOVA), [b]P < 0.001 (ANOVA) *vs* controls.

IBS features

Among IBS sufferers, 31 were classified as per the Rome Ⅱ criteria as diarrhea-predominant, 26 as constipation-predominant, and 43 as alternating. With respect to severity of IBS, 55 subjects were classified as mild, 37 as moderate, and eight as severe, with a mean FDSI score of 47.0 ± 49.4, and a mean current VAS pain of 29.2 ± 24.7. Seventy-seven subjects reported IBS symptoms of more than 2 years duration.

Intelligence, personality, psychopathology, anxiety and life events in IBS subjects

Comparison of intelligence, personality, and life events between controls and IBS subjects were shown in Table 1. Six subjects (four controls, one IBS consulter, and one IBS non-consulter) produced invalid CTI scores, so that they and their matched counterparts were excluded from the analysis. IBS subjects registered similar IQ test results

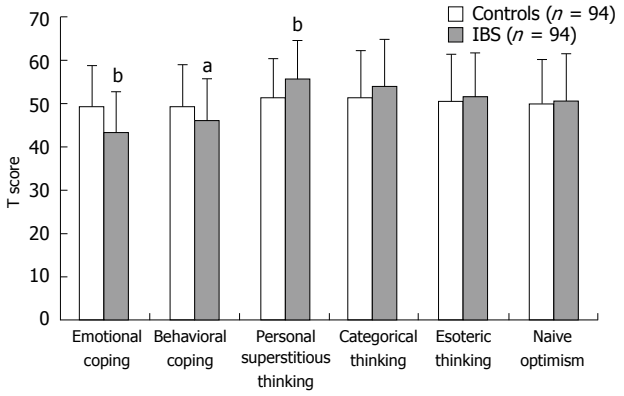

Figure 1 Subscales of constructive thinking in IBS and controls. [a]$P < 0.05$, [b]$P < 0.01$ *vs* controls.

Table 2 Regression models to evaluate the association of Global CTI and IQ with suffering IBS

	Model 1	Full model
Global CTI score	0.93 (0.90-0.97)[d]	0.92 (0.87-0.97)[b]
Total IQ	0.99 (0.96-1.03)	0.99 (0.97-1.04)
Stressful life events (SRRS)		1.05 (0.97-1.15)
State anxiety		1.05 (0.99-1.10)
1-Hypochondriasis		1.19 (1.11-1.27)[d]
2-Depression		0.95 (0.90-1.00)
3-Hysteria		0.96 (0.90-1.02)
4-Psychopathic deviate		1.02 (0.96-1.08)
6-Paranoia		1.04 (0.99-1.10)
7-Psychasthenia		1.01 (0.93-1.09)
8-Schizophrenia		0.93 (0.85-1.01)
9-Hypomania		1.02 (0.97-1.07)

Adjusted by age, sex and educational level. [b]$P < 0.01$ (ANOVA); [d]$P < 0.001$ (ANOVA). Hosmer and lemeshow goodness-of-fit test for the full model: $P = 0.16$. CTI: Constructive Thinking Inventory.

to the controls, without differences in total, verbal and performance IQ. However, IBS subjects scored lower in global constructive thinking and higher in neuroticism, and reported more stressful life events in the prior year than did controls. The detailed CTI subscale scores obtained by healthy controls and IBS subjects are shown in Figure 1.

Global CTI score was correlated to neuroticism ($r = 0.71$) and trait anxiety ($r = 0.71$), so these last variables were not included in the model. Table 2 shows the results of the logistic regression model. When neuroticism and trait anxiety were included, scale 1 of MMPI-2 (hypochondriasis) [OR 1.19 (1.11-1.27)] and global CTI score [OR 0.93 (0.87-0.99)] remained independently related, at the same magnitude, to suffering from IBS, without significant OR for any other variable.

IBS consulters vs non-consulters

Two subjects (one IBS consulter and one IBS non-consulter) produced invalid CTI scores and were thus excluded from the analysis. There were no significant differences between IBS consulters and non-consulters in terms of age, sex, educational level, marital status, work status and family monthly income. Furthermore, IBS features (distribution of subtypes, evolution of bowel symptoms) were similar in both groups, except for severity, with differences between consulters and non-consulters in FDSI scores (70.8 ± 57.8 *vs* 23.5 ± 21.7; $P < 0.001$) and current pain on VAS (34.4 ± 25.5 *vs* 23.5 ± 21.7; $P < 0.05$). IBS consulters displayed lower total and verbal IQ and lower global constructive thinking than did IBS non-consulters, without differences in personality traits and stressful life events in the preceding year (Table 3). CTI subscale scores obtained by IBS consulters and non-consulters are shown in detail in Figure 2.

In the logistic regression model, adjusted for age, sex and educational level, no variable was independently

Table 3 Comparison of intelligence, personality, and life events between IBS consulters and non-consulters

	IBS non-consulters (n = 49)	IBS consulters (n = 49)
Rational intelligence (WAIS-Ⅲ)		
Total IQ	103.8 ± 11.2	99.3 ± 10.5[a]
Verbal IQ	104.4 ± 11.0	100.1 ± 10.4[a]
Performance IQ	103.0 ± 11.7	99.1 ± 11.6
Experiential intelligence (CTI)		
Global CT score	46.6 ± 9.2	41.1 ± 9.0[d]
Personality traits (NEO-PI)		
Neuroticism	59.7 ± 7.5	62.1 ± 9.6
Extroversion	46.6 ± 11.5	43.3 ± 10.2
Openness	52.5 ± 11.0	48.6 ± 13.0
Agreeableness	45.5 ± 8.4	42.7 ± 9.2
Consciousness	35.9 ± 7.4	35.7 ± 8.9
Psychopathology (MMPI-2)		
1-Hypochondriasis	60.1 ± 12.0	64.6 ± 11.8
2-Depression	50.4 ± 9.8	55.8 ± 11.2[a]
3-Hysteria	58.6 ± 12.3	62.7 ± 10.5
4-Psychopathic deviate	52.5 ± 9.7	57.3 ± 11.9[a]
5-Masculinity-femininity	49.2 ± 11.2	50.9 ± 8.4
6-Paranoia	51.4 ± 9.5	53.1 ± 8.8
7-Psychasthenia	49.8 ± 8.4	54.1 ± 8.0[a]
8-Schizophrenia	50.5 ± 9.3	52.9 ± 10.4
9-Hypomania	51.7 ± 9.1	52.6 ± 11.3
0-Social Introversion	47.9 ± 8.5	49.4 ± 8.0
Anxiety (STAI)		
State anxiety	37.2 ± 10.0	43.6 ± 11.6[a]
Trait anxiety	41.4 ± 9.5	45.8 ± 11.4[a]
Stressful life events (SRRS)		
Number in prior 12 mo	10.3 ± 5.3	9.5 ± 4.4
Total emotional impact	539.2 ± 274.4	500.8 ± 233.3

[a]$P < 0.05$ (ANOVA), [b]$P < 0.001$ (ANOVA) *vs* non-consulters.

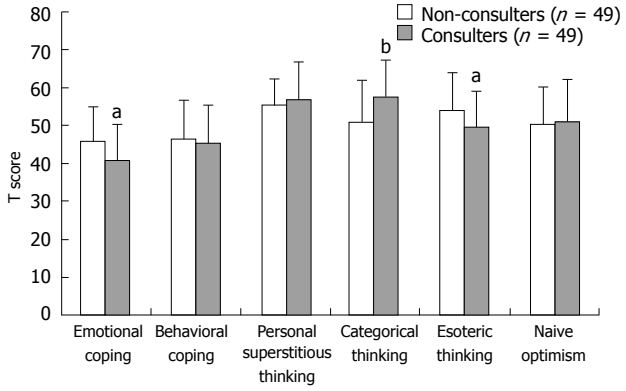

Figure 2 Subscales of constructive thinking in IBS consulters and non-consulters. [a]$P < 0.05$, [b]$P < 0.01$ *vs* non-consulters.

Table 4 Results of stepwise multiple linear regressions to predict number of physician visits among IBS consulters

Dimension	Variables entered in the model	Standardized coefficient (β)	Model
Step 1	1-Current pain (VAS)	-0.38[b]	$r^2 = 0.13$[d]
Step 2	1-Current pain (VAS)	-0.39[b]	$r^2 = 0.22$[d]
	2-Age	-0.33[a]	

[a]$P < 0.05$, [b]$P < 0.01$; [d]$P < 0.001$.

associated with IBS-related health care seeking, Among those who had sought health care, the number of physician visits correlated with age ($r = 0.31$; $P < 0.05$), current pain ($r = 0.38$; $P < 0.01$) and hypochondriasis ($r = 0.29$; $P < 0.05$). The number of physician visits among IBS consulters was predicted by current pain, evaluated by VAS, and age in the stepwise multiple linear regression model (Table 4).

DISCUSSION

The main finding of our study is that experiential rather than rational intelligence is associated with IBS *per se*, supporting the role of cognitive factors, specifically through generation of stress from daily life events.

Our results reject the hypothesis of IBS subjects having a lower rational intelligence. Attree *et al*[19] recently reported that IBS subjects had a full and verbal IQ slightly lower than controls and a deficit in verbal *vs* performance IQ. Differences in our results may be due to differences in the study sample. We studied a convenience sample of subjects with IBS covering the whole spectrum of the disease, without interest in psychological management or prior psychiatric diagnosis, while Attree *et al*[19] included a group of IBS subjects who had previously expressed an interest in participating in research at a school of psychology. Indeed, our finding that consulters displayed a slightly lower full and verbal IQ than non-consulters points to this explanation. Although a subtle cognitive impairment in some IBS patients cannot be ruled out, this would not seem to be true of the majority of IBS subjects.

Our results support the hypothesis of a lower level of constructive thinking among IBS subjects. The main CTI measure is defined as the ability to adapt the way of thinking to different situations (flexibility in thinking), while one of the main scales (emotional coping) is primarily described as the ability to appraise situations as a challenge rather than a threat. The relationship between physical symptoms and constructive thinking seems to be mediated by stress, mostly self-produced, through the generation of negative emotions[31]. Moreover, in a common stressful situation such as pregnancy, constructive thinking was shown to impact both on cognitive appraisal of stress (reducing the need for coping and adjustment) and on active coping responses, once the situation has been perceived as stressful[32]. Low constructive thinking suggests that subjects with IBS have a higher probability of experiencing daily life events that are not intrinsically stressful, as being stressful, due to cognitive appraisal.

Stress is thought to play a pre-eminent role in generating and sustaining IBS symptoms. Over 50% of IBS subjects-consulters[33] and non-consulters alike[34] report that stressful events precipitate or worsen their symptoms. While some previous studies have observed that IBS subjects experience a slightly greater number of stressful life events than do controls or patients with other digestive disease[35,36], other studies have observed no differences in this regard[6,37]. In a prospective study, Levy *et al*[38] failed to find differences between IBS subjects and controls in the number of positive or negative daily events, but self-reported daily stress was higher among IBS subjects. Hence, daily events are perceived as being more stressful by IBS subjects, and indeed, a recent Japanese study showed that IBS subjects scored higher on a perceived stress scale[39].

Emotional distress plays a role in IBS and may also impact on daily stress. The association between low constructive thinking and IBS does not seem to be explained by emotional distress, as an association in multivariate analysis persisted even when adjusting for emotional distress measures, like MMPI clinical scales. Although we excluded subjects with a psychiatric diagnosis requiring treatment, our sample of IBS subjects still suffer higher emotional distress than control subjects, to a level similar to that reported by Drossman *et al*[6].

Constructive thinking correlated to neuroticism; a correlation has been previously reported with the CTI emotional coping subscale, which has been deemed to represent the cognitive component of neuroticism[21]. Neuroticism is a broad concept that includes cognitive, affective, and behavioral traits[40]. Most[5,41-44], although not all[36] studies have reported an association between neuroticism and IBS. However, constructive thinking seems to account for the relationship between neuroticism and IBS, suggesting the relevance of cognitive factors in IBS.

Several studies have shown that IBS is associated with less effective strategies to cope with stress[45,46], specifically with symptoms. Thus, the difference between constructive thinking and coping response merits some comment. Constructive thinking would be mostly implicated in the automatic and holistic cognitive appraisal of any event, prior to the occurrence of emotion, and operates at a preconscious level in a way in which a person is unaware[47]. Coping is usually defined as cognitive and behavioural efforts to manage a troubled person-environment relationship; it is the reaction to a conscious appraisal of the situation[48]. Thus, interpretational activity may occur outside awareness and may be the non-conscious trigger of an emotional response[49]. Such a process is likely involved in the activation of the anterior cingulate cortex during subliminal and supraliminal stimulation[50]. Moreover, hypnotherapy is effective in IBS and, although the underlying mechanism is unknown[51], it might be hypothesized that it operates partly through a change in preconscious appraisal of internal events[52,53].

Another interesting finding is the association between constructive thinking and IBS-related medical consultation. In the 1980s and 1990s, psychological factors were regarded as predictors of health care sought by IBS subjects[6,54], but recent population-based studies suggest that such factors are not quite so important[7,39,55]. Our results are closer to these latter studies, inasmuch as severity of symptoms is seen as the leading factor accounting for medical consultation, without any role for psychological factors. Nevertheless, univariate analysis showed a lower level of constructive thinking in those who had sought health care compared to those who did not; consulters displayed lower emotional coping and higher categorical thinking scores than non-consulters, a finding that points to appraisal of events (i.e. symptoms) as a threat and a more rigid mindset inclined to simplistic solutions as the main differences in thinking between those seeking care and non-consulters. However, constructive thinking does not seem to have a significant role in illness behavior, in view of the lack of correlation with the number of physician visits.

The main limitation of our study lies in the fact that subjects were not extracted from the general population strictly at random. We opted for a semi-random approach owing to the difficulties posed by recruiting our target population in a purely random fashion. There were four requirements for inclusion, namely: presence of bowel symptoms as per Rome Ⅱ or, alternatively, absence of such symptoms; absence of any psychiatric diagnosis requiring treatment; consent to undergo in-depth evaluation, including a 2-h WAIS-Ⅲ interview and a number of self-administered questionnaires; and, the need to be matched by age, sex and educational level. The direct-invitation approach allowed for a brief 5-min conversation to assess these criteria, and subjects were recruited in different environments (work and leisure centers) unrelated to medical facilities. Indeed, results from control subjects support this approach, since test profiles proved quite similar to data expected from the general population (mean T-scores of around 50 ± 10; IQ around 100 ± 15). In the case of IBS non-consulters, the data were comparable to the results of Mearin *et al*[2] based on a random Spanish-population sample. Thus, the results in no way suggest that there was any selection bias.

COMMENTS

Background

Irritable bowel syndrome (IBS) has been related to psychological distress and consequences of the stress driven emotional outputs. Cognitive abilities drive the interaction of the subject with the environment, which is the basis for stress, and may participate in abnormal illness behaviour, hypervigilance, and less effective coping abilities. One study suggested a lower intelligence in IBS patients.

Research frontiers

This study is the first to comprehensively evaluate intelligence (rational and experiential intelligence) in an adequate sample of cases and controls.

Innovations and breakthroughs

The potential role of intelligence in the pathophysiology of IBS is tested. This arm (cognition ability) of the subject-environment interaction has not been formally tested.

Applications

This study provides support for the role of experiential intelligence in IBS. Moreover, experiential intelligence may be improved with psychological therapies.

Peer review

The authors exposed patients and controls to eight different psychological tests in an effort to identify correlations/associations for IBS and psychological phenotypes. The article is well written and the contents are credible.

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ORIGINAL ARTICLES

Enhancement patterns of pancreatic adenocarcinoma on conventional dynamic multi-detector row CT: Correlation with angiogenesis and fibrosis

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Abstract

AIM: To evaluate retrospectively the correlation between enhancement patterns on dynamic computed tomography (CT) and angiogenesis and fibrosis in pancreatic adenocarcinoma.

METHODS: Twenty-three patients with pancreatic adenocarcinoma underwent dynamic CT and tumor resection. In addition to the absolute and relative enhanced value that was calculated by subtracting the attenuation value on pre-contrast from those on contrast-enhanced CT in each phase, we defined one parameter, "tumor-aorta enhancement ratio", which was calculated by dividing enhancement of pancreatic cancer by enhancement of abdominal aorta in each phase. These enhancement patterns were correlated with the level of vascular endothelial growth factor (VEGF), microvessel density (MVD), and extent of fibrosis.

RESULTS: The absolute enhanced value in the arterial phase correlated with the level of VEGF and MVD (P = 0.047, P = 0.001). The relative enhanced value in arterial phase and tumor-aorta enhancement ratio (arterial) correlated with MVD (P = 0.003, P = 0.022). Tumor-aorta enhancement ratio (arterial) correlated negatively with the extent of fibrosis (P = 0.004). The tumors with greater MVD and higher expression of VEGF tended to show high enhancement in the arterial dominant phase. On the other hand, the tumors with a larger amount of fibrosis showed a negative correlation with the grade of enhancement during the arterial phase.

CONCLUSION: Enhancement patterns on dynamic CT correlated with angiogenesis and may be modified by the extent of fibrosis.

Key words: Computed tomography; Contrast media; Pancreatic cancer; Angiogenesis

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Hattori Y, Gabata T, Matsui O, Mochizuki K, Kitagawa H, Kayahara M, Ohta T, Nakanuma Y. Enhancement patterns of pancreatic adenocarcinoma on conventional dynamic multi-detector row CT: Correlation with angiogenesis and fibrosis. *World J Gastroenterol* 2009; 15(25): 3114-3121 Available from: URL: http://www.wjgnet.com/1007-9327/15/3114.asp DOI: http://dx.doi.org/10.3748/wjg.15.3114

INTRODUCTION

Pancreatic cancer is one of the leading causes of cancer-related death, with an overall 5-year survival rate of < 5%[1]. Surgical resection is still the only potentially curative treatment for pancreatic cancer. However, the resection rate is < 40%[2,3] because of the difficulty in achieving early detection. In addition, the results of other treatment methods including radiation therapy and chemotherapy are also poor.

Angiogenesis is the development of new blood vessels and is required for tumor growth. In the 1970s, Folkman reported that the development of neoplasms is angiogenesis-dependent[4,5], with this process induced by angiogenic factors such as vascular endothelial growth factor (VEGF). As a result, microvessel density (MVD) increases in neoplasms. Recently, it has been clarified that the grade of tumor angiogenesis is a useful prognostic marker in human cancers[6-9], including pancreatic cancer[10-14]. Generally, tumors with strong expression of angiogenesis show a poor prognosis. Therefore, anti-angiogenic treatment may be effective in improving the prognosis of patients with neoplasms including pancreatic cancer.

Evaluation of the grade of angiogenesis is important as a prognostic marker and is necessary for deciding the indications and evaluating the effect of anti-angiogenic treatment. For this, biopsy is necessary. However, because repeated biopsy is often difficult and invasive, and the specimen obtained does not always reflect the entire tumor, to establish the grade of tumor angiogenesis by non-invasive imaging may be important clinically. There have been several reports evaluating the correlation between angiogenesis and imaging findings in several types of cancers[15-18], but only a few such reports on pancreatic adenocarcinoma[19,20]. Recently, perfusion computed tomography (CT) has been used to measure the hemodynamic characteristics of various tumors, and many authors have reported the results of perfusion CT in this context[21-23]. The correlation of perfusion CT findings and MVD in lung cancer[24,25] and the evaluation of the effect of anti-angiogenic therapy by perfusion CT[26,27] have been described. However, this method requires an additional procedure for conventional CT examination and a special CT machine or software. In addition, its usefulness for pancreatic cancer is now under investigation. For the present, anti-angiogenesis agents are still not approved for the treatment of pancreatic cancer. However, as a preliminary investigation for future clinical application, to predict the grade of angiogenesis by conventional dynamic multidetector CT (MDCT), most commonly performed for the diagnosis of pancreatic cancer, would be useful clinically.

The purpose of the present study was to evaluate the validity of conventional dynamic MDCT findings to predict angiogenesis in pancreatic cancer. We analyzed retrospectively the correlation between the enhancement on CT and the histopathological findings, including the grade of tumor angiogenesis, with special reference to MVD and expression of VEGF, and the extent of fibrosis in surgically resected pancreatic adenocarcinoma.

MATERIALS AND METHODS

Patients

Thirty-six patients with pancreatic cancer underwent surgical resection between January 2003 and October 2004. Among them, 10 patients did not receive dynamic CT examination and were excluded from the study. Additionally, two with adenosquamous carcinoma and one with mucinous carcinoma were excluded. Finally, 23 patients (15 men and eight women; age range, 34-79 years; mean age, 62.6 years) with tubular adenocarcinoma of the pancreas were evaluated. All patients underwent dynamic CT, surgical resection, and histopathological examination. The range of tumor sizes was 20-48 mm and the mean was 40.5 mm.

Our institutional review board approved this retrospective study and informed consent for the use of medical records was obtained from the patients.

CT imaging

CT images were obtained using a multi-detector row CT scanner (LightSpeed Ultra 16; GE Medical Systems, Milwaukee, WI, USA). The scanning parameters were 2.5 mm section thickness, pitch of 1.5, 120 kV, and auto mA. After pre-contrast CT scans, arterial dominant phase images of dynamic CT were obtained starting 30 s after the beginning of the intravenous bolus injection (3 mL/s) of 100 mL of iodized contrast medium at 300 mg/mL. The pancreatic phase and the late phase (near equilibrium phase) were also obtained, starting at 50 and 90 s after injection, respectively.

Imaging analysis

Two radiologists (Y.H., T.G., one with > 7 years and the other with > 23 years of experience in pancreatic imaging) evaluated retrospectively all images and determined a decision with consensus circular regions of interest (ROI) were decided in the most enhanced area of the tumor in the pancreatic phase, excluding cystic or necrotic areas and adjacent pancreatic parenchyma, and in the abdominal aorta of the same slice, and ROIs in the other phases were drawn on the same site. Attenuation values were measured in Hounsfield units (HU) (absolute enhanced value). The relative enhanced value was calculated by subtracting the attenuation value on pre-contrast CT from those on contrast-enhanced CT in each phase. Furthermore, we defined one parameter as follows. The "tumor-aorta enhancement ratio" was calculated by dividing the attenuation value (HU) of pancreatic cancer by that of the abdominal aorta in each phase of contrast-enhanced CT, as a parameter of the grade of tumor enhancement. The ratio in arterial dominant phase was defined as the "tumor-aorta enhancement ratio (arterial)", and in the pancreatic phase "tumor-aorta enhancement ratio (pancreatic)" and in late phase "tumor-aorta enhancement ratio (late)", respectively.

Immunohistochemical staining

Surgical specimens of 5 mm thickness were cut in the axial plane for pancreatic head cancer and the sagittal plane for pancreatic body and tail cancer. Formalin-fixed, paraffin-embedded tissues were sectioned at 4 μm thickness. The sections were stained with hematoxylin and eosin. Immunohistochemical and elastica van Gieson (EVG) staining was performed in the section that corresponded to the particular CT slices employed for the evaluation of enhancement pattern of the tumor. Immunohistochemical

staining was performed using the dextran polymer system (EnVision+ System; DAKO, Glostrup, Denmark). Color development was performed using 3,3'-diaminobenzidine tetrahydrochloride (DAKO), followed by hematoxylin counterstaining. For the detection of VEGF, which is an angiogenic factor, we used rabbit polyclonal anti-VEGF antibodies (A-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:100. The sections were heated in citrate buffer (pH 6.0; 10 mmol/L) using microwaves at 95℃ for 20 min, and incubated at 4℃ overnight in humid chambers with primary antibodies. For the detection of CD34 expressed on small-vessel endothelial cells, we used mouse monoclonal anti-CD34 antibodies (clone GBEnd/10; IMMUNOTECH, Marseilles, France) at a dilution of 1:200. The sections were incubated at room temperature for 1 h with primary antibodies. Islets of Langerhans and endothelium of arterial branches were used as internal positive controls for VEGF and CD34, respectively.

Histopathological analysis

One author (Y.H.) evaluated the anonymous histological specimens without any information about the radiological images under assistance of one pathologist (Y.N., with > 30 years of experience). The level of VEGF staining was scored in comparison with that in the islets of Langerhans as a positive control: score 1, extremely weak; score 2, weak; score 3, mildly weak; score 4, almost equal. Each CD34-stained slide was scanned at a low magnification (× 40) to determine five "hot spot" areas of the largest number of microvessels. MVD was determined according to the mean number of microvessels counted in the five hot spots at high magnification (× 200). The extent of fibrosis was scored according to the ratio of fibrosis in the tumor with EVG staining in which elastic fibers were stained dark brown and collagen fibers were stained pink, with a score of 1, 0%-25%; 2, 25%-50%; and 3, 50%-100%.

Statistical analysis

Statistical software (Dr. SPSS Ⅱ for windows; SPSS, Chicago, IL, USA) was used for statistical analysis. The extent and dynamics of enhancement on dynamic CT were correlated with the level of VEGF, MVD and extent of fibrosis, to analyze whether the dynamic CT parameters defined above reflect the histopathological findings, including tumor angiogenesis. In addition, we also analyzed the correlation among the expression of VEGF, MVD and fibrosis. For these analyses, Spearman's rank correlation test was used. $P < 0.05$ was considered to indicate a significant difference.

RESULTS

Correlation between absolute and relative enhanced values and histopathological findings

Table 1 shows the averages of the absolute attenuation value (HU) of the tumor and abdominal aorta in each phase of dynamic CT, and Table 2 shows the averages of the relative enhanced value (HU).

Table 1 Absolute attenuation value (HU)

	Pre-contrast	Arterial phase	Pancreatic phase	Late phase
Pancreatic cancer				
mean ± SD	41 ± 4	61 ± 13	80 ± 13	86 ± 11
Median	41	56	81	85
Range	32-48	41-93	60-104	64-106
Abdominal aorta				
mean ± SD	45 ± 4	284 ± 50	185 ± 40	139 ± 21
Median	45	295	171	136
Range	38-51	205-409	127-307	107-208

Table 2 Relatively enhanced value (HU)

	Arterial phase	Pancreatic phase	Late phase
Pancreatic cancer			
mean ± SD	20 ± 14	39 ± 15	45 ± 13
Median	19	38	43
Range	6-54	15-70	23-74
Abdominal aorta			
mean ± SD	239 ± 51	140 ± 40	94 ± 22
Median	248	125	91
Range	157-365	86-269	69-171

Table 3 Tumor-aorta enhancement ratio

	mean ± SD	Median	Range
Tumor-aorta enhancement ratio (arterial)	0.835 ± 0.053	0.066	0.029-0.216
Tumor-aorta enhancement ratio (pancreatic)	0.291 ± 0.12	0.244	0.139-0.569
Tumor-aorta enhancement ratio (late)	0.487 ± 0.129	0.483	0.295-0.816

The absolute value of pre-contrast CT correlated significantly with none of the histopathological findings, or the level of VEGF, MVD or fibrosis. The absolute value in the arterial phase correlated significantly with the level of VEGF and MVD ($P = 0.047$, $P = 0.001$) (Figure 1A and B, Figures 2 and 3). The absolute value in the arterial, pancreatic and late phases correlated significantly and negatively with the extent of fibrosis ($P = 0.006$, $P = 0.018$, $P = 0.035$) (Figure 1C-E and Figure 4). None of the relatively enhanced values in any phase correlated significantly with the level of VEGF. The relatively enhanced value in the arterial phase correlated significantly with MVD ($P = 0.003$) (Figure 5A). All of the relatively enhanced values in the arterial, pancreatic and late phase correlated significantly and negatively with the extent of fibrosis ($P = 0.003$, $P = 0.020$, $P = 0.039$) (Figure 5B-D).

Correlation between tumor-aorta enhancement ratio and histopathological findings

The averages of the tumor-aorta ratio are shown in Table 3. None of tumor-aorta enhancement ratios in any phase correlated significantly with the level of VEGF. Tumor-aorta enhancement ratio (arterial) was correlated significantly with MVD ($P = 0.022$) (Figure 6A), and significantly and negatively with the extent of fibrosis ($P = 0.004$) (Figure 6B).

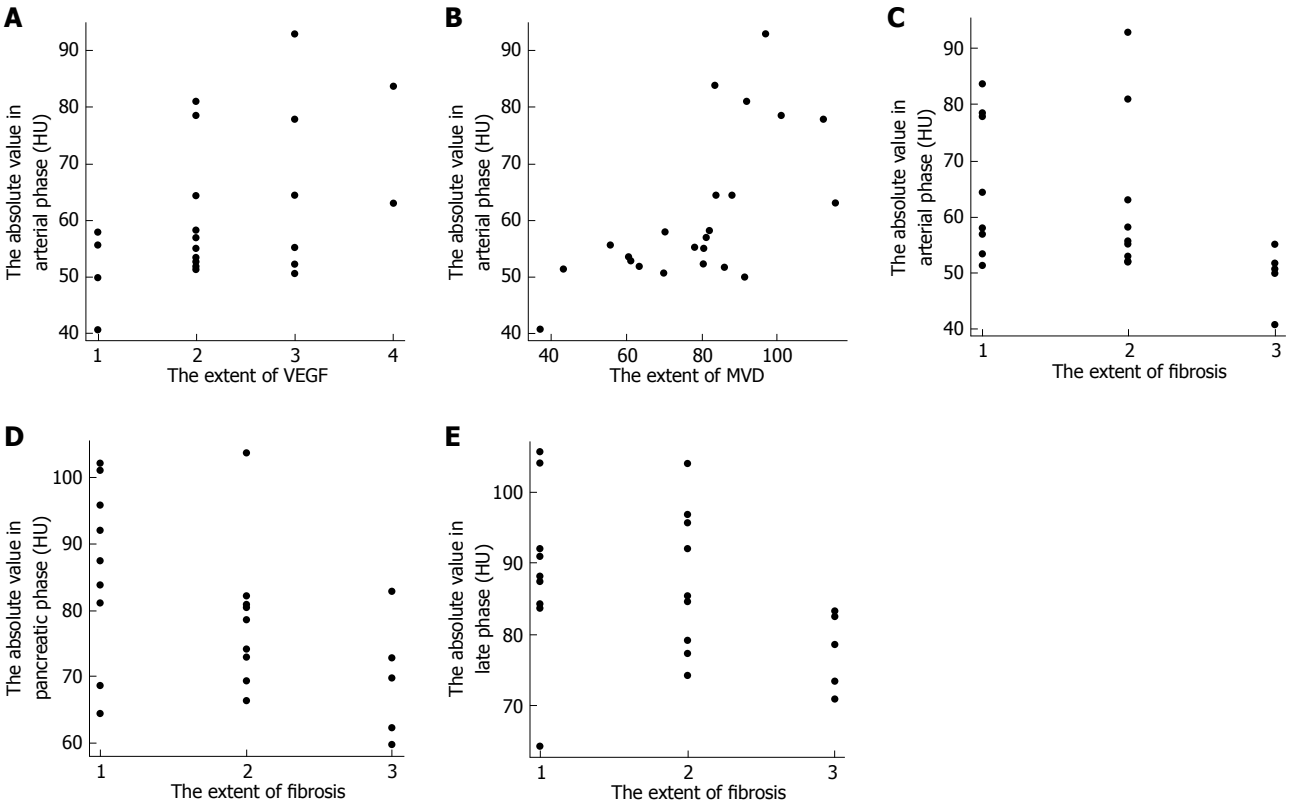

Figure 1 Scatter plots showing correlation between absolute values and histopathological findings. A: The absolute value in the arterial phase correlated significantly with the level of VEGF (r = 0.418, P = 0.047); B: The absolute value in the arterial phase correlated significantly with MVD (r = 0.649, P = 0.001); C: The absolute value in the arterial phase correlated significantly and negatively with the extent of fibrosis (r = -0.556, P = 0.006); D: The absolute value in the pancreatic phase correlated significantly and negatively with the extent of fibrosis (r = -0.488, P = 0.018); E: The absolute value in the late phase correlated significantly and negatively with the extent of fibrosis (r = -0.442, P = 0.035).

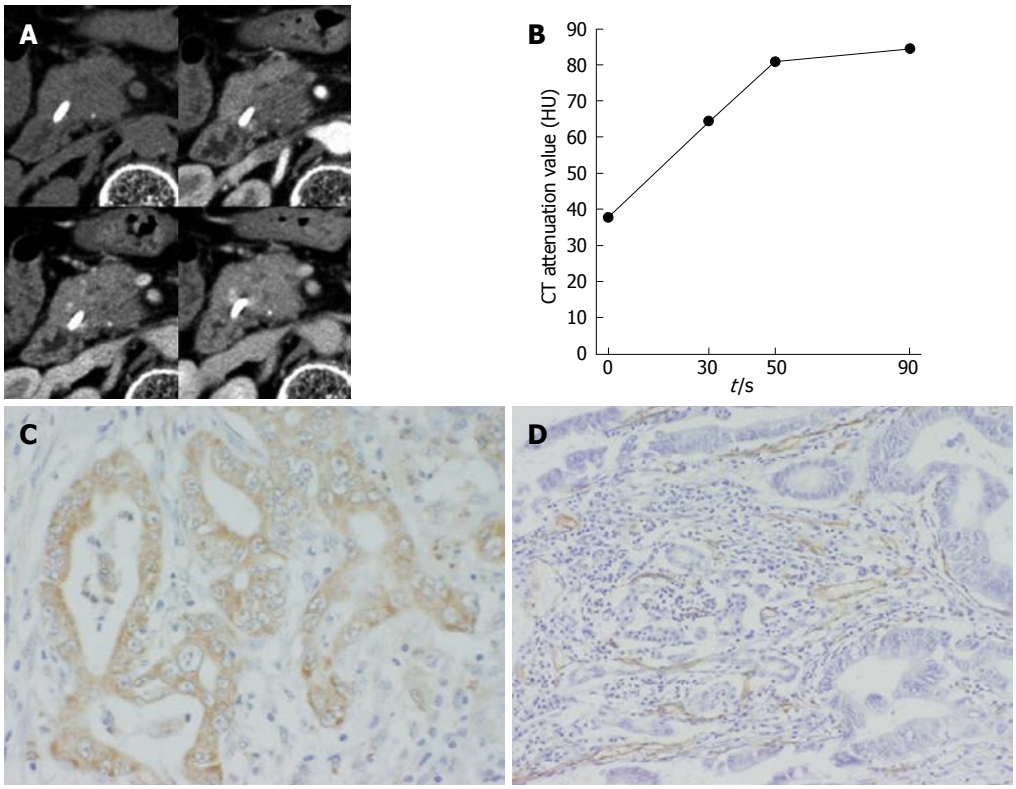

Figure 2 Moderately differentiated tubular adenocarcinoma in a 73-year-old woman. A: Transverse dynamic CT images; B: Time-attenuation curve. Dynamic CT scans showing marked enhancement in the arterial phase; C: Photomicrograph showing immunoreactivity to VEGF, which is depicted as brown cytoplasm. The score was 4 (high expression) (Anti-VEGF stain; original magnification, × 400); D: Photomicrograph showing abundant microvessels and depicting vessel walls that appeared brown (Anti-CD34 stain; original magnification, × 200).

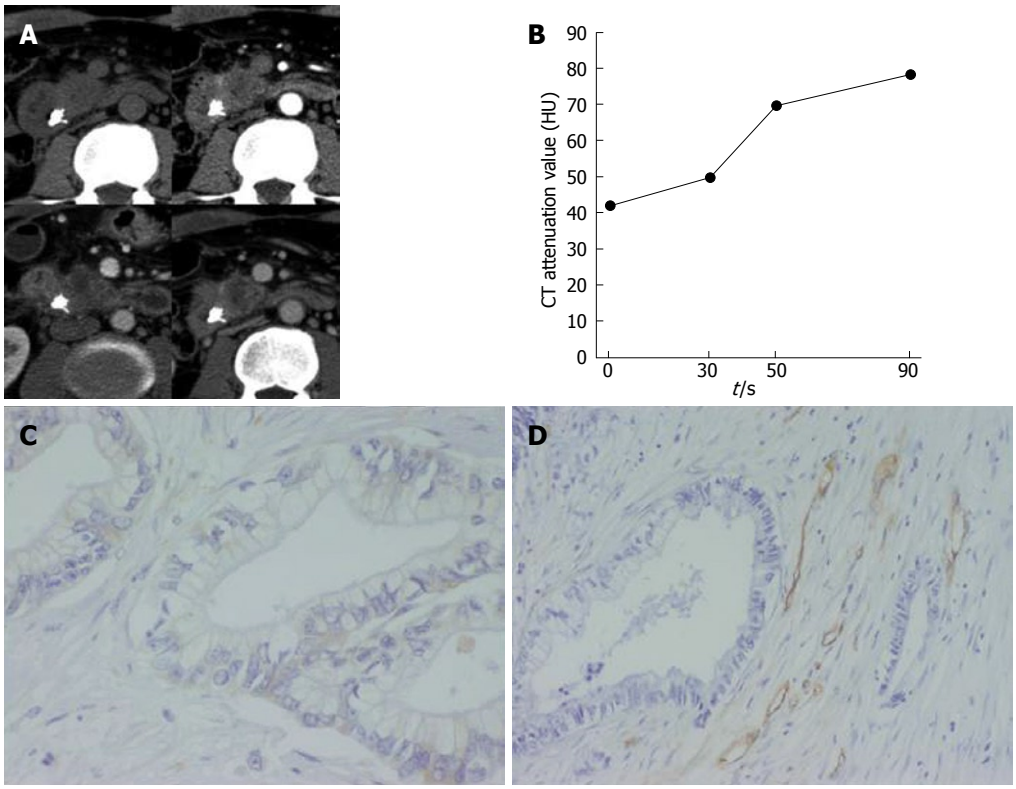

Figure 3 Well-differentiated tubular adenocarcinoma in a 44-year-old man. A: Transverse dynamic CT images; B: Time-attenuation curve. Dynamic CT scans showing low enhancement in the arterial phase; C: Photomicrograph showing immunoreactivity to VEGF, which is depicted as brown cytoplasm. The score was 1 (extremely weak) (Anti-VEGF stain; original magnification, × 400); D: Photomicrograph showing few microvessels and depicting vessel walls, which appear brown (Anti-CD34 stain; original magnification, × 200).

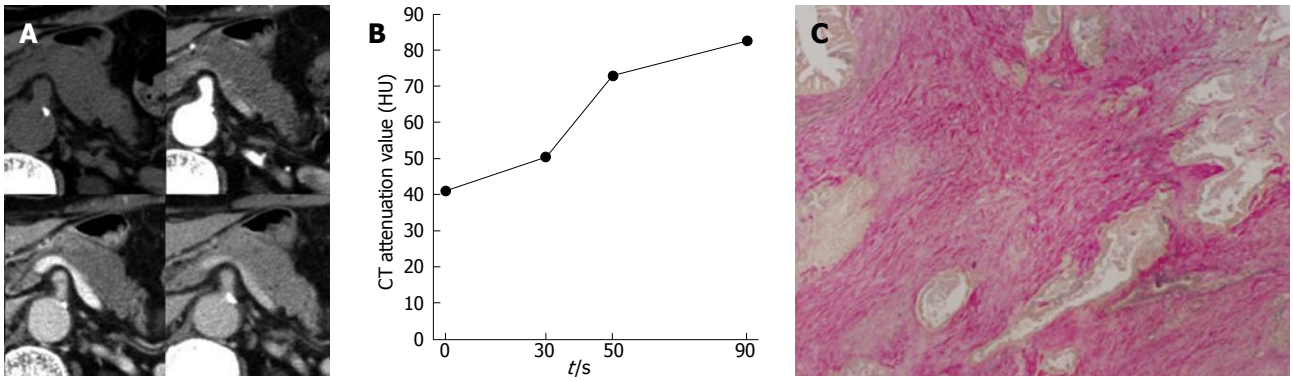

Figure 4 Moderately differentiated tubular adenocarcinoma in a 79-year-old man. A: Transverse dynamic CT images; B: Time-attenuation curve. Dynamic CT scans showing gradual enhancement; C: Photomicrograph showing abundant fibrosis and collagen fibers, which appear pink. The score was 3 (EVG stain; original magnification, × 40).

Correlation among histopathological findings

The level of VEGF was correlated significantly with MVD ($P = 0.037$). The extent of fibrosis was not correlated significantly with the level of VEGF and MVD.

DISCUSSION

The correlation between conventional dynamic MDCT findings and angiogenesis in lung[15] and renal cell[16] cancer has been reported previously. These studies have revealed that the attenuation value of the peak enhancement of the tumor and the enhancement ratio (peak enhancement value divided by time) are correlated positively with the extent of angiogenesis. However, it is not realistic to apply these results to pancreatic cancer, which usually has abundant fibrosis and tends to show gradual enhancement with the peak enhancement in the equilibrium phase[28,29]. To overcome this important problem in the common type of pancreatic cancer, we analyzed the correlation with enhancement of each phase and angiogenesis and fibrosis. In general, contrast agents have two-compartment pharmacokinetics with intravascular and extravascular-extracellular (interstitium) components. The enhancement of the tumor depends on the concentration of the injected agent, blood flow, blood volume, permeability, and extravascular-

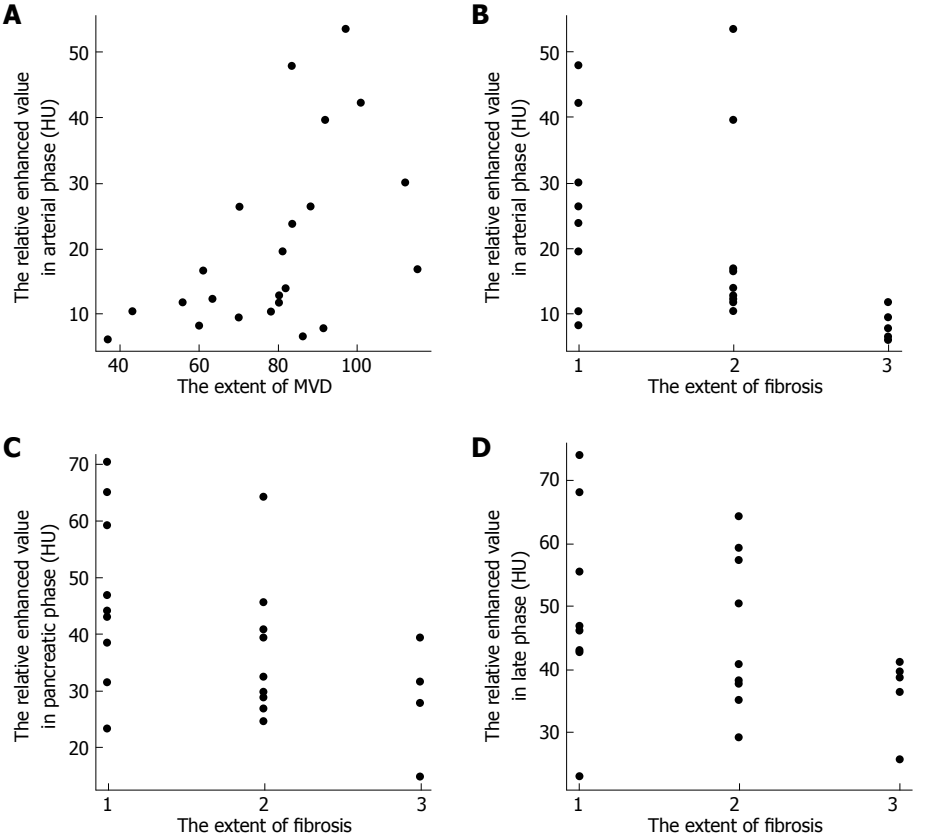

Figure 5 Scatter plots showing correlation between the relative enhanced values and histopathological findings. A: The relatively enhanced value in the arterial phase correlated significantly with the extent of MVD (r = 0.593, P = 0.003); B: The relatively enhanced values in the arterial phase correlated significantly and negatively with the extent of fibrosis (r = -0.590, P = 0.003); C: The relatively enhanced values in the pancreatic phase correlated significantly and negatively with the extent of fibrosis (r = -0.483, P = 0.020); D: The relatively enhanced values in the late phase correlated significantly and negatively with the extent of fibrosis (r = -0.433, P = 0.039).

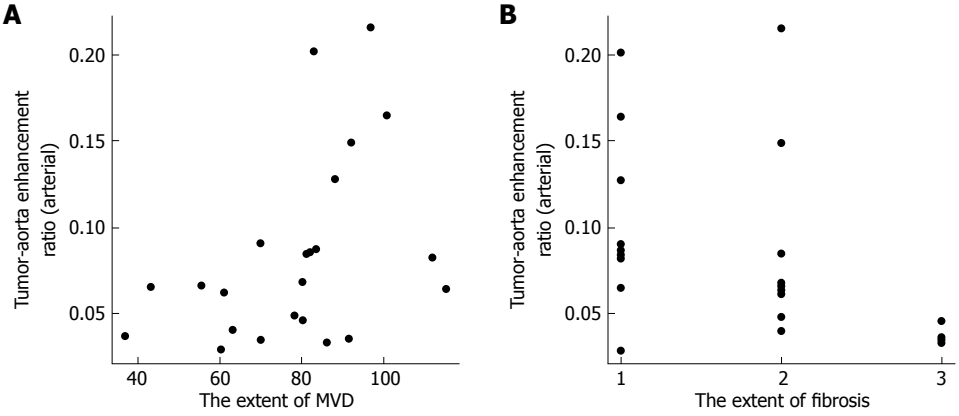

Figure 6 Scatter plots showing correlation between tumor-aorta enhancement ratio and histopathological findings. A: Tumor-aorta enhancement ratio (arterial) was correlated positively with MVD (r = 0.477, P = 0.022); B: Tumor-aorta enhancement ratio (arterial) was correlated negatively with the extent of fibrosis (r = -0.575, P = 0.004).

extracellular components. The contrast agents in the arterial dominant phase are predominantly the intravascular component. In the pancreatic phase (near portal dominant phase), they pass into the extravascular-extracellular components. The enhancement in this phase is considered to be a mixture of intravascular and extravascular-extracellular components. Tissues with adequate blood supply generally show the highest enhancement in this phase. The contrast agents in late phase (equilibrium phase) are both intravascular and extravascular-extracellular components, and the enhancement depends mainly on the extravascular-extracellular components. In addition to absolute and relative enhanced values, we employed the tumor-aorta enhancement ratio, which was calculated by dividing the attenuation value (HU) of pancreatic cancer by that of the abdominal aorta in each phase of contrast-enhanced CT, as a parameter of the grade of tumor enhancement. This parameter was decided in order to exclude the influence of the intravascular concentration of an injected contrast agent that is dependent on cardiac output and circular blood volume. We think that this parameter reflects tumor enhancement more exactly than the absolute and relative attenuation values. Tumor-aorta enhancement ratio (arterial) is considered to reflect mainly the amount of arterial blood flow and volume of intratumoral blood spaces. Tumor-aorta enhancement ratio (pancreatic) may depend on vascular permeability in addition to intratumoral blood flow and/or blood volume. Tumor-aorta enhancement ratio (late) may

reflect mostly the extravascular-extracellular component.

In the present study, several findings of dynamic CT showed significant correlations with the histopathological findings. MVD in the tumor correlated significantly with the absolute value in the arterial and pancreatic phases, relative enhanced value in the arterial phase and the tumor-aorta enhancement ratio (arterial). This may have resulted from the increased vascular space and/or increased blood flow in tumors with increased MVD. The absolute value in the arterial phase also correlated significantly with the level of VEGF, probably for the same reason as in the case of MVD. On the other hand, the absolute value and relatively enhanced value in the arterial and pancreatic phases and tumor-aorta enhancement ratio (arterial) correlated significantly and negatively with the extent of fibrosis. This may have resulted from the smaller intratumoral blood spaces and blood flow in the tumors, with abundant fibrosis resulting in an absolutely lower volume of contrast inflow into this kind of tumor. Both the absolute value and relatively enhanced value in the late phases correlated significantly and negatively with the extent of fibrosis. It is known that the tumors with abundant internal fibrosis show prominent delayed enhancement in the late-phase of dynamic CT because of an increased extravascular-extracellular component. Therefore, the results obtained in our tumor were not consistent with previous speculations. This may also have been caused by the smaller amount of blood inflow into the tumors with more abundant fibrosis. We need to study this issue further with a more delayed phase on dynamic CT.

Histologically, the extent of VEGF expression was correlated significantly with MVD. However, the extent of fibrosis was not correlated significantly with the level of VEGF or MVD. These results support the similarity of the findings of dynamic CT between tumors with increased MVD and expression of VEGF. However, these findings may be modified by the extent of intratumoral fibrosis, which has no direct correlation with VEGF expression and MVD.

There are several limitations in the present study. First, the protocol of dynamic CT was not entirely appropriate. The late phase was earlier than the widely accepted equilibrium phase of dynamic CT of the pancreas. We used a fixed amount of contrast material. The grade of enhancement might be influenced by several factors such as patient weight, cardiac output, and CT tube wear[30,31]. Since we analyzed the validity of conventional dynamic CT retrospectively and preliminarily, we did not adopt these factors in the present study. Second, the attenuation value (HU) reflects not only the vascular characteristics of immature vessels formed by tumor angiogenesis, but also those of pre-existing mature vessels. Third, there are several other factors that affect the enhancement pattern of pancreatic cancers, such as the shape of intratumoral blood spaces and vasoactive elements. Fourth, the attenuation value (HU) in each phase on conventional dynamic MDCT may reflect various levels of blood flow, blood volume, vascular permeability and extravascular-extracellular components. In particular, new capillaries formed by tumor angiogenesis are immature and have greater permeability than normal capillaries[32]. Further investigation, including by perfusion CT, is needed in this regard. In spite of these limitations, we think that our results provide some useful indication for the estimation of angiogenesis and intratumoral fibrosis in pancreatic cancer. After clinical application of anti-angiogenesis agents for pancreatic cancer, evaluation of the results obtained this study should be performed.

In conclusion, there was a significant correlation between the enhancement in conventional dynamic CT and angiogenesis and fibrosis in pancreatic adenocarcinoma. The tumors with greater MVD and expression of VEGF tended to show high enhancement in the arterial dominant phase. On the other hand, the tumors with a larger amount of fibrosis showed a negative correlation with the grade of enhancement during the arterial phase. Dynamic CT features that are caused by angiogenesis may be modified by the extent of intratumoral fibrosis.

COMMENTS

Background

Prognosis of pancreatic cancer is poor. Recently, it has been clarified that the grade of tumor angiogenesis is a useful prognostic marker in human cancer, including pancreatic cancer. Therefore, evaluation of the grade of angiogenesis by imaging may be important as a prognostic marker and is necessary for deciding the indications and evaluating the effect of anti-angiogenic treatment.

Research frontiers

To establish the grade of tumor angiogenesis by non-invasive imaging may be important clinically. However, there are only a few such reports on pancreatic adenocarcinoma. In the present study, the authors analyzed the correlation between enhancement on dynamic multidetector computed tomography (MDCT) and histopathological findings, including the grade of tumor angiogenesis, and the extent of fibrosis in surgically resected pancreatic adenocarcinoma.

Innovations and breakthroughs

This study predicted the grade of angiogenesis by conventional dynamic MDCT that is performed most often for the diagnosis of pancreatic cancer. The tumors with strong angiogenesis tended to show high enhancement in the arterial dominant phase. On the other hand, tumors with a larger amount of fibrosis showed a negative correlation with the grade of enhancement during the arterial phase.

Applications

Anti-angiogenesis agents are still not approved for the treatment of pancreatic cancer at present. However, as a preliminary investigation for future application, prediction of the grade of angiogenesis by conventional dynamic MDCT would be useful clinically.

Terminology

Angiogenesis is the process of new blood vessel formation. This consists of endothelial sprouts of preexisting vessels and is stimulated by angiogenic factors such as vascular endothelial growth factors. Recently, angiogenesis has been recognized as an important factor of tumor growth and metastasis.

Peer review

The author retrospectively evaluate the correlation between enhancement patterns on dynamic computed tomography and angiogenesis and fibrosis in pancreatic adenocarcinoma.

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BRIEF ARTICLES

Microscopic colitis: A large retrospective analysis from a health maintenance organization experience

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Abstract

AIM: To examine the demographic data on a large multi-ethnic population of patients with microscopic colitis (MC) in Southern California and to determine the association of MC with inflammatory bowel disease (IBD) and colorectal cancer.

METHODS: All patients diagnosed with MC by colonic biopsy from 1996-2005 were identified utilizing a pathology database. All biopsies were reviewed by experienced pathologists utilizing standard histologic criteria. Patients' medical records were reviewed and data regarding patient age, co-morbidities, sex, ethnicity, and medications were analyzed. An age- and sex-matched standard control group was also generated. Chi-square test was used to evaluate the associations of co-morbidities between lymphocytic colitis (LC), collagenous colitis (CC) and the control group.

RESULTS: A total of 547 cases of MC were identified, 376 patients with LC and 171 patients with CC. The female/male ratio was 3:1 in CC and 2.7:1 in LC patients. Celiac disease ($P < 0.001$), irritable bowel syndrome (IBS) ($P < 0.001$), and thyroid diseases ($P < 0.001$) were found to have a higher occurrence in MC compared to the control group. No statistical differences in the occurrence of colorectal cancer, diabetes and IBD were found between the MC group and the control group.

CONCLUSION: This is the largest group of patients with MC known to the authors that has been studied to date. Conditions such as celiac disease, IBS, and thyroid diseases were found to be related to MC. Furthermore, neither an increased risk of colorectal cancer nor IBD was associated with MC in this study.

Key words: Collagenous colitis; Inflammatory bowel disease; Lymphocytic colitis; Microscopic colitis

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INTRODUCTION

Microscopic colitis (MC) is a condition characterized by chronic, watery diarrhea with normal endoscopic appearance of colonic mucosa, yet abnormal histology. Further classification of this condition based on histology reveals two distinct entities: collagenous colitis (CC) and lymphocytic colitis (LC). Although both entities demonstrate colonic intraepithelial lymphocytosis, increased inflammatory cells within the lamina propria and preserved crypt architecture, the presence of a

thickened sub-epithelial collagen band is characteristic of CC.

The incidence of CC is approximately 0.6-2.3/100 000 per year with a prevalence of 10-15.7/100 000, while LC has an incidence of 3.1/100 000 per year and a prevalence of 14.4/100 000[1,2]. However, more recently published data from Olesen *et al*[3] suggests that the incidence of microscopic colitides are on the rise. Observational studies have also suggested associations between MC and various autoimmune diseases including thyroid, rheumatoid, celiac, and inflammatory bowel disease (IBD)[4-7]. Various medications have also been linked with MC in published case reports. Currently, little is known regarding cancer risk associated with MC.

With the number of reported cases clearly increasing, surprisingly, its etiology is still mostly unknown. Furthermore, the relationship between collagenous and LC and their association with other diseases has yet to be clearly defined. Should a connection between MC and colorectal cancer or IBD exist, this would have huge ramifications on the management of MC. Most of the currently available data on MC are from European studies with relatively small and homogeneous populations. There are even fewer comparative studies on the subtypes of MC.

The purpose of this study was to observe and report the demographic data on a large multi-ethnic population in Southern California, USA, with MC. We also report on the pattern of concurrent diseases and cancers in this group of patients.

MATERIALS AND METHODS

All patients diagnosed with MC (both collagenous and LC) from 1996 to 2005 by colonic biopsy were identified using the pathology database at Kaiser Permanente (a large health maintenance organization) in Southern California and the search words "MC, LC and CC". This region includes eight major Kaiser Permanente medical centers that serve over three million members throughout Southern California.

The histological criteria for inclusion in this study were defined as follows: (1) An increase in intraepithelial lymphocytes (more than 10 lymphocytes/100 epithelial cells); (2) Surface epithelial damage; (3) Absent or minimal crypt architectural damage; (4) An increase in sub-epithelial collagen band > 10 micrometers was required for the diagnosis of CC (Figure 1).

Experienced gastrointestinal pathologists (JM, SW, NK, RZ, MK, JP, KG) at Kaiser Permanente reviewed all biopsies.

Patients with ulcerations, erythema or any other visible abnormalities seen on the colonic mucosa during the endoscopic exam were excluded from the study. We included patients with a history of colorectal cancer.

We reviewed medical records using the Kaiser Permanente Database System and recorded data regarding patient age at diagnosis, sex, ethnicity, and concurrent medical conditions. All medications taken by patients, within 1 year prior to the diagnosis of collagenous or LC, were also recorded for analysis.

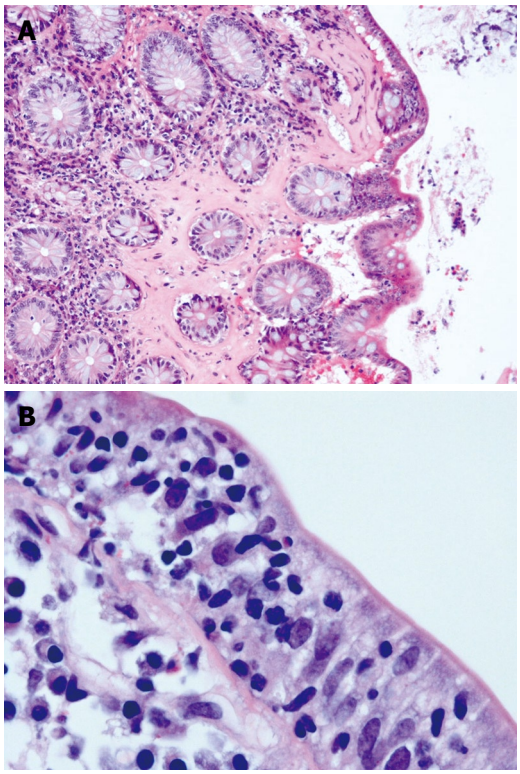

Figure 1 Collagenous colitis (A) and lymphocytic colitis (B).

Demographic data such as age at diagnosis, sex and ethnicity is presented as means and standard deviation. An age- and sex-matched standard control group was also randomly generated from the same Kaiser Permanente database. We used a chi-square test to evaluate the associations of co-morbidities between LC, CC and the control group.

Differences in the clinical characteristics between LC and CC were also evaluated using the chi-square test. Differences were assumed as significant at a P value of less than 0.05.

The study protocol was approved by the Internal Review Board of Kaiser Permanente in Southern California.

RESULTS

From January 1996 to July 2005 a total of 547 patients were identified with MC from our database. There were 27.1% male subjects, while 72.9% were female. The average age at diagnosis was 61.7 years for both men and women. We were able to identify the ethnicity in 465 cases. The percentage of each ethnic group was as follows: 72.4% Caucasian, 6.9% Hispanic, 2.6% Asian, 2.9% African American, and 0.2% Middle Easterners.

CC

Demographics: A total of 171 patients were identified as having CC. The average age at diagnosis in these patients was 63.8 years with a standard deviation of 13.5 years. The range for age at diagnosis was between

Table 1 Comparison of our demographic data against published demographic data in collagenous colitis (mean ± SD)

	Total number	Age at diagnosis (yr)	Gender ratio M/F
Goff *et al*[8] 1997	31	66	5/26
Fernandez-Banares *et al*[1] 1999	23	57.8 ± 2.9	4/19
Agnarsdottir *et al*[9] 2002	71	66.1 ± 14.3	8/63
Olesen *et al*[3] 2004	51	Not reported	6/45
Koskela *et al*[18] 2004	30	56.5 ± 12.7	10/20
Kao *et al* 2006	171	63.8 ± 13.5	42/129

29 to 93 years. The sex distribution was 24.6% male and 75.4% female. The ratio between male and female ratio was about 1:3. Of the 171 patients identified, we were able to identify the ethnicity of 152 patients. The percentage of each ethnic group was as follows: 80.7% Caucasian, 5.3% Hispanic, 2.3% African American and 0.6% Asian. No patients identified as Middle Easterners were observed[8,9] (Table 1).

Associated diseases: Of the CC population identified, the most common endocrinopathies were as follows: hyperlipidemia (42.7%), diabetes (14%) and thyroid disease, including hyperthyroidism, hypothyroidism and Grave's disease (21%). The three most common rheumatologic and other inflammatory disorders were rheumatoid arthritis (7%), fibromyalgia (3.5%) and Raynaud/CREST syndrome (2.9%). Other diseases diagnosed in these patients included giant cell/temporal arteritis (n = 3, 1.75%), polymyalgia rheumatica (n = 2, 1.16%), systemic lupus erythematosus (n = 1, 0.58%) and scleroderma (n = 1, 0.58%). Only five patients in the CC group were diagnosed with celiac disease (2.92%). One of these patients had the diagnosis of celiac disease prior to the diagnosis of CC. Thirty patients were diagnosed with irritable bowel syndrome (IBS) prior to the diagnosis of CC, while only one patient was diagnosed with irritable bowel after the diagnosis of CC was made. We observed one patient with an established diagnosis of IBD prior to the diagnosis of CC. During a median follow up of 3 years, none of our CC patients have subsequently been diagnosed with IBD.

Compared with the control group, patients with CC had higher rates of thyroid disease ($P < 0.001$). We also observed a higher rate of celiac disease ($P < 0.01$), IBS, ($P < 0.001$) rheumatoid arthritis ($P < 0.001$) and Rayanud/CREST syndrome ($P < 0.01$) in our patients with CC (Table 2).

Cancer: The three most common cancers found in CC patients were breast (n = 4, 2.34%), lung (n = 3, 1.75%) and colorectal cancer (n = 5, 2.82%). Four patients were diagnosed with cancer prior to the diagnosis of CC. Only one patient was diagnosed with colorectal cancer following the diagnosis of CC. This patient was a 79-year-old female diagnosed with colorectal cancer by screening colonoscopy 5 years after the diagnosis of CC was made.

Table 2 Comparison of concurrent diseases between collagenous colitis and the control group

Disease	Number of patients affected	Percentage (%)	Compared to control group
Hyperlipidemia	73	43.5	NS
Diabetes type Ⅰ and Ⅱ	24	15.8	NS
Thyroid diseases[1]	36	21	$P < 0.001$
Rheumatoid arthritis	12	7	$P < 0.001$
Fibromyalgia	6	3.51	NS
Raynaud/CREST syndrome	5	2.92	$P < 0.01$
Giant cell/temporal arteritis	3	1.75	NS
Polymyalgia rheumatica	2	1.16	NS
SLE	1	0.58	NS
Scleroderma	1	0.58	NS
Celiac disease	5	2.92	$P < 0.010$
IBS	30	17.5	$P < 0.001$
IBD	1	0.58	NS

[1]Thyroid diseases including hypothyroidism, hyperthyroidism and Grave's disease. SLE: Systemic lupus erythematosus.

Table 3 Comparison of our demographic data against published demographic data in lymphocytic colitis (mean ± SD)

	Total number	Age at diagnosis (yr)	Gender ratio M/F
Fernandez-Banares *et al*[1] 1999	37	65.3 ± 2.4	10/27
Agnarsdottir *et al*[9] 2002	54	68.7 ± 12.7	5/45
Olesen *et al*[3] 2004	199	59	59/140
Koskela *et al*[18] 2004	54	55.4 ± 13.2	8/46
Kao *et al* 2006	376	60.7 ± 16.1	106/270

No cancer demonstrated a statistical difference compared with the control group, including colorectal cancer.

LC

Demographics: A total of 376 patients were identified as having LC. The average age at diagnosis in this group was 60.7 years, with a standard deviation of 16.1 years. The age range at diagnosis was between 19 to 98 years. Sex distribution was 28.1% male and 71.8% female. The ratio between men and women was about 1:2.5. Of the 376 patients identified, ethnicity was determined in 313 cases. The percentage of each ethnic group was as follows: 68.8% Caucasian, 7.71% Hispanic, 3.45% Asian, 3.19% African American and 0.26% Middle Eastern (Table 3).

Associated diseases: Of the total LC population, the most common endocrinopathies were as follows: hyperlipidemia = 44.1%, diabetes = 14.6% and thyroid disease (including hyperthyroidism, hypothyroidism and Grave's disease) = 18.8%. Rheumatoid arthritis was seen in 3.99% of the LC patients. Fibromyalgia was observed in 4.52%, while Raynaud/CREST syndrome was observed in 1.33% of the LC population. Others diseases such as giant cell/temporal arteritis (n = 2, 0.53%), polymyalgia rheumatica (n = 6, 1.6%), systemic lupus erythematosus (n = 3, 0.8%) and scleroderma (n = 1, 0.27%) were also

Table 4 Comparison of concurrent diseases between lymphocytic colitis and the control group

Disease	Number of patients affected	Percentage (%)	Compared to control group
Hyperlipidemia	166	44.10	NS
Diabetes type I and II	55	14.60	NS
Thyroid diseases[1]	71	18.80	$P < 0.01$
Rheumatoid arthritis	15	3.99	$P < 0.01$
Fibromyalgia	17	4.52	$P < 0.01$
Raynaud/CREST syndrome	5	1.33	$P < 0.01$
Giant cell/temporal arteritis	2	0.53	$P < 0.025$
Polymyalgia rheumatica	6	1.60	NS
SLE	3	0.80	$P < 0.025$
Scleroderma	1	0.27	NS
Celiac disease	13	3.46	$P < 0.001$
IBS	43	11.40	$P < 0.001$
IBD	1	0.27	NS

[1]Thyroid diseases including hypothyroidism, hyperthyroidism and Grave's disease.

observed. Thirteen patients were diagnosed with celiac disease (3.46%) and six of these patients had a diagnosis of celiac disease prior to the diagnosis of LC. Forty three patients were thought to have IBS prior to the diagnosis of LC, while seven were diagnosed with IBS after the diagnosis of LC was made. Overall, 11.4% of patients with LC were diagnosed with IBS. We observed one patient with an established diagnosis of IBD prior to the diagnosis of LC. During a median follow up of 3 years, none of our LC patients have subsequently been diagnosed with IBD.

Patients with LC had a higher rate of thyroid diseases ($P < 0.01$) compared with the control group. Diseases thought to be autoimmune-related, such as rheumatoid arthritis ($P < 0.01$), Raynaud/CREST syndrome ($P < 0.01$), SLE ($P < 0.025$), fibromyalgia ($P < 0.01$), and temporal arteritis ($P < 0.025$) also had statistically higher rates of occurrence in patients with LC. IBS ($P < 0.001$) and celiac disease ($P < 0.001$) also occurred more often in patients with LC (Table 4).

Cancer: The three most common cancers found in our LC patients were breast ($n = 20$, 5.31%), prostate ($n = 7$, 1.86%) and lung ($n = 4$, 1.06%). Five patients were diagnosed with colorectal cancer prior to the diagnosis of LC. None were diagnosed with colorectal cancer after the diagnosis of LC had been made.

No cancer was noted to have a statistical difference compared with the control group, including colorectal cancer.

CC vs LC

Despite a significant population size difference, we found little statistical difference between collagenous and LC, in terms of their associated diseases. Interestingly, we found a higher rate of irritable bowel disease diagnosed concurrently with CC than with LC ($P < 0.05$).

DISCUSSION

Increasingly, MC has become more and more recognized worldwide, yet little is know about its pathophysiology and the long term outcome of the disease. Currently, multiple hypotheses exist for the pathophysiology of MC and include immune dysregulation/autoimmunity. This hypothesis is further supported by findings that human leukocyte antigen haplotype is increased in A1 and DRW53 in LC[10]. While others have found increases in DQ2 and DQ1, three in LC and CC[11]. Autoantibodies such as ANA have also been found to be higher in patients with MC[12]. Indeed, multiple retrospective clinical studies have noted a higher incidence of autoimmune diseases in patients diagnosed with MC. This suspected relationship between immune dysregulation/autoimmunity and MC has led to the question of whether MC is a pre-IBD entity? If so, does the patient with MC require a higher level of surveillance and more aggressive treatment to prevent IBD from developing? These concerns were further illustrated by multiple case reports that seemed to convincingly suggest that MC may be the initial phase of a full blown IBD. However, the above theory has never been formally addressed in a large population study. In our investigation we were able to validate the previously known association between LC, CC and various autoimmune diseases, celiac disease and thyroid disorders in a large population study. Yet, interestingly, we found no significant correlation between IBD and CC/LC. Therefore, LC and CC may not be an indicator for intense surveillance to detect progression to IBD. To date, none of our LC or CC patients have been diagnosed with IBD.

Another theoretic risk for patients with MC is colorectal cancer. This concern is based on the IBD model where chronic inflammation can lead to increased dysplasia and malignancy. Thus far, to the authors' knowledge, only one study has evaluated the risk of colorectal cancer in MC. Chan *et al*[13] studied 117 patients diagnosed with CC during a mean follow-up period of 7 years to examine the risk of colorectal cancer. A similar risk of colorectal cancer was found in MC patients and the general population in that study. Only one patient was diagnosed with colorectal cancer after being diagnosed with MC. This was confirmed in our study as no increase in colorectal cancer occurrence was found between our patients with MC and the control group.

Interestingly, we found that IBS was associated with MC in approximately 13% of our MC patients. Other studies have also shown a correlation between IBS and MC, with IBS diagnosed in up to 23% of patients with LC[14,15]. The relationship is unclear, however it could be due to a similar clinical presentation between MC and IBS, as suggested by Barta *et al*[16]. There is also increasing evidence to support an inflammatory process in the pathogenesis of IBS. Furthermore, studies have shown that increasing amounts of intraepithelial lymphocytes can be seen in patients diagnosed with post infectious

IBS[17]. Usually, the number of lymphocytes does not reach that needed for the diagnosis of MC in most post infectious IBS patients, however, there have been cases where patients with a clinical history of post infectious IBS have been diagnosed with MC. To accurately distinguish these entities is important, as their management and treatment may be very different. For example, currently, colonoscopy is rarely performed for the diagnosis of IBS. However, as a result of our findings, colonoscopy with random biopsy may be warranted as a part of the workup for suspected diarrhea predominant IBS in the future.

Endocrinopathies such as thyroid dysfunction and diabetes have been known to have correlations with MC as noted in previously published studies. Several studies showed that diabetes was found in up to 8.3% of CC patients and in up to 13.5% of LC patients[18,19]. Despite reports of these associations, our study did not show a statistical significance when compared with our control group. One possible explanation for this is that LC, CC and diabetes tend to occur more frequently in elderly populations and thus could easily be thought of as related diseases. In a recent published study by Williams *et al*[20] after risk stratification of their data by age, the risk association between MC and the general population was no longer significant. Another possible explanation is that most of the previous studies were smaller in size which may have led to sampling error. However, the same argument can not be used to explain the high occurrence of thyroid dysfunction previously reported[21]. Indeed, we found a statistically higher occurrence of thyroid dysfunction in LC and CC patients when compared to the control group. The association between thyroid dysfunction, LC and CC is still unknown, although some suggest autoimmunity as a possible cause.

There are several limitations to our study. For example, because routine screening for other disorders that may be related to MC (such as celiac disease) were not performed in a prospective format, the prevalence of these associated disorders may be underestimated. Another limitation is the lack of long term follow up. Because of this lack of long term follow up, it is possible that colorectal cancers and IBD may not have had sufficient time to manifest. Due to this, we continue to actively follow these patients and long term data should be forthcoming in the future. Another limitation involves the retrospective nature of this study for which selection bias is commonly seen. However, we hope that the large patient population would minimize such bias.

Our analysis produced many interesting observations and provoked thoughtful questions regarding the etiology, disease mechanism, and associations with other diseases and medications in the largest study to date on MC. Several of these findings have been previously noted, however, some other relationships have yet to be reported. We believe that the large number of patients in our study makes this study less vulnerable to sampling error and provides a good representation of MC.

COMMENTS

Background

The prevalence of microscopic colitis (MC) is on the rise worldwide, yet little is known about the natural history and etiology of this disease. Currently, it is believed that the disease follows a benign course; however, cases have been reported in association with inflammatory bowel disease (IBD). Its association with colorectal cancer is also poorly understood.

Research frontiers

Case reports have suggested that MC can evolve into IBD, but no studies have been carried out to thoroughly evaluate this relationship.

Innovations and breakthroughs

This is the largest study published to-date that addresses the important question of whether patients with MC have a higher prevalence of IBD compared to the general population. It also provided additional clinical information regarding this rarely-studied disease.

Applications

This study, which evaluated the largest population to-date, demonstrated that there is no increase in the incidence of IBD or colorectal cancer in patients with MC. It also showed that MC can mimic symptoms of irritable bowel syndrome which should be considered during evaluation.

Terminology

MC is a condition characterized by chronic, watery diarrhea with normal endoscopic appearance of the colonic mucosa. It is further divided into lymphocytic colitis and collagenous colitis based on its histologic appearance.

Peer review

This is an interesting paper addressing a topic which often remains scarcely investigated. The article is well balanced and the discussion is clear and exhaustive.

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BRIEF ARTICLES

Portal hypertension secondary to myelofibrosis with myeloid metaplasia: A study of 13 cases

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Abstract

AIM: To describe the clinical presentation and complications of portal hypertension (PH) secondary to myelofibrosis with myeloid metaplasia (MMM).

METHODS: Medical records for 123 patients with MMM were reviewed.

RESULTS: Thirteen patients with PH secondary to MMM were identified. Median ages at time of MMM and PH diagnosis were 61 and 66 years, respectively. The interval from MMM diagnosis to presentation with one of the PH features ranged from 1 to 11 years. Variceal bleeding and ascites were the most common presentations. Of the eight patients who presented with variceal bleeding, six patients underwent endoscopic variceal ligation (EVL) with no variceal recurrence or hematological worsening during a 12-mo follow up period.

CONCLUSION: Patients with MMM might develop PH. Exact mechanisms leading to PH in MMM are still controversial. As in other etiologies, variceal bleeding and ascites are the most common presentations. Anemia may correlate with, and/or predict, the severity of the PH presentation in these patients. EVL can successfully control variceal bleeding in MMM. Further clinical studies are required.

Key words: Portal hypertension; Myelofibrosis; Myeloid metaplasia; Ascites; Variceal bleeding

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INTRODUCTION

The term "myelofibrosis with myeloid metaplasia (MMM)" is usually referred to for patients with chronic idiopathic myelofibrosis (CIM), also known as angiogenic myeloid metaplasia (AMM), and for those with advanced phases of polycythemia vera [post polycythemic myeloid metaplasia (PPMM)] and essential thrombocythemia [post thrombocythemic myeloid metaplasia (PTMM)]. All three represent chronic stem cell-derived clonal myeloproliferative diseases that, in the case of myeloid metaplasia, are accompanied by an intense reactive bone marrow fibrosis that leads to ineffective erythropoiesis and extramedullary hematopoiesis in multiple organs, predominantly the spleen. However, extramedullary hematopoiesis may also occur at sites other than the spleen, including lymph nodes causing lymphadenopathy and also in the liver resulting in hepatomegaly and possibly, with other contributing factors, portal hypertension (PH)[1,2].

Patients with MMM present with variable clinical and histomorphologic features. The typical clinical features include hypermetabolic symptoms (fever, fatigue and weight loss), marked splenomegaly and anemia[3]. PH with subsequent ascites and gastrointestinal hemorrhage from ruptured varices has been described in patients with MMM[4-8]. Only a few published studies have discussed this issue extensively. Furthermore, these reports described and focused on PH in CIM which represents a subgroup of MMM. In this retrospective study, we describe 13 patients with PH secondary to MMM.

MATERIALS AND METHODS

After obtaining approval from the Institutional Review Board of our institution, the study patients were identified through the use of a comprehensive institutional database of medical diagnoses and procedures. Between January 1, 1995 and December 31, 2007, an estimated number of 123 patients with MMM were evaluated at the Mayo Clinic, Rochester, MN. The diagnosis of MMM was confirmed on the basis of traditional criteria that included bone marrow fibrosis associated with splenomegaly and leukoerythroblastosis (immature granulocytes and nucleated red cells). Patients with bone marrow fibrosis resulting from other disorders were excluded. This included patients with myelodysplastic syndrome, acute myelofibrosis, or chronic myelogenous leukemia.

From this sample, a subgroup of 13 patients with clinical evidence of PH was identified. Although the gradient between wedged and free hepatic venous pressure was not assessed in many patients, the diagnosis of PH was based on clinical criteria [e.g. esophageal varices by endoscopy, ascites with Serum-Ascites Albumin Gradient (SAAG) > 1.1] in conjunction with imaging studies. Patients with liver disease and patients having risk factors for liver disease such as chronic alcohol consumption were excluded. Patients with PH due to other diseases were also excluded. The medical records for these 13 patients with PH secondary to MMM were comprehensively reviewed. Pertinent clinical and laboratory variables were recorded for all patients.

Table 1 Clinical characteristics, presentation and laboratory values within 1 wk of PH diagnosis for the 13 patients

Variable	
Age (yr), median (range)	
All patients	67 (49-88)
At time of diagnosis of MMM	61 (45-79)
At time of diagnosis of PH	66 (47-84)
Interval till presentation with PH	5 (1-11)
Sex *n* (%)	
Male	9 (69)
Female	4 (31)
Type of MMM *n* (%)	
AMM	6 (46)
PPMM	6 (46)
PETMM	1 (8)
Initial presentation *n* (%)	
GI bleeding only	3 (23)
Ascites only	3 (23)
GI bleeding and ascites concurrently	2 (15)
Jaundice (with GI bleeding)	1 (8)
Encephalopathy	0 (0)
Abdominal pain (with ascites)	1 (8)
Splenomegaly	13 (100)
Hepatomegaly	9 (69)
Portal vein thrombosis	3 (23)
CBC, median (range)	
Hemoglobin (g/dL)	10 (6.6-13.9)
WBC ($\times 10^9$/L)	8.8 (2.1-49)
Platelet count ($\times 10^9$/L)	225 (47-694)
Liver function, median (range)	
AST (U/L)	69 (26-172)
Alkaline phosphatase (U/L)	338 (74-850)
Total bilirubin (mg/dL)	0.9 (0.3-2.2)
PT (s)	12.4 (10.8-14.1)
Albumin (g/dL)	4 (2.8-4.4)
SAAG	2 (1.9-2.4)

PT: Prothrombin time.

RESULTS

Thirteen patients with PH secondary to MMM were identified. Table 1 shows their pertinent clinical characteristics, presentation and laboratory values within 1 wk of PH diagnosis. Nine male (69%) and four female patients (31%) were identified: six patients (46%) had AMM, six patients (46%) had PPMM, and one patient (8%) had PTMM. The age of the patients ranged from 49 to 88 years (median, 67 years). Median ages and range at time of MMM and PH diagnoses were 61 (45-79) years and 66 (47-84) years respectively. The interval from MMM diagnosis to presentation with one of the PH features ranged from 1 to 11 years (median, 5 years).

Variceal bleeding and ascites were the most common presentations. Six patients (46%) initially presented with GI bleeding (five patients presented with acute upper GI bleeding and one presented with melena). Among those six patients; three presented with GI bleeding only, two presented with both GI bleeding and ascites, and one presented with GI bleeding and jaundice. All the six patients were diagnosed as having mild to moderate esophageal varices (Grade 1 to 2). Five patients (38%) presented initially with ascites. Among these five patients; three had only ascites at presentation, two presented with GI bleeding with the ascites, and one patient presented with abdominal pain in addition to ascites. SAAG was calculated in patients with ascites; all had SAAG above 1.1 which was considered as indicative of PH. Amongst all 13 patients, 12 of them (92%) eventually developed at least one episode of ascites within 6 mo from initial presentation. Jaundice was never the sole presenting feature. One patient presented with jaundice in addition to GI bleeding. Another patient developed jaundice afterwards, during the course of the disease. None presented with encephalopathy.

All patients (100%) had splenomegaly; nine patients (69%) had hepatomegaly and consequently nine patients (69%) had both. Eleven patients (84%) had elevated alkaline phosphatase levels at time of PH diagnosis, nine patients (69%) had elevated aspartate aminotransferase levels, and eight patients (62%) had both enzyme levels elevated. Median and range values for all liver tests are displayed in Table 1. Hyperbilirubinemia was present in four patients (31%) at time of PH diagnosis but jaundice was uncommon. Four patients (31%) had low albumin, with a median albumin level of 4 g/dL (2.8-4.4 g/dL). Ten patients (77%) were anemic at the time of PH diagnosis. Median hemoglobin level was 10 mg/dL (6.6-13.9 mg/dL). Three patients (23%) were diagnosed with portal vein thrombosis at time of

PH diagnosis, indicated by either abdominal doppler ultrasound (US) or CT or MRI. Among these three patients with portal vein thrombosis, one patient had high white blood cell count and low hemoglobin, while the other two patients had white blood counts and hemoglobin levels within normal limits but had decreased portal blood flow velocity indicated by abdominal doppler US. Among the 10 patients who did not have thrombosis, eight patients have undergone liver biopsy: all eight showed non cirrhotic liver parenchyma with varying degrees of extramedullary hematopoiesis, and infiltration of liver sinusoids with hematopoietic cells (myeloid metaplasia). Two of these eight patients also had mild fibrotic changes but none of them had truly cirrhotic features. All patients, including those without thrombotic etiology, had splenomegaly. Of the eight patients presenting with variceal bleeding, six patients underwent endoscopic variceal ligation (EVL) requiring 1-4 sessions. These six patients were followed up by endoscopies at 1, 3 and 12 mo to inspect for the re-appearance of varices. Varices were completely obliterated with no recurrence at the 12 mo time point. No hematologic worsening was recorded during the 12 mo follow-up period. The other two patients underwent endoscopic variceal sclerotherapy (EVS) but were not followed up. Interestingly, three patients (23%) had pulmonary hypertension concurrent with PH, with no cardiopulmonary causes.

DISCUSSION

PH has been reported in 7%-18% of AMM, which represents a subgroup of MMM[8,9]. In this study, simple mathematical calculation revealed an 11% prevalence of symptomatic PH in MMM. However, as in other most etiologies, PH secondary to MMM is usually asymptomatic and diagnosis of PH in these patients is often not made until they become symptomatic, which sometimes does not become apparent for up to 11 years. In the present study we found that the median interval for our patients to present with one of the PH features is 5 years, ranging from 1-11 years. Taking into consideration that the median survival for patients with MMM is 4 years[10-15], this suggests that many MMM patients die due to the underlying disease before they become symptomatic from PH. Therefore, we believe that a prevalence of 11% represents the prevalence of symptomatic PH and that the true prevalence of PH in MMM, including the asymptomatic PH, is higher.

When patients become symptomatic, they usually present either with acute upper GI bleeding from ruptured varices or in the form of melena, or they present with ascites. Other presentations such as jaundice and encephalopathy are unlikely.

Exact mechanisms leading to PH in MMM are still controversial. In the absence of portal and/or hepatic vein thromboses two theories have been proposed. The first theory states that PH develops in MMM patients due to sinusoidal narrowing and intrahepatic obstruction caused by extramedullary hematopoiesis and infiltration of the liver by myeloid cells leading to increased intrahepatic resistance[6,16,17], while the other theory states that PH develops in such patients due to increased portal blood flow through the enlarged spleen[8,18,19].

Portal vein thrombosis is a major cause of PH in hematological disorders[6,16,18]. It is still unclear whether portal vein thrombosis in MMM is caused by a hypercoagulable state and hyperviscosity related to underlying disease or as a consequence of stasis due to elevated sinusoidal pressure. In our study, PH in three of our patients (23%) was due to thrombosis of the portal and/or hepatic veins as indicated by imaging studies. The three patients had prothrombin time within normal limits. However, one of these patients had a very high white blood cell count (49 000 cells/mL), possibly contributing to thrombosis by increasing the blood viscosity. When thrombosis is absent, PH has been related to an increased intrahepatic resistance and sinusoidal narrowing due to the myeloid metaplasia[6,16,17] and/or due to a marked increase in portal flow as a consequence of marked splenomegaly[8,18,19]. In our report, the 10 patients who did not have portal vein thrombosis had splenomegaly; six of them had dilated splenic veins with increased portal blood flow indicated by abdominal doppler ultrasound. The increase in portal flow itself may explain PH. In fact, few cases of PH in myelofibrosis patients secondary to increased splenic and/or portal flow with minimal hematopoiesis have been reported[20,21]. On the other hand, Sikuler *et al*[22] experimentally demonstrated that in the absence of structural alteration of the liver, PH does not develop as a consequence of an increased portal flow. They proposed that the main contributory factor is the increased intrahepatic resistance caused by obstruction due to extramedullary hematopoiesis of the liver. In our group of patients, liver biopsy in those six patients with increased portal flow and another two patients without increased portal flow showed infiltration of liver sinusoids with hematopoietic cells and myeloid metaplasia. Therefore six of our patients, without thrombotic etiology, have both enhanced portal flow and increased intrahepatic resistance as contributing factors. Hence, it seems that in absence of thrombosis, both enhanced portal flow from the enlarged spleen and intra-hepatic sinusoidal obstruction have synergistic effects, so that even a slight increase in resistance in the face of enhanced portal flow might produce clinically significant PH.

Patients with PH characteristically exhibit a hyperdynamic circulation with increased cardiac output and decreased peripheral resistance[23]. Overactivity of some vasodilator factors has been proposed and there is a growing body of evidence suggesting that endogenous NO accounts for much of this activity[24-26]. Anemia, which is a very common feature in patients with MMM, has been shown to further worsen the hyperdynamic circulation associated with PH[27-29]. In this group of patients, 10 patients (77%) were anemic at the time of PH

diagnosis. At presentation, a total of four patients had a picture of severe PH. Three of these four patients had profound anemia (hemoglobin 6.6, 6.9 and 7.1 mg/dL) suggesting that anemia, possibly by worsening the hyperdynamic circulation, might have a role in exacerbating PH and that anemia may correlate with and/or predict the severity of the PH presentation in these patients. Experimental studies have demonstrated that increasing blood hemoglobin levels partially correct PH hyperdynamic circulation in rats[30]. However, clinical data focusing on the role of anemia on the hyperdynamic state are scant and more studies are needed.

The optimal management of PH secondary to MMM and management of subsequent variceal hemorrhage and ascites have not been well established. Based on the theory of increased portal flow due to splenomegaly as a mechanism for the PH in MMM patients, splenectomy would be a reasonable choice. In fact, splenectomy, which is commonly performed for patients to relieve abdominal discomfort and early satiety caused by the mass effect of the enlarged spleen, has effectively reversed PH in selected patients[4,31]. However, increased blood flow from an enlarged spleen is not the sole mechanism for development of PH in MMM patients. Also, the enlarged spleen may possibly be the main or even the only site for red cell production in patients with advanced MMM. Moreover, there are some unique post-operative complications such as massive hepatomegaly due to extramedullary hematopoiesis in 16%-24% of splenectomized MMM patients leading to liver failure in some cases, post-splenectomy extreme thrombocytosis in up to 50% of splenectomized MMM patients, and the major concern of leukemic transformation. All these factors, together with the knowledge that splenectomy has not been shown to improve overall survival in MMM patients, must be strongly considered before proceeding for splenectomy to manage PH[3,32-34].

PH caused by intra-hepatic or portal obstruction requires interventional or surgical portosystemic shunting. Relief of intra-hepatic PH in patients with MMM can be accomplished by implantation of a transjugular intrahepatic portosystemic shunt (TIPS)[35-37]. TIPS is an effective and well established procedure that involves creation of a side-to-side portocaval shunt in the liver and it has very good efficacy for intractable ascites. However, such a procedure needs ideal candidates who display normal liver synthetic function with little interventional risk. Only a few reports have been published regarding the use of TIPS for PH secondary to MMM, but these have proved to be effective[3,35-39]. Few data exist regarding the outcomes of this procedure in MMM patients and more studies are needed to examine whether it prolongs survival or just alleviates variceal hemorrhage and recurrent ascites.

Managing the acute bleeding episodes consists of general resuscitative measures such as volume and blood replacement, and specific measures to stop bleeding. EVL[40-42] as well as EVS[7] have been utilized successfully for the management of GI bleeding in MMM patients. EVL has been reported to have very good efficacy, with fewer therapeutic sessions and complications when compared to EVS in variceal bleeding due to other etiologies[43]. In our report, six out of eight patients who had variceal bleeding underwent EVL with no variceal recurrence or hematological worsening during a brief 12 mo follow up period. Nevertheless, there is a paucity of data regarding the use of EVL in patients with MMM-associated PH and further studies are required. We report our successful experience with EVL in this small group of patients.

In conclusion, patients with MMM might develop PH. Exact mechanisms leading to PH in MMM are still controversial. In the absence of portal vein thrombosis, both increased intrahepatic resistance due to sinusoidal narrowing caused by extramedullary hematopoiesis and a rise in portal pressure, *via* an increase in portal blood flow secondary to increased splenic blood flow from an enlarged spleen, might play a role in the pathogenesis of PH. Clinical presentation is similar to PH due to other etiologies with variceal bleeding and ascites being the most common presentations. Anemia may correlate with, and/or predict the severity of, the PH presentation in these patients. EVL can successfully control variceal bleeding in MMM. Further clinical studies are required.

COMMENTS

Background

Patients with myelofibrosis with myeloid metaplasia (MMM) present with variable clinical features. The typical clinical features include constitutional symptoms, splenomegaly and progressive anemia. Portal hypertension (PH) with subsequent variceal bleeding and ascites has been described.

Research frontiers

Few published studies have discussed PH in patients with MMM. However, these small reports described and focused on PH in chronic idiopathic myelofibrosis (CIM) which represents a subgroup of MMM.

Innovations and breakthroughs

In this retrospective study, the authors describe clinical presentation and complications of 13 patients with PH secondary to MMM. The article also discusses the possible mechanisms leading to PH in these patients and the possible treatment options.

Applications

Patients with MMM might develop PH. As in other etiologies, variceal bleeding and ascites are the most common presentations. Exact mechanisms leading to PH in MMM are still controversial. Interestingly, anemia may correlate with and/or predict the severity of the PH presentation in those patients. Endoscopic variceal ligation can successfully control variceal bleeding in these patients. However, further clinical studies and trials are required.

Terminology

PH is an increase in the pressure within the portal vein and its tributaries. It is defined as a portal pressure of 12 mmHg or more compared with a normal figure of 5-8 mmHg. Myelofibrosis with myeloid metaplasia is a term referred to for patients with CIM, also known as angiogenic myeloid metaplasia, and advanced phases of polycythemia vera and essential thrombocythemia.

Peer review

This is a retrospective study of patients with myelofibrosis and PH. The authors describe the clinical presentation and complications of this unusual condition. The paper is well written and includes a discussion of the pathogenesis of PH in myelofibrosis.

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BRIEF ARTICLES

Serum bile acid profiling reflects enterohepatic detoxification state and intestinal barrier function in inflammatory bowel disease

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Abstract

AIM: To determine free and conjugated serum bile acid (BA) levels in inflammatory bowel disease (IBD) subgroups with defined clinical manifestations.

METHODS: Comprehensive serum BA profiling was performed in 358 IBD patients and 310 healthy controls by liquid chromatography coupled to electrospray ionization tandem mass spectrometry.

RESULTS: Serum levels of hyodeoxycholic acid, the CYP3A4-mediated detoxification product of the secondary BA lithocholic acid (LCA), was increased significantly in Crohn's disease (CD) and ulcerative colitis (UC), while most other serum BA species were decreased significantly. Total BA, total BA conjugate, and total BA glycoconjugate levels were decreased only in CD, whereas total unconjugated BA levels were decreased only in UC. In UC patients with hepatobiliary manifestations, the conjugated primary BAs glycocholic acid, taurocholic acid, and glycochenodeoxycholic acid were as significantly increased as the secondary BAs LCA, ursodeoxycholic acid, and tauroursodeoxycholic acid compared to UC patients without hepatobiliary manifestations. Finally, we found that in ileocecal resected CD patients, the unconjugated primary BAs, cholic acid and chenodeoxycholic acid, were increased significantly compared to controls and patients without surgical interventions.

CONCLUSION: Serum BA profiling in IBD patients that indicates impaired intestinal barrier function and increased detoxification is suitable for advanced diagnostic characterization and differentiation of IBD subgroups with defined clinical manifestations.

Key words: Bile acids; Liquid chromatography; Tandem mass spectrometry; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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INTRODUCTION

The pathophysiology of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) is not yet completely understood. In recent years, it has become obvious that genetic, immunological, environmental and microbial factors contribute to the etiology of IBD[1-5]. The concept of multilevel dysfunction of the intestinal detoxification system is accepted as an important aspect of the pathophysiology of IBD[6]. Intestinal epithelial cells are of major importance as a physiological barrier against components of the intestinal lumen such as bacteria, nutrients and toxins. Protective mechanisms that maintain intestinal barrier function include detoxification and biotransformation of luminal substances, as well as the control of junctional proteins in cell-cell contacts. These processes are influenced by lipids and the availability of adequate cellular lipid processing[7].

Bile acids (BAs) are involved in these processes. First of all, intestinal reabsorption is a critical step in enterohepatic circulation (EHC) of BAs[8]. Once synthesized in the liver and secreted *via* the bile duct into the duodenum, BAs are effectively absorbed in the distal ileum and transported back to the liver *via* the portal vein, which contributes to the pool of BAs in the blood[9,10]. Absorption in the distal ileum may be hampered in CD by terminal ileitis or by ileocecal resection, which tends towards decreased fasting and slightly increased postprandial blood BA levels[11-15]. There have also been indications of abnormal blood BA levels in UC[16,17]. Furthermore, EHC and enterohepatic detoxification of BAs depend on a carefully adjusted regulatory network of BA-binding transcription factors, including farnesoid X receptor (FXR) and pregnane X receptor (PXR)[9,18]. For instance, like ursodeoxycholic acid (UDCA), the endogenous toxic lithocholic acid (LCA) belongs to a group of potent PXR agonists[19-21] that comprises steroid hormones, vitamins and β-carotene[22]. Several detoxification genes and ATP-binding cassette transporters are down-regulated in intestinal cells of IBD patients[23], and PXR as a major transcriptional regulator of these detoxification genes is decreased in UC patients[23]. Finally, despite their potential toxic activities, BAs can also confer gut mucosal protection against bacteria and other cell damaging constituents of the gut lumen by two mechanisms[10]. In the proximal small intestine, BAs inhibit bacterial growth directly *via* their pharmacological properties, whereas in the distal small intestine, BAs mediate their antimicrobial effect indirectly *via* activation of FXR[24,25]. For instance, binding of chenodeoxycholic acid (CDCA) to FXR and subsequent activation of the receptor is followed by up-regulation of genes that are involved in the prevention of bacterial overgrowth and promotion of epithelial integrity[26].

In the present multicenter study, serum BA profiling was performed retrospectively in 358 IBD patients and in 310 age-matched healthy controls to assess the influence of different IBD phenotypes with various clinical manifestations on BA composition. The results further elucidate the intestinal contribution to BA homeostasis and detoxification, which is much less understood compared to corresponding processes in the liver[24,27].

MATERIALS AND METHODS

Patients and specimens

Blood samples of IBD patients and healthy volunteers were from the University Hospitals of Regensburg and Heidelberg (Germany), the Konventhospital Barmherzige Brueder Linz (Austria), and the Innsbruck Medical University (Austria). Informed consent was obtained from all patients, and the study was approved by the respective ethics committees. Samples of the Regensburg patients were from the serum bank of the German IBD network of excellence ("Kompetenznetz CED"). Blood samples were collected irrespective of the individual diet. For BA analysis, sera and clinical data were obtained from 197 CD patients (62% females; aged 16-84 years, mean age, 40 years; 46 with active disease, 43 with chronic active disease, 108 in remission) and 161 UC patients (63% females; aged 17-90 years, mean age, 40 years; 42 with active disease, 40 with chronic active disease, 79 in remission). The diagnosis was based on clinical, radiological and pathological criteria according to the guidelines of the German Gastroenterological Association (DGVS). CD patients were subgrouped according to the Vienna Classification with respect to disease behavior and localization. A CD activity index (CDAI) > 150 was regarded as "active CD", duration of CDAI > 150 for > 3 mo as "chronic active CD", and CDAI < 150 as "CD in remission". UC patients were classified according to the Truelove-Witts index: A Truelove-Witts index > 6 was regarded as "active UC", a Truelove-Witts index > 6 for > 3 mo as "chronic active UC", and a Truelove-Witts index < 6 as "UC in remission". Sera from 310 healthy blood donors (60% females; aged 19-66 years, mean age, 40 years) served as controls. All samples were stored frozen at -20℃ until analysis.

Materials for BA analysis

Cholic acid (CA), CDCA, deoxycholic acid (DCA), LCA, UDCA, hyodeoxycholic acid (HDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), glycoursodeoxycholic acid (GUDCA), glycohyodeoxycholic acid (GHDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), taurolithocholic acid (TLCA), taurour-

sodeoxycholic acid (TUDCA), taurohyodeoxycholic acid (THDCA) standard substances, as well as deuterated BA internal standard (IS) substances (D_4-CA, -CDCA, -DCA, -LCA, -UDCA, -GCA, -GCDCA) were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), Steraloids Inc. (Newport, RI, USA), Campro Scientific GmbH (Berlin, Germany), Larodan Fine Chemicals AB (Malmø, Sweden), and were at least of 95% purity. Ammonium acetate (98%), hydrochloric acid (p.a.), as well as HPLC grade methanol and acetonitrile were purchased from VWR Int. GmbH (Darmstadt, Germany). Ultra pure water (18.2 MΩcm) was obtained from a Milli-Q Plus system (Millipore GmbH, Schwalbach, Germany).

BA extraction

For serum BA extraction, the method of Tagliacozzi *et al*[28] was applied with some modifications. Twenty-five microliters of a mixed IS BA solution (6-140 μmol/L in methanol) was pipetted into a 1.5-mL reaction tube and vacuum-evaporated. Two hundred and fifty microliters serum and 30 μL 1 mol/L hydrochloric acid were added (pH < 1), and the mixture was shaken for 1 min. After addition of 1 mL acetonitrile, the mixture was shaken for 2 min and centrifuged at 14000 *g* for 15 min. The acetonitrile supernatant was transferred to a new reaction tube and vacuum-evaporated. The residue was dissolved in 250 μL methanol/water 1:1 (v/v) that contained 10 mmol/L ammonium acetate by shaking and sonication, and centrifuged at 14000 *g* for 5 min. Ten microliters of the clear methanolic supernatant was used for analysis. Calibration samples were prepared by spiking pooled control serum with 0, 25, 50, 75 and 100 μL of a combined BA standard solution that contained appropriate amounts of each BA (0.5-70.5 μmol/L).

Liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) determination of BAs

BAs were analyzed by LC-ESI-MS/MS using the following instrumentation. An HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), an Agilent 1100 series binary HPLC pump (Agilent Technologies Sales & Services GmbH & Co KG, Waldbronn, Germany) combined with a Micromass Quattro Ultima tandem MS (Waters GmbH, Eschborn, Germany) operated in negative-ion mode. BA separation was performed on a Symmetry C18 reversed-phase HPLC column (50 mm × 2.1 mm, 3.5 μm particle size; Waters GmbH) by gradient elution at a flow rate of 0.3 mL/min. The 25-min elution cycle consisted of a stepwise linear change from 90% solvent A (methanol/water 1:1, v/v, 10 mmol/L ammonium acetate) + 10% solvent B (methanol, 10 mmol/L ammonium acetate) to 100% B; in detail: 0-6 min A/B = 90/10; 6-12 min A/B = 72/28; 12-16 min A/B = 60/40; 16-22 min A/B = 0/100; 22-25 min A/B = 90/10. The mass spectrometer was operated in multiple-reaction monitoring (MRM) with a cone voltage of 80 V and a collision gas pressure of 250 kPa argon. Unconjugated BAs were detected unfragmented using a collision energy of 10 eV. Glycine- and taurine-conjugated BAs were analyzed by their specific product ions at m/z 74 and 80 using collision energies of 35 and 85 eV, respectively. In detail, the retention times and MRM transitions were as follows: TCA (6.0 min, 514→80), TUDCA (4.0 min, 498→80), THDCA (4.8 min, 498→80), TCDCA (9.3 min, 498→80), TDCA (10.2 min, 498→80), TLCA (13.7 min, 482→80), GCA (6.2 min, 464→74), GUDCA (4.1 min, 448→74), GHDCA (4.9 min, 448→74), GCDCA (9.5 min, 448→74), GDCA (10.4 min, 448→74), GLCA (13.9 min, 432→74), CA (7.1 min, 407→407), UDCA (5.1 min, 391→391), HDCA (6.0 min, 391→391), CDCA (11.0 min, 391→391), DCA (11.8 min, 391→391), LCA (14.8 min, 375→375). Quantification was performed by peak ratios of BA peak areas and corresponding IS peak areas. BAs without identical deuterated ISs were related to the IS with the nearest retention time, as well as the similar MRM transition.

Statistical analysis

The significance of differences in BA concentrations was determined between cohorts with $n \geqslant 10$ by Mann-Whitney *U* test for non-normally distributed data using SPSS for Windows version 14.0 (SPSS Inc., Chicago, IL, USA). A two-sided *P* value < 0.05 was considered statistically significant.

RESULTS

Differentiation of CD, UC and controls by characteristic BA profiles

Based on previous findings on BA metabolism in IBD and our earlier results on dysregulation of xenobiotic nuclear receptors including PXR in IBD[23], serum BA levels and composition were determined in the two major IBD phenotypes, CD and UC, and in healthy controls. The most significant differences in serum BA concentrations were found in comparison to controls. BA concentrations were decreased predominantly in both IBD subgroups, CD and UC (Tables 1-3). Considering individual BA species, as in CD, most BAs were decreased significantly in UC patients compared with controls, but several BA conjugates, for instance TCDCA, GCDCA and GDCA, were decreased more significantly in CD than in UC patients (Table 1).

While serum levels of LCA, known to be the strongest PXR agonist, were significantly lower, serum levels of HDCA, the CYP3A4-mediated detoxification product of LCA, were always significantly higher in CD and UC patients compared to controls (Figure 1).

If total serum BA levels were considered (Table 4), we found that total unconjugated and total BA tauroconjugate levels, respectively, were decreased significantly in UC patients, but other than in CD patients, there was no decrease in total BA, total BA conjugate, and total BA glycoconjugate levels compared to controls. Moreover, total BA conjugate levels, as well as total BA glycoconjugate and

Table 1 Serum bile acids and conjugates in CD, UC and control cohorts (nmol/L)

BA class	BA/BA-conjugate	Control	CD	UC
Primary	CA	62.5	72.0	58.0
	TCA	23.6	0.0[a]	0.0[a,b]
	GCA	383.5	234.0[a]	377.0[b]
	CDCA	196.5	190.0	145.0[a,b]
	TCDCA	230.5	46.0[a]	93.0[a,b]
	GCDCA	1446.0	848.0[a]	1243.0[a,b]
Secondary	DCA	239.8	53.0[a]	64.0[a]
	TDCA	48.2	0.0[a]	0.0[a]
	GDCA	238.6	26.0[a]	80.0[a,b]
	LCA	15.0	6.2[a]	8.0[a]
	TLCA	0.0	0.0[a]	0.0[a]
	GLCA[1]	17.4	0.0[a]	0.4[a]
	UDCA	28.5	22.0[a]	17.0[a]
	TUDCA	0.0	0.0[a]	0.0[b]
	GUDCA[1]	137.9	75.6[a]	60.6[a]
Tertiary	HDCA	0.0	16.0[a]	10.0[a]
	THDCA	0.0	0.0[a]	0.0[a]
	GHDCA[1]	0.0	0.0[a]	0.0[a]

Control: n = 310; CD: n = 197, n = 73[1]; UC: n = 161, n = 44[1]. Data expressed as medians. [a]P < 0.05 *vs* control, [b]P < 0.05 *vs* CD (Mann-Whitney U test).

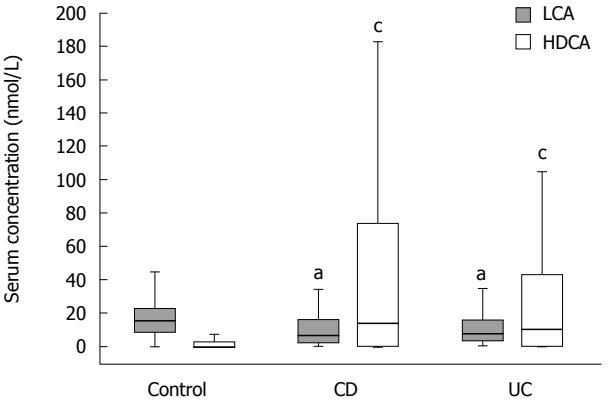

Figure 1 Decrease of serum LCA but increase of serum HDCA in CD and UC *vs* healthy control cohorts. LCA and HDCA were analyzed using LC-ESI-MS/MS. Box plots represent interquartile ranges containing medians (boxes) and minimum/maximum bars. [a]P < 0.05 *vs* LCA in controls; [c]P < 0.05 *vs* HDCA in controls (Mann-Whitney U test).

tauroconjugate levels alone, were increased significantly in UC *vs* CD patients. However, if levels relative to total BA conjugate levels were considered, total BA tauroconjugate levels were increased significantly but total BA glycoconjugate levels were decreased significantly in UC *vs* CD patients. In addition, UC patients were characterized by a significantly decreased ratio of total deoxy-BA, LCA, and LCA conjugate levels to total BA levels compared to CD patients and controls (Table 4).

Hepatobiliary manifestations influence BA composition in UC

IBD is often accompanied by extraintestinal manifestations (EMs), such as hepatobiliary diseases, and eye, joint and skin affections[29,30]. We therefore investigated whether IBD patients with EMs showed different serum

Table 2 Effect of extraintestinal manifestations on serum bile acids and conjugates in UC cohorts (nmol/L)

BA class	BA/BA-conjugate	Control	No EM	Arthralgia/arthritis	Hepatobiliary diseases
Primary	CA	62.5	67.5	88.5	59.0
	TCA	23.6	0.0	0.0	67.9[b,c]
	GCA	383.5	360.0	408.5	722.5[a,b]
	CDCA	196.5	37.5	175.5	252.0
	TCDCA	230.5	140.0[a]	92.5[a]	163.0[c]
	GCDCA	1446.0	1327.0	1281.5	2403.0[b,c]
Secondary	DCA	239.8	78.0[a]	28.0[a]	62.5[a]
	TDCA	48.2	0.0[a]	0.0[a]	0.0[a]
	GDCA	38.6	82.2[a]	14.0[a]	131.7[c]
	LCA	15.0	8.0[a]	6.1[a]	18.0[b,c]
	TLCA	0.0	0.0[a]	0.0[a]	0.0
	GLCA[1]	17.4	2.0[a]	0.0	6.5
	UDCA	28.5	18.0[a]	7.0[a,b]	131.5[b,c]
	TUDCA	0.0	0.0	0.0[a,b]	30.9[a,b,c]
	GUDCA[1]	137.9	96.5	54.1	75.1
Tertiary	HDCA	0.0	9.5[a]	8.0[a]	28.0[a]
	THDCA	0.0	0.0[a]	0.0	0.0
	GHDCA[1]	0.0	0.0	0.0	0.0

Control: n = 310; No EM: n = 50, n = 21[1]; Arthralgia/arthritis: n = 30, n = 7[1]; Hepatobiliary diseases: n = 16, n = 6[1]. Data expressed as medians. [a]P < 0.05 *vs* control, [b]P < 0.05 *vs* no EM, [c]P < 0.05 *vs* arthralgia/arthritis (Mann-Whitney U test).

BA profiles. While we found no influence of EMs on serum BA levels in CD patients (data not shown), UC patients with hepatobiliary diseases, e.g. primary sclerosing cholangitis (PSC), hepatitis, or cholelithiasis, had significantly increased BA concentrations compared to UC patients without EMs, especially levels of the primary BAs TCA, GCA and GCDCA, as well as the secondary BAs LCA, UDCA and TUDCA (Table 2).

Previous bowel resection influences BA composition in CD

Intestinal reabsorption of BAs is a physiological function of the terminal ileum, therefore, surgical interventions in this region may influence serum BA levels caused by impaired EHC. While there were no UC patients with surgical interventions included in the present study, CD patients showed significant variations in serum BA concentrations correlated to previous bowel resection (Table 3). Overall, compared to controls and CD patients without surgical interventions, ileocecal resection alone was associated with the most intensive decrease of primary and secondary BA conjugates, such as TCDCA, TDCA, GCA and GDCA, as well as a marked increase in the unconjugated primary BAs, CA and CDCA. In addition, CD patients with ileocecal resection and other surgical interventions, e.g. ileostomy, sigmoidostomy, transversostomy, fistula excision, and hemicolectomy, had significantly decreased TCDCA compared to controls and CD patients without surgical interventions. Furthermore, in CD patients with colectomy, TCDCA, as well as the secondary BAs GDCA and DCA, were decreased significantly compared to those in controls and patients without surgical interventions.

Table 3 Effect of surgical interventions on serum bile acids and conjugates in CD cohorts (nmol/L)

BA class	BA/BA-conjugate	Control	No surgery	Ileocecal resection	Colectomy	Other surgery	Ileocecal resection + other surgery
Primary	CA	62.5	52.0	160.0[a,b]	71.0	57.5[c]	89.0[b]
	TCA	23.6	0.0[a]	0.0[a,b]	9.5[c]	8.0[c]	0.0
	GCA	383.5	240.5[a]	156.0[a,b]	375.0[c]	212.0	221.5[a]
	CDCA	196.5	144.0[a]	505.0[a,b]	174.5	153.0	121.0
	TCDCA	230.5	81.0[a]	19.0[a,b]	37.5[a,b]	55.5[a]	17.0[a,b]
	GCDCA	1446.0	848.5[a]	767.0[a]	1059.0	663.5[a]	772.0
Secondary	DCA	239.8	90.5[a]	11.0[a]	6.5[a,b]	17.5[a]	53.0[d]
	TDCA	48.2	5.9[a]	0.0[a,b]	0.0[a]	0.0[a,b]	0.0[a]
	GDCA	238.6	64.0[a]	0.0[a,b]	0.0[a,b]	0.0[a,b]	43.5[a]
	LCA	15.0	7.0[a]	5.9[a]	3.0[a]	4.7[a]	2.9[a]
	TLCA	0.0	0.0	0.0[a,b]	0.0[a,b]	0.0	0.0
	GLCA[1]	17.4	3.6[a]	0.0[a]	0.0	2.3	0.0
	UDCA	28.5	14.5[a]	26.0[b]	12.7	53.5	22.5
	TUDCA	0.0	0.0	0.0[a]	0.0[a]	0.0[a]	0.0[a]
	GUDCA[1]	137.9	95.2[a]	59.7	92.1	323.5	75.6
Tertiary	HDCA	0.0	27.0[a]	10.0[a]	9.5[a]	9.0[a]	2.0[a]
	THDCA	0.0	0.0[a]	0.0	0.0	0.0	0.0
	GHDCA[1]	0.0	0.0	0.0	0.0	2.0	0.0

Control: n = 310; No surgery: n = 64, n = 31[1]; Ileocecal resection: n = 41, n = 16[1]; Colectomy: n = 22, n = 8[1]; Other surgery: n = 12, n = 4[1]; Ileocecal resection + other surgery: n = 12, n = 9[1]. Data expressed as medians. [a]P < 0.05 *vs* control, [b]P < 0.05 *vs* no surgery, [c]P < 0.05 *vs* ileocecal resection, [d]P < 0.05 *vs* colectomy (Mann-Whitney U test).

Table 4 Total serum bile acids and conjugates in CD, UC and control cohorts

	Control	CD	UC
Total BAs (nmol/L)	3752.0	2563.3[a]	3010.2
Total unconjugated BAs[1] (nmol/L)	644.1	631.1	471.0[a,b]
Total conjugated BAs (nmol/L)	2763.6	1526.9[a]	2529.6[b]
Total BA glycoconjugates (nmol/L)	2423.6	1407.6[a]	2298.9[b]
Total BA glycoconjugates/total conjugated BAs (%)	87.4	95.7[a]	92.7[a,b]
Total BA tauroconjugates[1] (nmol/L)	344.2	68.0[a]	145.0[a,b]
Total BA tauroconjugates/total conjugated BAs (%)	12.6	4.3[a]	7.3[a,b]
(Deoxy-BAs + TLCA + GLCA + LCA)/total BAs (%)	83.7	84.1	77.4[a,b]

Control: n = 310; CD: n = 73, n = 197[1]; UC: n = 44, n = 161[1]. Data expressed as medians. [a]P < 0.05 *vs* control, [b]P < 0.05 *vs* CD (Mann-Whitney U test).

No effects of disease activity and medical treatment on BA composition in CD and UC

We also investigated whether serum BA composition was influenced by disease activity and different therapeutic medications in IBD patients, since mucosal inflammation, as well as pharmacologically induced changes in the inflammatory process in IBD may influence BA reabsorption, which results in changed serum BA profiles. Overall, serum BA composition in CD and UC were independent of disease activity and medical treatment (data not shown).

DISCUSSION

During the past three decades, BA analysis in IBD has been achieved in serum, bile or feces from small patient cohorts by radioimmunoassay or gas-liquid chromatography detecting total BAs and selected individual BAs, respectively[17,31-34]. In the present study, we applied a sensitive high-throughput LC-ESI-MS/MS method with minimal sample preparation steps for simultaneous determination of 18 different BA species as serum BA profiles in a large cohort of IBD patients and controls. We analyzed the main unconjugated human primary, secondary and tertiary BAs, i.e. CA, CDCA, DCA, LCA, UDCA, HDCA, and their respective glycine and taurine conjugates. Comparing a whole profile of BA subspecies in various IBD phenotypes may reflect intestinal malfunction and disease states more sensitively than just considering total or selected individual BA levels.

Thus, we showed that decreased serum BA levels were not restricted to CD alone, as previously reported[13-15,35], but were also found in UC if a defined set of specific BAs were considered. This is contradictory to the few reports on abnormal blood BA levels in UC patients. Ejderhamm and Strandvik[17] have reported increased primary serum BAs, CA and CDCA, in juvenile active UC patients compared to healthy controls, while there were no significant differences for CA, but decreased CDCA levels in our UC cohort compared to controls. Kostic *et al*[16] have reported decreased total plasma BA levels in CD and UC patients compared to controls, but we did not find significant differences in total BA, total BA conjugate, and total BA glycoconjugate levels between UC patients and the control group. Only total BA tauroconjugate and unconjugated BA levels were reduced significantly in UC patients *vs* controls. However, because of their relatively low contribution to total BA and BA conjugate levels compared to the most abundant BA glycoconjugates, this effect was not dominant and may explain the missing reports on decreased total BA levels in UC patients. In summary, our data confirm studies that have shown a decrease in serum

BA levels in CD patients, which reflects the strongest impact on intestinal BA reabsorption during EHC. This can be explained by the fact that BA reabsorption takes place predominantly in the distal small intestine[10], which is usually more affected in CD in contrast to colon-restricted UC. Therefore, most serum BA levels in UC patients are not decreased as much as in CD patients.

Furthermore, it is noteworthy that the levels of the unconjugated primary BAs, CA and CDCA, in CD and UC patients were not significantly different from the controls, except for decreased CDCA in UC patients. The explanation that there is an increased compensatory synthesis of primary BAs in IBD, as suggested by Rutgeerts *et al*[13] in Crohn's ileitis, assumes an accelerated bacterial deconjugation of the respective glyco- and tauroconjugates in the intestine. Indeed, the reduced serum levels of TCA, TCDCA, GCA, and GCDCA shown in Table 1 support this assumption, which is in agreement with previous findings of unusually high intestinal BA deconjugation in CD and UC[36,37]. Apart from deconjugation, the quantitatively most important bacterial biotransformation of BAs is 7α-deoxidation of CA and CDCA by *Eubacteria* in the colon, which yields DCA and LCA, respectively[38,39]. In UC patients, we found significantly reduced ratios of total deoxy-BA (including DCA, LCA, and its conjugates) to total BA levels compared to those in CD patients and controls, which may reflect an abnormal colonic bacterial flora with reduced deoxidation capacity. In addition, bacterial overgrowth in the small intestine and colon of IBD patients may enhance the described BA biotransformation processes and contribute to the imbalance of BA species distribution in the EHC. Decreased intestinal BA levels, especially of conjugated BAs, may promote bacterial overgrowth because of a loss of their antimicrobial properties[10,26]. Since IBD patients included in this study were not stratified according to the use of antibiotics, this effect has not been evaluated and needs further systematic investigation in well-defined patient cohorts.

The invariably increased HDCA and decreased LCA in IBD compared to control sera, irrespective of the clinical findings (EMs, surgical interventions, disease activity or medication), indicates accelerated enterohepatic LCA detoxification *via* CYP3A4[18]. Whether serum HDCA elevation is additionally caused by increased intestinal reabsorption or impaired hepatic excretion cannot be resolved by the present data and has to be further investigated.

Moreover, the influence of hepatobiliary EMs on serum BA levels in IBD is demonstrated clearly in UC patients, who showed a significant increase in primary and secondary BAs compared to EM-free patients. This confirms previous observations when elevated serum levels of total primary BA conjugates have been seen in IBD patients with liver diseases[31,40]. Although serum LCA levels in UC patients with hepatobiliary EMs were found to be normal compared to controls, in accordance with the findings of Dew *et al*[40], they were significantly higher compared to those in UC patients without EMs and with arthralgia/arthritis. Elevated BA levels are particularly found in patients with PSC, which is often associated with IBD[29,41]. However, it cannot be ruled out that the significantly increased serum levels of TUDCA, UDCA and LCA found in our UC cohort with hepatobiliary EMs were caused by therapeutic administration of UDCA, which is being used increasingly for the treatment of cholestatic liver diseases[42-44] and PSC-associated UC[45,46]. UDCA medication not only causes increased primary BA biosynthesis, but UDCA is also metabolized to additional TUDCA and LCA[47], which yields increased serum levels in these patients. Nonetheless, we assume that disturbed EHC of BAs in IBD is highly susceptible to additional hepatobiliary EMs.

With surgical interventions predominantly appearing in CD patients, we found that ileocecal resection exerts the strongest impact on serum BA levels in CD patients, since BA reabsorption is located predominantly in the terminal ileum[10]. Compared to patients without surgical interventions, the finding that patients with ileocecal resection showed significantly decreased conjugated BAs but increased unconjugated primary BAs, i.e. CA and CDCA, may be explained by an increased compensatory synthesis of primary BAs in IBD, as previously suggested[13], associated with an enhanced bacterial deconjugation of the respective glyco- and tauroconjugates in the remaining intestinal sections[36,37]. In addition, concerning the elevated CA levels in patients with ileocecal resection, we confirm the previous findings of Tougaard *et al*[48]. As expected, unlike ileocecal resection, the influence of colectomy on serum BA levels in CD was less significant, since only small amounts of BAs are reabsorbed in this intestinal region.

To summarize, using mass spectrometric BA species profiling instead of total BA determination, we showed the characteristic impact of different IBD phenotypes with intestinal and hepatobiliary manifestations on BA homeostasis and detoxification. Further prospective studies on prominent BAs in well-defined IBD cohorts are necessary to confirm their diagnostic and prognostic value.

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COMMENTS

Background

Inflammatory bowel disease (IBD) is a multifactorial disorder with as yet incompletely elucidated causes. Since bile acids (BAs) derived from the liver are directly involved in intestinal processes primarily by facilitating lipid digestion, IBD has an impact on BA metabolism. This correlation may be reflected in unusual BA blood levels that differentiate between the two clinical IBD phenotypes, Crohn's disease (CD) and ulcerative colitis (UC), as well as between CD and UC subgroups with diverse clinical manifestations.

Research frontiers

Besides their digestive functions, BAs have recently been found to play an important regulatory role in numerous metabolic processes, e.g. energy and lipid balance and elimination of harmful substances. They are mediated by binding appropriate nuclear receptors in the cell that depend on the molecular type of BA, which can be differentiated by means of high performance mass spectrometry. Thus, quantifying diverse BAs simultaneously, a characteristic profile of main and rare BAs is available that reflects medical conditions far better than measuring total BA levels or individual abundant BAs.

Innovations and breakthroughs

Applying BA profiling in IBD patients, the authors showed that most but not all BA species are decreased in CD and UC patient sera, but with different intensity. BA decrease is highly pronounced in CD patients with surgical interventions in the gut except for unconjugated primary BAs. On the other hand, UC patients with additional liver and gallbladder diseases show clearly increased levels of primary and secondary BAs. Finally, the authors found a marked decrease in the toxic BA lithocholic acid, together with a marked increase in its physiological detoxification product, hyodeoxycholic acid, irrespective of the IBD phenotype or clinical manifestation, which shows accelerated detoxification activity in IBD patients.

Applications

Serum BA profiling may serve as an additional diagnostic tool for IBD characterization and differentiation. In combination with expression profiles of pregnane X receptor (PXR) -regulated genes, it may allow us to estimate the BA detoxification potential of IBD patients.

Terminology

Primary BAs are directly synthesized in the liver and secondary BAs are derived from primary BAs by biochemical modification by intestinal bacteria. BAs can be conjugated, mainly with the amino acids glycine and taurine, or unconjugated. The enterohepatic circulation leads to a maximum physiological recycling of BAs and comprises liver synthesis, intestinal excretion *via* the bile duct, intestinal reabsorption, and return transport to the liver *via* the portal vein. Liquid chromatography tandem mass spectrometry is a sensitive analytical method for simultaneous determination of structural related biomolecules like BAs. The nuclear BA receptors farnesoid X receptor and PXR mediate most of the physiological effects of BAs, e.g. expression of detoxification genes by PXR.

Peer review

This work expand the knowledge about the role of BA metabolism in IBD. It is a well-conducted study.

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BRIEF ARTICLES

Human papilloma virus and esophageal carcinoma in a Latin-American region

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Abstract

AIM: To investigate the presence of high-risk human papilloma virus (HPV) in esophageal squamous cell carcinomas (ESCCs) in a non-selected Mexican population.

METHODS: Cases with a pathological diagnosis of squamous cell carcinoma of the esophagus were obtained from Department of Pathology files, at the National Cancer Institute in Mexico City during the period between 2000 and 2008. Slides from each case were reviewed and cases with sufficient neoplastic tissue were selected for molecular analysis. DNA was extracted from paraffin-embedded tissue samples for polymerase chain reaction analysis to detect HPV DNA sequences. Demographic and clinical data of each patient were retrieved from corresponding clinical records.

RESULTS: HPV was detected in 15 (25%) of ESCCs. HPV-16 was the most frequently observed genotype, followed by HPV-18; HPV-59 was also detected in one case. Unfortunately, HPV genotype could not be established in three cases due to lack of material for direct sequencing, although universal primers detected the presence of HPV generic sequences. No low-risk HPV genotypes were found nor was HPV-16/18 co-infection. HPV presence in ESCC was not significantly associated with gender, age, alcohol consumption, smoking, anatomic location, or histologic grade. All patients belonged to low and very low socioeconomic strata, and were diagnosed at advanced disease stage. Male patients were most commonly affected and the male:female ratio in HPV-positive ESCC increased two-fold in comparison with HPV-negative cases (6.5:1 *vs* 3.1:1).

CONCLUSION: High prevalence of high-risk HPV in ESCC in Mexico does not support the hypothesis that HPV-associated ESCC is more common in areas with higher ESCC incidence rates.

Key words: Esophagus; Human papilloma virus; Squamous cell carcinoma; High-risk human papilloma virus

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INTRODUCTION

With regard to incidence and prevalence, esophageal cancer exhibits striking geographical variations due to unknown factors between countries, as well as between different regions of the same country. According to the World Health Organization, incidence-rate spectra are located between Western Africa -at the low-risk end, and China at the high-risk end, including the so-called "Asian esophageal cancer belt"[1].

Among Latin American countries, Mexico and Peru have the lowest mortality rates for esophageal carcinoma, in both males and females, whereas Brazil, Argentina and Chile, have the highest mortality rates[2]. Mexico has a low esophageal cancer risk, with mortality rates in male and female inhabitants *ca* 2 and *ca* 1 per 100 000, respectively[3].

Esophageal carcinoma has been related to tobacco, alcohol, fungal toxins, nutritional deficiencies, and hot food and beverages[4]. In addition, human papilloma virus (HPV), a major cause of carcinoma of the cervix uteri throughout the world, is suspected of being related to the development of this carcinoma. Meta-analyses by Syrjänen[5] (2002) showed that HPV DNA was detected in 15.2% of esophageal squamous cell carcinomas (ESCCs). To date, total or partial sequencing identified > 200 genotypes of HPV, which are categorized into the following four cervical oncogenicity-based risk groups: high, probably high, low and undetermined risks[6]. High-risk HPV is considered to cause neoplastic transformation of normal epithelial cells, through expression of early transforming viral proteins E6 and 7, resulting in cell cycle-machinery deregulation and expression of several transforming oncogenes[7].

High-risk HPV-associated ESCC is hypothesized to be consistently more common in countries with higher ESCC risk[8]. In a recent study by Castillo *et al*[9], HPV DNA was detected in 34% and 19% of esophageal carcinomas in Colombia and Chile, respectively; HPV-16 was the most frequent genotype in both countries. It is noteworthy that the HPV detection rate in ESCCs was found to be two-fold higher in Colombia than in Chile, whereas the esophageal cancer mortality rate showed an inverse relationship.

The aim of the present study was to investigate the presence of high-risk HPV DNA jn ESCCs, among a non-selected Mexican population.

MATERIALS AND METHODS

Subjects

Cases with a pathological diagnosis of squamous cell carcinoma of the esophagus were obtained from Department of Pathology files, at the National Cancer Institute in Mexico City during the period between 2000 and 2008. Slides from each case were reviewed and cases with sufficient neoplastic tissue were selected for molecular analysis. Because the majority of the cases of esophageal cancer seen at our Institution correspond to advanced clinical stages, elective surgery is not performed. Subsequently, DNA was extracted from paraffin-embedded tumor samples, obtained during a panendoscopic procedure; adjacent normal esophageal mucosa was not sampled. Demographic and clinical data of each patient were retrieved from corresponding clinical records.

DNA extraction and HPV detection

Twenty micrometer sections of formalin-fixed and paraffin-embedded tumors were de-waxed by incubation with N-octane and washings with 100% ethanol. This process was repeated twice, and the pellet was dried. Deparaffinized samples were digested with 1 mL of lysis buffer (Tris-HCl 10 mmol/L pH 8.0, EDTA 0.1 mol/L pH 8-0, SDS 0.5%, Proteinase K 200 μg/mL, RNase A 20 μg/mL) at 55℃ for 3 h. DNA was extracted with phenol/chloroform precipitations as described by Sambrook *et al*[10]. To test DNA suitability for polymerase chain reaction (PCR) amplification the DNA obtained was amplified for: β-globin gene (PCO4/GH2O) under conditions described by Resnick *et al*[11]. Samples were later submitted to HPV amplification with three sets of the following universal primers recognizing distinct size fragments of L1 gene: L1C1/L1C2, MY09/MY11, and GP5/GP6[12-14]. HPV type-specific amplification was also performed with primers designed to amplify the E6 gene of HPV type 16 and 18 as described by Lizano *et al*[15].

HPV PCR products were electrophoresed in a 1.2% agarose gel and visualized by ethidium bromide staining. HPV typing was carried out by direct sequencing of PCR products by means of the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The resulting sequences were analyzed in BLAST data bank for comparison with known HPV sequences. HPV 16 and 18 specific amplification was conducted for every DNA sample. DNA extracted from CaSki and Hela HPV containing cell lines were used as positive controls.

Statistical analysis

Fisher's exact test was applied for categorical variables. A probability value $P < 0.05$ was considered statistically significant.

RESULTS

In 60 cases of ESCC, DNA quality was adequate for PCR analysis to detect HPV DNA sequences, as demonstrated by β-globin gene amplification. There were 47 male and 13 female patients, with a mean age of 62.7 years (range, 27-85 years) and 61.2 years (range, 47-80 years), respectively. Thirty-six subjects consumed alcohol (ranging from occasional to 60 years drinkers) but frequency and alcoholic-beverage type could not be precisely assessed. In 14 subjects, previous history of alcohol consumption was unknown and ten patients denied alcohol consumption. Smoking habit was recorded in 28 patients (from occasional to up to 60 years of smoking; frequency was not known), whereas in 16 subjects smoking habit was unknown; 16 patients did not smoke. Clinical history of esophageal achalasia was not recorded in any case, and there were previous symptoms of gastro-esophageal reflux disease (GERD) in two patients. Finally, oesophageal surgery was performed in one patient 26 years previously due to direct trauma. All patients belonged to low and very-low socioeconomic strata, the majority of these were agricultural workers.

All patients were diagnosed at advanced disease stage. Among these, 42 patients (70%) were treated with chemo- and/or radiotherapy and three subjects underwent esophagectomy immediately after neoadjuvant

Table 1 HPV detection frequency according to subjects' demographic factors and lifestyles, and the clinicopathological features of esophageal carcinoma

	Total (%)	HPV- (%)	HPV+ (%)	*P* value
Total	60 (100)	45 (75)	15 (25)	
Gender				0.485
Female	13 (100)	11 (85)	2 (15)	
Male	47 (100)	34 (72)	13 (28)	
Alcohol consumption				0.377 (0.420)
No	10 (100)	9 (90)	1 (10)	
Yes	36 (100)	27 (75)	9 (25)	
Unknown	14 (100)	9 (64)	5 (36)	
Smoking				0.274 (0.450)
No	16 (100)	14 (88)	2 (12)	
Yes	28 (100)	21 (75)	7 (25)	
Unknown	16 (100)	10 (63)	6 (37)	
Esophageal location				0.330
Upper third	14 (100)	9 (64)	5 (36)	
Upper/middle third	4 (100)	2 (50)	2 (50)	
Middle third	28 (100)	23 (82)	5 (28)	
Middle/lower third	10 (100)	7 (70)	3 (30)	
Lower third	4 (100)	4 (100)	0 (0)	
Histologic grade				0.222
Basaloid/poorly differentiated	1 (100)	1 (100)	0 (0)	
Poorly differentiated	20 (100)	17 (85)	3 (15)	
Moderately-differentiated	38 (100)	27 (71)	11 (29)	
Well-differentiated	1 (100)	0 (0)	1 (100)	

P values in parentheses are those without "unknown" category.

Table 2 HPV genotypes detected in ESCCs

HPV genotype	*n* (%)
HPV-16	6 (40)
HPV-18	5 (33)
HPV-59	1 (7)
Unknown	3 (20)
Total	15 (100)

ESCCs: Esophageal squamous cell carcinomas.

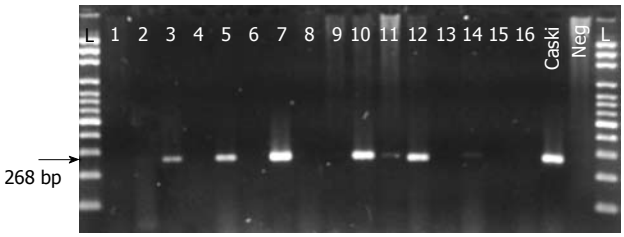

Figure 1 Identification of HPV with general primers L1C1/C2. PCR products were run in 2% agarose gel. Lanes 1-16 correspond to representative positive and negative cases. DNA extracted from Caski cell lines were used as a positive control. Neg: PCR mixture without DNA; L: 100 bp ladder.

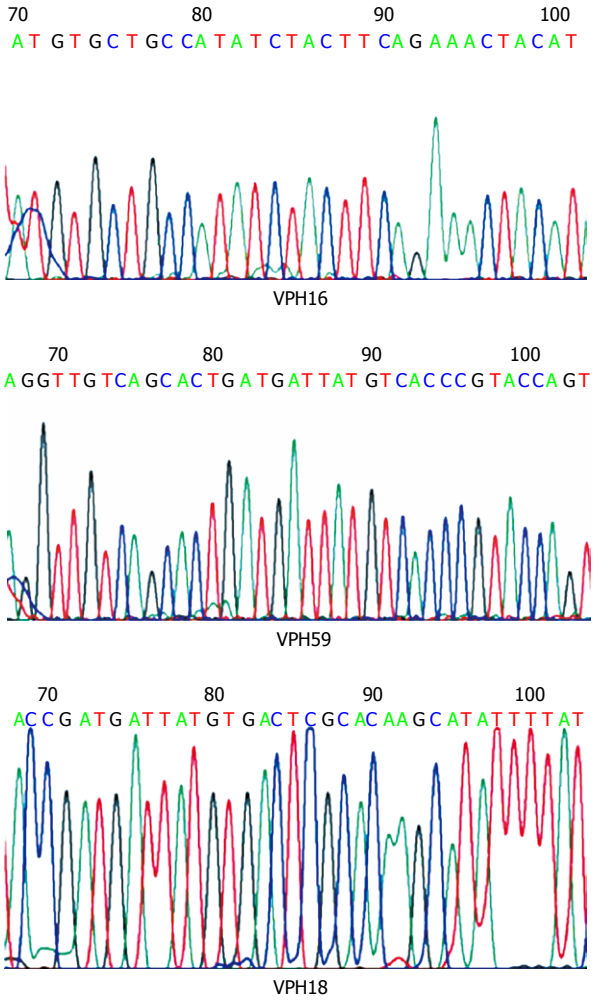

Figure 2 Representative electropherograms showing fragments of HPV-16, -18 and -59 genomic sequences, obtained from the analysis of PCR products L1C1/C2 from HPV-positive samples.

therapy. Finally, 18 patients (30%) refused any treatment. During follow-up, seven patients (11.7%) died of oesophageal carcinoma, whereas one of these died of causes not related to the neoplastic disease. The majority of patients were alive but with disease at 1 year or less of follow-up, but did not return for subsequent medical care. Regardless of the presence of HPV, all patients with ESCCs belonged to low and very-low socioeconomic strata, the majority of these were agricultural workers (data not shown). HPV presence in ESCCs was also unrelated to anatomic location, histologic grade or patient condition (alive or dead) at hospital discharge.

HPV universal primers and HPV-16 and -18 specific primers detected the presence of HPV DNA in 15 (25%) of 60 ESCCs. Cases of HPV DNA consisted of 13 males and two females with a mean age of 63.4 years (range, 37-76 years) and 62.5 years (range, 47-78 years), respectively. There were no statistical differences or associations ($P > 0.05$) between HPV status and gender, age, previous history of alcohol consumption and smoking, anatomic location, histologic grade or patient condition (alive or dead) at hospital discharge (Table 1).

Direct PCR-product sequencing detected 12 cases of high-risk HPV. As summarized in Table 2, HPV-16 was the most frequently detected genotype, followed by HPV-18; interestingly HPV-59 was also detected in one case (Figures 1 and 2). In three of 15 HPV-positive cases (20%), HPV genotype could not be specified although universal primers detected the presence of HPV generic sequences. Low-risk HPV DNA sequences were not detected and we found no HPV-16/18 co-infections among the cases under study.

DISCUSSION

PCR analysis in this study demonstrated the presence of high-risk HPV in 25% of ESCCs from a small set of Mexican patients; HPV-16 was the most frequent viral genotype followed by HPV-18. Interestingly, HPV-59 was also detected in one case; to our knowledge, this HPV genotype has not been previously reported in other ESCC studies. No low-risk HPV genotype was detected. In addition, HPV-16/18 co-infection was not found.

High-risk HPV-genotypes distribution in Mexican ESCCs observed in the present study is in agreement with that reported in the studies of cervical carcinomas in Mexico, in which HPV-16 is the most frequent genotype, followed by HPV-18, then by other high-risk HPV genotypes[16]. A similar high-risk HPV distribution was observed in a South American study of ESCC in Colombia and Chile[9], as well as a small Mexican series of lung carcinoma[17]. HPV-16 is not only the most prevalent high-risk genotype in cervical carcinomas[18] and in young females with normal Papanicolaou smears[19], but also in HPV-associated ESCC, worldwide[5].

HPV prevalence in Mexican women, as analyzed in cervical smears, has been reported to range 16.7%-23%, depending on the age group[20], and detected in 92% of cervical cancers[21]. A study of HPV prevalence in men showed a 62% HPV global positivity in samples from external genitalia of Mexican men, with 13% oncogenic types[22].

Since the Syrjänen[5] studies in the early 1980s, several studies have been conducted in different countries and in different geographical regions of the same country, to identify HPV DNA in ESCC. Utilizing molecular methods, the majority of these studies have shown the presence of high-risk HPV in a variable proportion of cases[23-27]; others, however, have failed to demonstrate HPV traces in EC, even from highly prevalent regions[28,29]. Differences in such figures could be attributed, first, to the sensitivity and specificity of molecular methods employed to detect HPV DNA[5]; it is widely accepted that PCR is the most sensitive method for detecting HPV DNA, and can detect as few as 20 copies or less[30].

According to the concept of "Condemned Mucosa Syndrome" proposed by Pillai and Nair[31], HPV promotes neoplastic transformation in a previously damaged mucosa with the aid of other carcinogenic agents. With regard to alcohol consumption and/or smoking, there were no substantial differences between HPV-positive and HPV-negative ESCCs, in this study. The present study also examined the possible association between the presence of HPV DNA and esophageal disorders involving membrane damage, such as achalasia and GERD. However, patients in the present study had no clinical history of esophageal achalasia. In two cases of ESCC in which there were previous symptoms of GERD and in another case with direct trauma-related esophageal surgery 26 years previously, HPV DNA was not detected.

The majority of our Mexican patients suffering from ESCC, were native to rural and semi-urban areas of Mexico, thus indicating low yearly income and a low level of education. Indeed, low socioeconomic status is characterized by, among others, nutritional deficiencies, poor hygiene habits and lack of health and public services, including drinking water supply, conditions that taken together could also predispose to esophageal carcinogenesis. Low socioeconomic status has also been associated with an increased risk of cervical intraepithelial neoplasia and cervical invasive carcinoma, among HPV-positive Mexican women[32].

Interestingly, the male:female ratio in HPV-positive ESCC increased two-fold in comparison with HPV-negative cases (6.5:1 *vs* 3.1:1). This ratio is higher than that reported from Colombia and Chile (0.75:1 *vs* 1.1:1)[9]. However, the gender-ratio between HPV-positive and -negative cases was not statistically significant. This finding does not allow us to draw any conclusion, due in part, to the small number of cases under study. No such comparison has been explored in other ESCC series, but its meaning warrants further research.

The etiological role of HPV in mucosal malignancy development has been supported on the basis of high-risk HPV localization and expression of viral oncoproteins in neoplastic cell nuclei, the characteristic squamous and basaloid morphology of malignant tumors, the occurrence of HPV-associated malignant neoplasms in anatomic sites where HPV is known to cause benign papillomas and where HPV direct exposure is suspected, and the elevation of serum antibodies against E6/E7 in patients harboring HPV-associated invasive cancers, among others[33]. The etiological significance of HPV detected in ESCCs is yet to be established. It is noteworthy that the present study was unable to find the association of basaloid morphology with HPV presence in ESCC. Although HPV-positive ESCC frequency was high, the mere presence of viral DNA does not necessarily imply its etiological involvement. It is necessary to conduct further studies to examine viral DNA integration into host carcinoma cells, localization in malignant cells, and monoclonality of HPV-positive cells.

The HPV transmission route detected in ESCCs is also of interest. HPV infection of the esophageal mucosa is highly suspected to occur in a direct fashion. In a recent case-control study[34], HPV oral infection was strongly associated with a sub-group of oropharyngeal squamous cell carcinomas, in which high-risk sexual behaviors (i.e. oral, vaginal) were recorded, regardless of alcohol and tobacco use. Notwithstanding the association between oral HPV infection and sexual behavior, a Finnish HPV Family Study[35] has shown that persistent high-risk HPV infection in a mother is a major risk factor for oral and genital infections by this virus in her offspring; this susceptibility appears to be modulated by the immune system. Thus, it could be argued that previous high-risk HPV oral infection, may predispose asymptomatic carriers for further ESCC development.

In conclusion, one fourth of ESCCs diagnosed in a Mexican population were found to harbor high-risk

HPV DNA. Elevated high-risk HPV prevalence of ESCC in Mexico, where ESCC incidence is relatively low, does not support the hypothesis that HPV-associated ESCC is more common in areas with higher ESCC incidence rates. Further studies are warranted to evaluate the etiological significance of HPV detected in Mexican ESCCs.

COMMENTS

Background

Esophageal carcinoma is a dismal disease which exhibits striking geographical variations in incidence and prevalence between countries and between different regions of the same country, due to unknown factors. Western Africa has the lowest incidence rates and China the highest. Among Latin America countries, Mexico and Peru have low mortality rates for esophageal carcinoma, whereas Brazil, Argentina and Chile have the highest. In Mexico, mortality rates in male and female inhabitants are *ca* 2 and *ca* 1 per 100 000, respectively.

Research frontiers

Esophageal carcinoma is multifactorial in origin; tobacco, alcohol, fungal toxins, nutritional deficiencies, hot food and beverages, as well as infectious agents, are related to esophageal carcinogenesis. Among infectious agents, high risk human papilloma virus (HPV), a major cause of carcinoma of the cervix uteri throughout the world, is strongly implicated in the etiology of esophageal carcinoma. High-risk HPV DNA sequences have been detected in approximately 15.2% of esophageal squamous cell carcinomas (ESCCs) worldwide, HPV-16 and -18 being the most frequent genotypes.

Innovations and breakthroughs

In this study, the authors found the presence of high-risk HPV DNA in 25% of ESCCs. HPV-16 and -18 were the most frequent genotypes, but they also demonstrated the presence of HPV-59 in one of the cases, a genotype not previously reported in ESCCs anywhere. On the other hand, the results do not support the hypothesis that HPV-associated ESCC is more frequent in areas with higher ESCC incidence rates, because ESCC is not a common cancer in Mexico. Interestingly, the male:female ratio in HPV-positive ESCC increased two-fold in comparison with HPV-negative cases (6.5:1 *vs* 3.1:1).

Applications

By knowing the prevalence of high-risk HPV-associated ESCC, this study among others, may contribute to the design of future strategies for the prevention of HPV-related malignancies, through the development of effective vaccines.

Terminology

HPVs are DNA viruses that infect basal skin and mucosal cells. HPVs are categorized according to their cervical oncogenicity-based risk, with high, probably high, low and undetermined risks.

Peer review

This study examined HPV infection in ESCCs in Mexican patients. This is an interesting study and the manuscript is written well.

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BRIEF ARTICLES

Is percutaneous endoscopic gastrostomy tube placement safe in patients with ventriculoperitoneal shunts?

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Abstract

AIM: To investigate whether percutaneous endoscopic gastrostomy (PEG) tube placement is safe in patients with ventriculoperitoneal (VP) shunts.

METHODS: This was a retrospective study of all patients undergoing PEG insertion at our institution between June 1999 and June 2006. Post-PEG complications were compared between two groups according to the presence or absence of VP shunts. VP shunt infection rates, the interval between PEG placement and VP shunt catheter insertion, and long-term follow-up were also investigated.

RESULTS: Fifty-five patients qualified for the study. Seven patients (12.7%) had pre-existing VP shunts. All patients received prophylactic antibiotics. The complication rate did not differ between VP shunt patients undergoing PEG (PEG/VP group) and non-VP shunt patients undergoing PEG (control group) [1 (14.3%) *vs* 6 (12.5%), P = 1.000]. All patients in the PEG/VP group had undergone VP shunt insertion prior to PEG placement. The mean interval between VP shunt insertion and PEG placement was 308.7 d (range, 65-831 d). The mean follow-up duration in the PEG/VP group was 6.4 mo (range, 1-15 mo). There were no VP shunt infections, although one patient in the PEG/VP group developed a minor peristomal infection during follow-up.

CONCLUSION: Complications following PEG placement in patients with VP shunts were infrequent in this study.

Key words: Percutaneous endoscopic gastrostomy; Ventriculoperitoneal shunt; Complication; Ventriculoperitoneal shunt infection; Prophylactic antibiotic

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INTRODUCTION

Percutaneous endoscopic gastrostomy (PEG) tube placement has been widely used for long-term nutritional support in patients with severe neurological impairment ever since it was first described by Gauderer *et al*[1] in 1980. However, patients requiring PEG tube placement may have concomitant hydrocephalus requiring insertion of a ventriculoperitoneal (VP) shunt, and VP shunts themselves are frequently associated with complications, such as shunt infection, obstruction, and migration with or without erosion into nearby structures. Shunt infection is a relatively common complication, occurring in 3% to 29% of patients[2-6]; its mortality rate is 30% to 40%[6]. There are a number of factors that can expose intraperitoneally placed catheters to bacterial pathogens in PEG patients with pre-existing VP shunts. Therefore, the presence of a VP shunt in a patient requiring PEG placement raises concerns about potential life-threatening complications such as VP shunt infection and VP shunt malfunction. A few studies have evaluated the safety of PEG placement in patients with VP shunts, but the results have been inconclusive. Furthermore, the study design, methods of PEG placement, control groups, and the use of antibiotics in these studies have been highly diverse.

Thus, to date, controversy still exists as to whether PEG

placement is safe in patients with VP shunts. This study was therefore designed to report our single center experience with PEG placement in patients with VP shunts, looking specifically at PEG-related complications and VP shunt infections. Relevant publications were also reviewed.

MATERIALS AND METHODS

Study design and patients

We performed a retrospective study on all patients who underwent PEG tube placement for enteral feeding at Uijeongbu St. Mary's Hospital between June 1999 and June 2006. A preliminary chart review identified the subset of patients with endoscopic records indicating PEG tube placement. A total of 55 patients were identified. Those patients with VP shunts were identified and assigned to the combined PEG and VP shunt (PEG/VP) group. The patients undergoing PEG tube placement (but without VP shunts) were assigned to the control group. A more detailed chart review was performed, evaluating patient ages at the time of the procedure, underlying disorders, comorbid diseases, number of PEG placements, and PEG-related complications. Adjustment for comorbidity was carried out for patients in this study using Charlson's comorbidity index[7]. Post-PEG placement complications were compared between the two groups. Furthermore, the incidence of VP shunt infections, interval between PEG placement and VP shunt catheter insertion, position of the abdominal shunt catheter, follow-up duration, and outcome of long-term follow-up were investigated in the PEG/VP group.

The requirement for informed consent was waived, because the study design was retrospective.

PEG tube placement

All PEGs were placed by gastroenterologists. A commercially available gastrostomy tube (US Endoscopy, Mentor, Ohio, USA) was introduced by standard pull-through technique. Enteral feeding was discontinued 12 h before PEG tube placement. All patients received prophylactic or perioperative antibiotics and received intravenous sedation and topical pharyngeal anesthesia. In each patient, the stomach was endoscopically inflated with air, and following satisfactory transillumination of the stomach in the left hypochondrium or epigastrium, the needle was passed through this site directly into the stomach. A guide wire was advanced through the needle, and the commercially available gastrostomy tube was placed over the wire from the aerodigestive tract, through the stomach, to the abdominal wall. In each patient with a pre-existing VP shunt, the shunt tract was carefully demarcated so it could be avoided during PEG tube placement.

Statistical analysis

With respect to demographic data and complications in the two groups, continuous variables were compared using Student's *t*-test, and discrete variables were compared using the Chi-square test or Fisher's exact probability test.

Table 1 Baseline patient characteristics *n* (%)

	PEG/VP (*n* = 7)	Control (*n* = 48)	*P* value
Age (yr)	55.3 ± 12.3	61.0 ± 16.6	0.387
Sex (M/F)	5/2	31/17	1.000
Primary diagnosis			0.897
Cerebrovascular disease	7 (100)	36 (75)	
Amyotrophic lateral sclerosis		4 (8.3)	
Hypoxic brain damage		2 (4.2)	
Parkinson's disease		2 (4.2)	
Malignancy		2 (4.2)	
Aspiration pneumonia		1 (2.1)	
Pharyngeal paralysis		1 (2.1)	
Diabetes mellitus	2 (28.6)	10 (20.8)	0.639
Tracheostomy	6 (85.7)	25 (52.1)	0.122
Mean number of PEG placements	1.3 ± 0.5	1.6 ± 1.1	0.459
Charlson's index score	3.0 ± 1.6	3.5 ± 1.9	0.504

PEG: Percutaneous endoscopic gastrostomy; VP: Ventriculoperitoneal; PEG/VP: Patients with PEG tubes and VP shunts; Control: Patients with PEG tubes alone.

A probability value of < 0.05 was considered statistically significant. All data were analyzed using SPSS 11.0 (SPSS Inc, Chicago, Illinois, USA).

RESULTS

Over a 7-year period, 55 patients underwent PEG tube placement at our hospital. Selected clinical characteristics of the patients are provided in Table 1. Seven patients (12.7%) had pre-existing VP shunts at the time of PEG placement (PEG/VP group), and 48 patients had no VP shunts (control group). There was no difference in the mean age between the PEG/VP and control groups (55.3 ± 12.3 *vs* 61.0 ± 16.6 years, $P = 0.387$) and no difference in the sex ratio between the two groups (male/female: 5/2 *vs* 31/17, $P = 1.000$). The primary diagnosis in all patients in the PEG/VP group was cerebrovascular disease, and all patients underwent VP shunt placement for hydrocephalus secondary to cerebral hemorrhage. In the control group, reasons for PEG tube placement included cerebrovascular disease in 36 patients (75%), amyotrophic lateral sclerosis in 4 (8.3%), hypoxic brain damage in 2 (4.2%), Parkinson's disease in 2 (4.2%), malignancy in 2 (4.2%), aspiration pneumonia in 1 (2.1%), and pharyngeal paralysis in 1 (2.1%). There were two patients (28.6%) with diabetes mellitus in the PEG/VP group and 10 (20.8%) in the control group ($P = 0.639$). Six patients (85.7%) in the PEG/VP group had tracheostomies at the time of PEG tube placement, as did 25 patients (52.1%) in the control group ($P = 0.122$). A total of 88 PEG tube placements were performed in 55 patients. The mean number of PEG placements per patient was 1.3 ± 0.5 in the PEG/VP group and 1.6 ± 1.1 in the control group ($P = 0.459$). There was no difference in Charlson's comorbidity index score between the two groups (3.0 ± 1.6 *vs* 3.5 ± 1.9, $P = 0.504$). All patients received prophylactic or periprocedural antibiotics.

There was one complication (14.3%) after PEG tube placement in the PEG/VP group, and there were six complications (12.5%) in the control group ($P = 1.000$).

Table 2 Diagnosis of complications *n* (%)

	PEG/VP (*n* = 7)	Control (*n* = 48)	*P* value
Complications	1 (14.3)	6 (12.5)	1.000
Wound infection	1	3	
Stomal leakage		1	
Bleeding		1	
Gastroesophageal reflux disease		1	
VP shunt infection	No	-	

Complications in the control group included three peristomal infections, one stomal leak, one case of bleeding, and one case of gastroesophageal reflux. There was no post-PEG VP shunt infection, malfunction, neurologic deterioration, or meningitis in patients with pre-existing VP shunts (Table 2). Because no VP shunt infections were identified based on clinical features (signs and symptoms), a detailed definition of shunt infection (CSF culture or leukocyte count) was not needed.

Long-term outcomes in patients with pre-existing VP shunts are shown in Table 3. In the PEG/VP group, the interval between VP shunt insertion and PEG tube placement ranged from 65 to 831 d (mean, 308.7 ± 260.5 d). The abdominal end of the VP shunt catheter was positioned in the right abdomen in five patients and in the left abdomen in two patients. Of the seven patients with pre-existing VP shunts, two had diabetes mellitus and six had tracheostomies. The mean follow-up duration was 6.4 ± 4.5 mo (range, 1-15 mo). One patient in the PEG/VP group had only a minor peristomal infection during follow-up. Four patients did well, and two required PEG tube replacement due to self-removal. One patient resumed eating and was able to have the PEG tube removed 96 d after placement. No patient died during follow-up.

DISCUSSION

In this study, patients with pre-existing VP shunts accounted for 7 (12.7%) of the 55 patients having PEG tubes inserted over a 7-year period. The incidence of PEG-related complications was 14.3% (1/7) among patients with VP shunts. The incidence of PEG-related complications was 12.5% (6/48) among patients without VP shunts. There was no difference between the groups with regard to complication rate and when disregarding the primary underlying disorder, presence of diabetes mellitus, and tracheostomy state. No VP shunt infections were identified in the patients with both PEG tubes and VP shunts during the mean follow-up duration of 6.4 mo. The mean interval between VP shunt insertion and PEG tube placement was 308.7 ± 260.5 d.

Ever since Gauderer *et al*[1] introduced the endoscopic placement of feeding gastrostomy tubes in 1980, clinicians have been able to perform the PEG procedure with a shorter operative time and without the need for laparotomy. This procedure has been shown to have fewer complications and lower cost compared to the traditional open gastrostomy originally described by Stamm in 1894[8-10]. However, PEG-related complications, including wound infection, bleeding, gastric leakage, tube dysfunction, and aspiration pneumonia, occur in approximately 10% of all cases[10]. Stomal site infections occur in 2.9% to 8.8% of patients[11-13], and peritonitis occurs in 0.5% to 6.6% of patients[12,14-16]. Major complications requiring surgical intervention, including intraperitoneal abscess and fistula formation, occur in 2% to 3% of all patients[10]. In this study, the incidence of PEG-related complications was 14.3% in the PEG/VP group and 12.5% in the PEG alone group. All complications were manageable with conservative therapy. Despite the small numbers of patients, especially in the PEG/VP group, these incidences were similar to those seen in previous reports, and no other major complications occurred.

VP shunt placement is the major neurological procedure required in the treatment of hydrocephalus. However, VP shunts are frequently associated with serious complications, including shunt obstruction, meningitis, and intraperitoneal infection. According to the available literature, the rate of shunt infection ranges from 3% to 29% after VP shunting procedures[2-6]. Many of these complications occur at the abdominal sites of VP shunts. Patients with indwelling peritoneal shunts could be at risk for infection, even without PEG tubes.

Therefore, we hypothesized that the incidence of VP shunt infection would be higher in those patients with VP shunt catheters and PEG tubes. However, the question is, do PEG tubes increase VP shunt complication rates? To date, there have been seven reports addressing the safety of PEG tubes in patients with VP shunts (Table 4)[17-23]. There is only one prospective study in the literature[17]. The number of patients in these studies with both PEG tubes and VP shunts ranged from 6 to 55, and the VP shunt infection rate ranged from 0% to 50%. Most patients have had their VP shunts placed first, followed by PEG insertion. Two separate studies looked at VP shunt infection rates in patients undergoing VP shunt placement before PEG tube placement and in patients undergoing PEG tube placement before VP shunt placement. Infection rates were higher in patients undergoing PEG tube placement first, although not to a statistically significant degree[22,23]. In the study of Taylor *et al*[19], PEG tubes and VP shunts were simultaneously placed in 16 patients; VP shunt infections occurred in eight patients (50%). Therefore, the investigators recommended that simultaneous PEG tube/VP shunt insertion be avoided. The VP shunt infection rate was higher in tracheostomy patients in the study of Taylor *et al*[19], but it was not higher in our study. With regard to the time interval between PEG tube and VP shunt insertion, Graham *et al*[17] insisted that a 1-wk interval is safe. However, this interval has been more than 1 mo in most previous reports, and Nabika *et al*[22] recommended a 1-mo interval because three of four patients developing VP shunt infections in their study had PEG tubes and VP shunts placed within 1 mo of each other. In our study, the mean interval between the two was very long (308.7 d). We think this may have contributed to the absence of

Table 3 Long-term outcomes in patients with PEG tubes and VP shunts

No. of case	Sex/age (yr)	PEG-VP shunt interval (d)	Position of abdominal shunt catheter	DM	Complication	Follow-up (mo)	Outcome
1	F/67	409	Right	-	Wound infection	7	Doing well
2	M/57	65	Right	+	-	15	Doing well
3	M/57	256	Left	+	-	8	PEG change due to self-removal
4	F/62	831	Right	-	-	6	PEG change due to self-removal
5	F/67	274	Right	-	-	5	Doing well
6	F/36	259	Right	-	-	1	Doing well
7	F/42	67	Left	-	-	3	PEG removal

Table 4 Summary of published data on infections related to gastrostomy placement in patients with ventriculoperitoneal shunts

Investigator	Study design	Method of gastrostomy	Order of PEG & VP shunt	*n*	VP shunt infection rate	Interval between PEG & VP shunt	Control group	VP shunt infection rate in control group	Antibiotic used
Graham *et al*[17]	Prospective	Percutaneous endoscopic	VP→PEG	15	0%	2.2 wk	None	-	Cefazolin
Sane *et al*[18]	Retrospective	Fluoroscopic	VP→PEG	23	9% (2/23)	At least 4 wk	None	-	None
Taylor *et al*[19]	Retrospective	Percutaneous endoscopic	Simultaneous	16	50% (8/16)	-	VP shunt and tracheostomy without PEG	0% (0/21)	Yes (unspecified)
Baird *et al*[20]	Retrospective	Percutaneous endoscopic	VP→PEG	6	0%	33 d	None	-	Cefazolin
Schulman *et al*[21]	Retrospective	Percutaneous endoscopic	VP→PEG	39	5% (2/39)	43.1 d	None	-	72% received (unspecified)
Nabika *et al*[22]	Retrospective	Percutaneous endoscopic	Both	23	17.4% (4/23)	29.3 d	Only VP shunt	4.9% (6/123) (P = 0.0519)	Cefazolin
			PEG→VP	12	25% (3/12)	27.2 d			
			VP→PEG	11	9.1% (1/11)	39.2 d			
Roeder *et al*[23]	Retrospective	Percutaneous endoscopic and surgical	Both	55	12.7% (7/55)	-	Only PEG	-	90.9% received (unspecified)
			PEG→VP	30	16.6% (5/30)				
			VP→PEG	25	8% (2/25)				
This study	Retrospective	Percutaneous endoscopic	VP→PEG	7	0% (0/7)	308.7 d	Only PEG	-	Yes (unspecified)

VP shunt infections in our study. Concerning the control group, there have been two studies with VP shunt patients serving as the control group[19,22]. The VP shunt infection rates were 50% (8/16) in the PEG/VP group and 0% (0/21) in the control group in one study due to simultaneous insertion[19], but the VP shunt infection rates were 17.4% (4/23) in the PEG/VP group and 4.9% (6/123) in the control group (P = 0.0519) in the other study[22]. Therefore, except for simultaneous insertion, the VP shunt infection rates of patients with PEG and VP shunts are not significantly different from those seen in control patients with VP shunts. Only one report has addressed the question of mortality[23]. In this report, the all-cause mortality at 1 year after PEG tube placement in patients with VP shunts was 21%, and PEG tube placement in patients with VP shunts was not associated with excessive mortality compared to PEG tube placement alone. Prophylactic antibiotics were given in all studies, except for one. That study used percutaneous fluoroscopic antegrade technique in 23 children, 2 (9%) of whom developed VP shunt infections[18].

The limitations of our study are similar to those of previously published studies. Firstly, our study was retrospective. Secondly, the number of study patients was small; specifically, there were only seven patients with PEG tubes and VP shunts. Thirdly, the control group in our study was composed of patients with PEG tubes alone, not patients with VP shunts. However, despite these limitations, our study and literature review suggest that PEG tube placement is safe in patients with VP shunts, especially those in whom the VP shunt is inserted first, those in whom the interval between PEG tube and VP shunt insertion is greater than 1 mo, and those in whom prophylactic antibiotics are used.

COMMENTS

Background

Percutaneous endoscopic gastrostomy (PEG) tube placement has been widely used for long-term nutritional support in patients with severe neurological impairment. These patients requiring PEG tube placement may have concomitant hydrocephalus requiring insertion of a ventriculoperitoneal (VP) shunt. However, the presence of a VP shunt in a patient requiring PEG placement raises concerns about potential life-threatening complications such as VP shunt infection and VP shunt malfunction. Therefore, we aimed to investigate if PEG tube placement is safe in patients with VP shunts.

Research frontiers

To date, controversy still exists as to whether PEG placement is safe in patients with VP shunts. There have been seven reports addressing the safety of PEG tubes in patients with VP shunts. There is only one prospective study in the literature.

Innovations and breakthroughs

This study suggests that PEG tube placement is safe in patients with VP shunts, especially those in whom the VP shunt is inserted first, those in whom the interval between PEG tube and VP shunt insertion is greater than 1 mo, and those in whom prophylactic antibiotics are used.

Applications

To confirm whether PEG placement is safe in patients with VP shunts, a large scale prospective study including a control group which has patients with VP shunts is needed.

Peer review

This paper presents a series of patients with preexisting ventriculoperitoneal shunt, who needed a percutaneous endoscopic gastrostomy. The authors conclude that percutaneous endoscopic gastrostomy after previous ventriculoperitoneal shunt is safe. The paper may help to support the indication even in this group, if a gastrostomy is needed.

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What is the most cost-effective strategy to screen for second primary colorectal cancers in male cancer survivors in Korea?

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Abstract

AIM: To identify a cost-effective strategy of second primary colorectal cancer (CRC) screening for cancer survivors in Korea using a decision-analytic model.

METHODS: A Markov model estimated the clinical and economic consequences of a simulated 50-year-old male cancer survivors' cohort, and we compared the results of eight screening strategies: no screening, fecal occult blood test (FOBT) annually, FOBT every 2 years, sigmoidoscopy every 5 years, double contrast barium enema every 5 years, and colonoscopy every 10 years (COL10), every 5 years (COL5), and every 3 years (COL3). We included only direct medical costs, and our main outcome measures were discounted lifetime costs, life expectancy, and incremental cost-effectiveness ratio (ICER).

RESULTS: In the base-case analysis, the non-dominated strategies in cancer survivors were COL5, and COL3. The ICER for COL3 in cancer survivors was $5593/life-year saved (LYS), and did not exceed $10 000/LYS in one-way sensitivity analyses. If the risk of CRC in cancer survivors is at least two times higher than that in the general population, COL5 had an ICER of less than $10 500/LYS among both good and poor prognosis of index cancer. If the age of cancer survivors starting CRC screening was decreased to 40 years, the ICER of COL5 was less than $7400/LYS regardless of screening compliance.

CONCLUSION: Our study suggests that more strict and frequent recommendations for colonoscopy such as COL5 and COL3 could be considered as economically reasonable second primary CRC screening strategies for Korean male cancer survivors.

Key words: Cost-effectiveness; Second primary colorectal cancer; Screening; Cancer survivor

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INTRODUCTION

A recent improvement in cancer survival owing to early diagnosis and advances in treatment has raised the issue of second primary cancers (SPCs) in cancer survivors after their primary treatment[1,2]. Due to carcinogenic effects of cancer-related treatment, genetic susceptibil-

ity or unhealthy behavior such as smoking, alcohol and obesity, cancer survivors are at increased risk for SPCs, not only at the original site but at other sites as well[3,4]. Recent studies have shown that the age-standardized incidence rate was 2.3 times higher for an SPC than for a first cancer in the Korean general male population[5]. Specifically, the age-standardized incidence rate was about four times higher for second than for first primary colorectal cancers (CRCs)[5]. It is well-known that screening for CRC reduces mortality through detection of malignancy at an earlier, more treatable stage as well as by identification and removal of the precursor lesion, the adenomatous polyp[6]. These findings suggest that more thorough surveillance and screening for second primary CRC is needed for the cancer survivors.

Many previous studies have focused on the cost-effectiveness (CE) of CRC screening in the general population[6-9], and several panels have recommended CRC screening for the general population[10-12]. As the risk of CRC and life expectancy are quite different between cancer survivors and the general population, screening guidelines for the general population could not be applied to the cancer survivors. However, until now, there have been few recommendations for CRC screening for cancer survivors. To suggest a feasible economic strategy of second primary CRC screening for cancer survivors in Korea, we constructed a decision-analytic model, and compared the CE results of cancer screening in cancer survivors and in the average-risk general population.

MATERIALS AND METHODS

The natural history of a simulated male cancer survivors' cohort was modeled with and without second primary CRC screening until age of 75 years (Figure 1). For simplicity and comparison with results from the general population, we assumed that all male cancer survivors enter at age 50 years, which most guidelines for the general population recommended for starting CRC screening[10-12]. We developed a Markov model using TreeAge-Pro 2007 software (TreeAge Software Inc., Williamstown, Massachusetts). The Markov model estimated the clinical and economic consequences of eight different screening strategies as follows: (1) no screening, (2) fecal occult blood test (FOBT) annually (FOBT1), (3) FOBT every 2 years (FOBT2), (4) sigmoidoscopy every 5 years (SIG5), (5) double contrast barium enema every 5 years (DCBE5), (6) colonoscopy every 10 years (COL10), (7) colonoscopy every 5 years (COL5), and (8) colonoscopy every 3 years (COL3).

Individuals were placed into health states defined by the presence or absence of a colorectal polyp or second primary CRC (early or advanced) 1 year after the index cancer diagnosis. Cases of positive screening test results were worked up with a colonoscopy, and individuals diagnosed with polyps underwent polypectomy. The probability of perforation was assigned to colonoscopy, sigmoidoscopy, DCBE and polypectomy[8,13,14]. Mortality caused by the risk of perforation was assumed to be

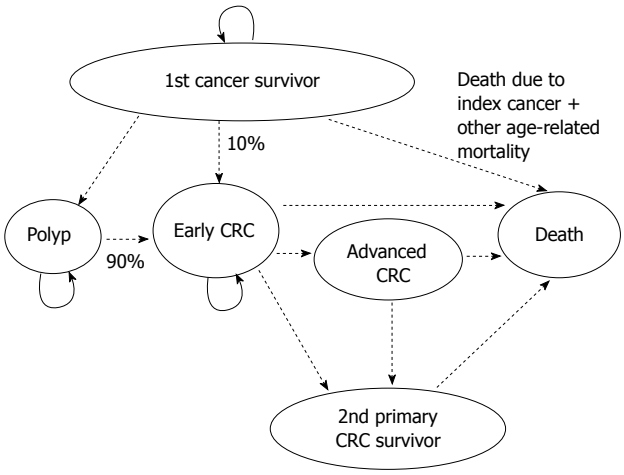

Figure 1 Markov model of second colorectal cancer (CRC) screening in Korean male cancer survivors.

0.02%[9,14]. Colonoscopy was repeated every 3 years for surveillance after polypectomy[15]. We assumed that 80% of male cancer survivors underwent the initial screening test, independent of whether they were compliant with past tests. The compliance of follow-up or surveillance colonoscopy was assumed to be 100%. We also assumed that 90% of CRCs develop from polyps[15,16], and the latent period between early stage and advanced stage was assumed to be 2 years[9]. The relative risk of CRC in Korean male cancer survivors was assumed to be four times higher than that in the general population[5]. Age-specific transition probabilities and prevalence were calculated between normal, polyp, and CRC to yield an incidence rate of polyp and CRC derived from previous literature and the Korea Central Cancer Registry[7,17-19]. The stage-specific CRC mortality were applied uniformly to all malignancies regardless of the means of detection (by symptoms or screening) or the state of detection (diagnosed *vs* undiagnosed cancer). Age-specific mortality from index cancer and other causes was estimated based on the above sources combined with statistics published by the National Center for Health Statistics[20] and Korea Central Cancer Registry[21]. As there have been few studies on mortality from second primary CRC, we calculated the additional yearly probability of dying from a second primary CRC based on cancer stage from previous studies of CRC as the first index cancer[21-23].

We obtained the data on the costs of CRC treatment by stage and time period from the National Health Insurance Corporation (social insurer of the national health insurance (NHI) with a universal coverage of population)[24]. Costs of screening tests were obtained from the fee schedule of the National Health Insurance Corporation (the NHI of Korea has a fee schedule applied to all insured services)[25]. Costs were expressed in US dollars and the exchange rate was 955 Korean Won for one US dollar in 2006[26]. As the indirect costs of cancer screening are not established in Korea, we included only direct medical costs.

Our main outcome measures were discounted lifetime costs, life expectancy, incremental cost-effectiveness

Table 1 Summary of assumptions

	Parameter	Base case value (range)	Ref.
Sensitivity & specificity of colorectal screening tests	Sensitivity of FOBT for colorectal polyps/cancer	0.1/0.5	[27-30]
	Sensitivity of colonoscopy for colorectal polyps/cancer	0.9/0.95	[6,31,32]
	Sensitivity of double contrast barium enema for colorectal polyps/cancer	0.5/0.8	[6,33,34]
	Sensitivity of sigmoidoscopy for colorectal polyps/cancer	0.46/0.52	[31,32,35]
	Specificity of FOBT	0.9	[28-30]
	Specificity of colonoscopy	1	[6,31,32]
	Specificity of double contrast barium enema	0.9	[6,33,34]
	Specificity of sigmoidoscopy	0.95	[6,31,32]
Natural history of colorectal polyp/cancer sequence	Prevalence of polyps at age 50 yr	0.20 (0.1-0.4)	[14,35]
	Annual polyp incidence rate in cancer survivors	Age specific	[14,35,36]2
	Percent of cancers originating as polyps	90%	[37,38]
	Relative risk of colorectal cancer in cancer survivor compared with the general population	4 (1-5)	[5]
	Age specific incidence rate of colorectal cancer without polypoid precursors in cancer survivors	Age specific	[5,39,40]2
	Age specific incidence rate of colorectal cancer with polypoid precursors in cancer survivors	Age specific	[5,39,40]2
	Dwelling time of colorectal cancer in early stages	2 yr	[29,41]
	Percent of colorectal cancers detected in early stages with no screening	5% (2%-10%)	[23]
	Five-year all cause survival for early 2nd primary colorectal cancer	90% (80%-95%)	[18,22,23]
	Five-year all cause survival for advanced 2nd primary colorectal cancer	60% (40%-70%)	[18,22,23]
Natural history of cancer survivors	Five-year survival for index cancer	40% (20%-80%)	[18]
	Age specific mortality except the index cancer	Age specific	[21]
	Age of cancer survivors for starting colorectal cancer screening, year	50 (40-60)	[10-12]
	Compliance of 2nd colorectal cancer screening	80% (60%-100%)	
Complications and unintended consequences	Rate of perforation of colon in colonoscopy	0.20% (0.1%-0.3%)	[13,14,42]
	Rate of perforation of colon in polypectomy	0.40% (0.2%-0.5%)	[13,14,42]
	Rate of perforation from sigmoidoscopy	0.01% (0.005%-0.05%)	[13,14,42]
	Rate of perforation from double contrast barium enema	0.005% (0.001%-0.01%)	[42]
	Death rate due to perforated colon	0.2% (0.1%-5%)	[19,39,42]
Cost (dollar[1]) & discount rate	Sigmoidoscopy	31.3	[25]
	Colonoscopy	61.7	[25]
	Double contrast barium enema	68.5	[25]
	FOBT	2.7	[25]
	Polypectomy, biopsy and pathologic exam	189	[25]
	Treatment of early cancer for first year	7330 (5860-8800)	[7,25]
	Treatment of advanced cancer for first year	14660 (10050-15080)	[7,25]
	Treatment of cancer after first year	2094 (1670-2510)	[7,25]
	Cost to repair the endoscopic perforation	3141 (2510-3770)	[7]
	Discount rate	0.03 (0-0.05)	

[1]Exchange rate: 955 Korean Won for one US dollar in 2006; [2]Estimated by calibration to national data on colorectal polyp and cancer incidence.

ratio (ICER), which were compared for different CRC screening strategies. Because there are uncertainties with respect to quality of life associated with CRC screening, colorectal polyp, and second CRC, we conducted the base case analysis using increase in life expectancy as the primary outcome. Incremental CE analysis was performed by ranking the 16 strategies in order of increasing effectiveness. After eliminating strategies that were more or equally costly and less effective than a competing strategy [i.e. ruled out by simple dominance), we calculated the ICER for each strategy (additional cost divided by life-year saved (LYS)] compared with the next least expensive strategy. Strategies exhibiting extended dominance were eliminated from the rank-ordered list, and ICERs of the remaining strategies were recalculated[27]. Future costs and life-years were discounted at an annual rate of 3%. We compared the results from male cancer survivors with that from general male population.

Sensitivity analyses were performed to assess the stability of the results of a plausible range of several parameters, such as prevalence of colorectal polyps at age 50 years, 5-year survival rates of second primary CRC, complications of screening test or polypectomy *etc* (Table 1). We performed detailed analyses by changing key variables of the index cancer such as 5-year survival rate of first cancer, relative risk of CRC in cancer survivors compared with that in general population. In addition, we evaluated the effects of changing age of subjects for starting second primary CRC screening and compliance rate on the CE of our results.

RESULTS

Base case

In the base-case analysis at 80% screening compliance, all screening strategies extended life expectancy both in male cancer survivors and the general population (Table 2). The strategies which were not ruled out by simple

Table 2 Cost-effectiveness of colorectal screening strategies among male cancer survivors and the general population in Korea (80% compliance)

Male general population				Male cancer survivors			
Strategy (abbreviation)	Lifetime cost per person (dollar)[1]	Life expectancy (yr)	Incremental C/E[2], dollar[1] per life-year gained	Strategy (abbreviation)	Lifetime cost per person (dollar)[1]	Life expectancy (yr)	Incremental C/E[2], dollar[1] per life-year gained
COL every 10 yr	437.3	17.260	…	COL every 5 yr	463.5	7.572	
No screening	448.0	17.243	…	COL every 10 yr	480.1	7.568	…
COL every 5 yr	478.3	17.263	14456.8	COL every 3 yr	480.2	7.575	5592.9
DCBE every 5 yr	542.3	17.256	…	DCBE every 5 yr	563.4	7.562	…
SIG every 5 yr	542.4	17.255	…	SIG every 5 yr	571.8	7.560	…
COL every 3 yr	554.4	17.265	38876.8	No screening	632.2	7.544	…
FOBT every 2 yr	810.0	17.252	…	FOBT every 2 yr	735.2	7.557	…
FOBT every 1 yr	1130.9	17.257	…	FOBT every 1 yr	842.3	7.564	…

COL: Colonoscopy; SIG: Sigmoidoscopy; DCBE: Double contrast barium enema; FOBT: Fecal occult blood test. Ellipse indicates no data (incremental CR ratios not calculated for these strategies because they were dominated). [1]Exchange rate, 955 Korean Won for one US dollar in 2006; [2]Incremental CE ratio (dollar/year) = Incremental cost per person/incremental years of life gained.

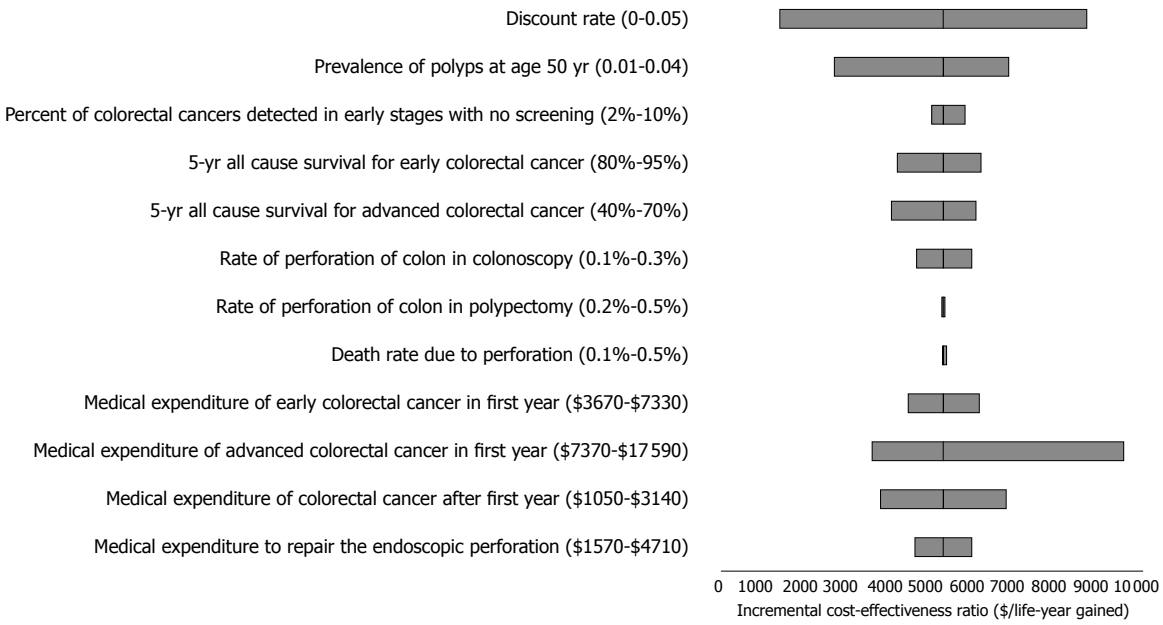

Figure 2 Sensitivity analyses on cost-effectiveness from the perspective of colonoscopy every 3 years *vs* colonoscopy every 5 years in male cancer survivors.

dominance nor extended dominance (non-dominated strategies) in the general population were COL10, COL5, and COL3, while those in cancer survivors were COL5 and COL3. The ICER for COL3 in cancer survivors was $5593 per LYS. In cancer survivors, the lifetime total cost per person associated with "FOBT annually" was larger than that associated with no screening, while COL5 and COL3 were less costly than no screening.

Sensitivity analyses

Figure 2 shows the results of one-way sensitivity analyses on CE from the perspective of COL3 *vs* COL5 in male cancer survivors. In most cases, COL5 and COL3 were non-dominated strategies, and the ICER of COL3 ranged between $1480 and $9192.

Table 3 shows the results of two-way sensitivity analyses by changing risk of second CRC and 5-year survival rate of index cancer in Korean male cancer survivors. If the risk of CRC in cancer survivors was at least three times higher than that in the general population, screening with COL5 in cancer survivors had an ICER of less than $4000 per LYS in the entire range of 5-year survival of index cancer between 20% and 80%. If the risk of CRC in cancer survivors was two times higher than that in the general population, COL5 in cancer survivors had an ICER of less than $10500 per LYS in both types of index cancer with poor and good prognosis. If the risk of CRC in cancer survivors was the same as that in the general population, non-dominated strategies were no screening, COL10, COL5, and COL3, and the ICER of COL5 was more than $25000 per LYS, while the ICER of COL10 ranged between $2315 in index cancer with good prognosis and $19650 in index cancer with poor prognosis.

Table 4 shows the results of sensitivity analysis by changing compliance of CRC screening and age of cancer

Table 3 Two-way sensitivity analysis by changing variables of index cancer such as 5-yr survival rate of first cancer and relative risk of colorectal cancer in Korean male cancer survivors

Relative risk of colorectal cancer in cancer survivor compared with that in general population	5-yr survival of index cancer = 20%				5-yr survival of index cancer = 40%				5-yr survival of index cancer = 80%			
	Non-dominated strategy	Lifetime cost per person (dollar)[1]	Life expectancy (yr)	Incremental C/E^2 (dollar)[1] per life-year gained	Non-dominated strategy	Lifetime cost per person (dollar)[1]	Life expectancy (yr)	Incremental C/E^2 (dollar)[1] per life-year gained	Non-dominated strategy	Lifetime cost per person (dollar)[1]	Life expectancy (yr)	Incremental C/E^2 (dollar)[1] per life-year gained
5	COL5	329.0	4.512		COL5	532.5	7.567		COL5	957.9	13.829	
	COL3	339.2	4.514	4921.7	COL3	542.2	7.571	2634.4	COL3	962.6	13.836	658.2
4	COL5	306.7	4.516		COL5	463.5	7.572		COL10	826.0	13.837	
	COL3	329.0	4.517	18078.3	COL3	480.2	7.575	5592.9	COL5	831.1	13.843	835.2
									COL3	879.7	13.847	11508.2
3	COL10	266.6	4.517		COL10	401.4	7.576		COL10	682.9	13.847	
	COL5	270.3	4.518	2685.2	COL5	409.6	7.579	3365.6	COL5	701.9	13.851	4104.9
	COL3	295.5	4.519	27126	COL3	447.3	7.580	22760.2	COL3	761.9	13.854	18922.1
2	No screening	185.4	4.512		COL10	324.4	7.582		COL10	533.5	13.857	
	COL10	224.5	4.52	4912	COL5	340.1	7.583	9506.4	COL5	566.7	13.860	10725.2
	COL5	232.3	4.521	8378.3	COL3	383.7	7.584	39469.5	COL3	638.6	13.862	34075.6
	COL3	260.4	4.522	45556.4								
1	No screening	96.1	4.519		No screening	175.9	7.580		No screening	342.6	13.853	
	COL10	180.5	4.523	20568.2	COL10	244.0	7.587	9196.3	COL10	377.2	13.867	2424.5
	COL5	192.4	4.524	25817.6	COL5	267.2	7.588	28343.9	COL5	425.1	13.869	31064.6
	COL3	223.5	4.524	103111.0	COL3	316.8	7.589	91808.0	COL3	509.3	13.870	81681.8

COL10: Colonoscopy every 10 years; COL5: Colonoscopy every 5 years; COL3: Colonoscopy every 3 years; Dominated strategy is a strategy that is more or equally costly and less effective than a competing strategy. [1]Exchange rate, 955 Korean won for one US dollar in 2006; [2]Incremental CE ratio (dollar/year) = Incremental cost per person/Incremental years of life gained.

Table 4 Two-way sensitivity analysis on cost-effectiveness of 2nd primary colorectal cancer screening by compliance of screening and age of Korean male cancer survivors for starting screening

Age for starting screening	Compliance of 2nd colorectal cancer screening = 60%				Compliance of 2nd colorectal cancer screening = 80%				Compliance of 2nd colorectal cancer screening = 100%			
	Non-dominated strategy	Lifetime cost per person (dollar)[1]	Life expectancy (yr)	Incremental C/E^2 (dollar)[1] per life-year gained	Non-dominated strategy	Lifetime cost per person (dollar)[1]	Life expectancy (yr)	Incremental C/E^2 (dollar)[1] per life-year gained	Non-dominated strategy	Lifetime cost per person (dollar)[1]	Life expectancy (yr)	Incremental C/E^2 (dollar)[1] per life-year gained
40	COL5	370.0	8.633		COL10	341.0	8.326		COL10	352.2	8.328	
	COL3	388.9	8.636	7584.1	COL5	343.7	8.329	872.5	COL5	369.2	8.330	7375.9
					COL3	383.7	8.330	26483.2	COL3	424.1	8.331	50842.3
50	COL3	479.2	7.665		COL5	463.5	7.572		COL10	475.0	7.571	
					COL3	480.2	7.575	5592.9	COL5	476.0	7.574	330.8
									COL3	508.1	7.577	14571.1
60	COL3	528.3	7.668		COL3	522.1	6.057		COL5	519.5	6.057	
									COL3	529.5	6.059	4186.7

COL10: Colonoscopy every 10 years; COL5: Colonoscopy every 5 years; COL3: Colonoscopy every 3 years. Dominated strategy is a strategy that is more or equally costly and less effective than a competing strategy. [1]Exchange rate: 955 Korean Won for one US dollar in 2006. [2]Incremental CE ratio (dollar/year) = Incremental cost per person/Incremental years of life gained.

survivors for starting CRC screening. If the age of cancer survivors for starting CRC screening was 50 years or older, COL3 had an ICER of less than $14600 per LYS, regardless of the screening compliance. If age for starting CRC screening in cancer survivors was 40 years, the ICER of COL5 was less than $7400 per LYS saved in all cases of CRC screening compliance.

DISCUSSION

We constructed a computer simulation to suggest economic strategies of second primary CRC screening for cancer survivors in Korea, and compared the CE results of CRC cancer screening in cancer survivors and in the average-risk general population. As all non-dominated strategy were those using colonoscopy in both cancer survivors and the general population, more strict and frequent recommendation of colonoscopy such as COL5 and COL3 could be considered economic strategies for male cancer survivors.

Until now, there has been no explicit threshold of CE below which policy makers will consider accepting the strategy. In the US, a figure of $50000 per Quality Adjusted Life-Year (QALY) has frequently been quoted

for many years as being cost-effective[43]. The World Health Report 2002 suggested that interventions costing less than three times Gross Domestic Product (GDP) per capita for each Disability Adjusted Life-Year (DALY) averted represented good value for money[44], which is usually well in excess of $50 000 per QALY in many high-income countries[43]. In our study, the ICER for COL3 was less than $6000 per LYS in a base line analysis, and did not exceed $10 000 per LYS in one-way sensitivity analyses. Our findings also suggest that if the risk of CRC in cancer survivors is at least two times higher than that in the general population, COL5 could be a cost-effective strategy for second primary CRC screening for cancer survivors of both good and poor prognosis of index cancer, with an ICER of less than $10 500 per LYS. As our primary outcomes are not QALY or DALY, direct comparisons might be difficult. The GDP per capita of Korea was more than $20 000 in 2006. When we approximately applied these CE thresholds, in most cases, COL5 and COL3 could be considered a cost-effective method for second primary CRC screening for Korean male cancer survivors, regardless of the index cancer. Even if the index cancer were CRC, our finding could be applied and be consistent with the CRC surveillance guidelines of the American Society of Clinical Oncology (ASCO)[45]. In ASCO guidelines, routine annual colonoscopies are not recommended for all CRC patients, and colonoscopy every 3-5 years could be sufficient to detect new CRCs and polyps[45].

It is also important to consider the changes in CE of these strategies according to age of starting second primary CRC screening in cancer survivors. Little is known about the CE if cancer survivors start second primary CRC screening above or below age 50, at which most guidelines recommend starting CRC screening in the general population. Our findings suggest that for male cancer survivors in older age, COL3 had more favorable CE results, regardless of screening compliance. For younger cancer survivors aged 40 years old, COL5 could be considered a CE strategy with ICER of less than $7600 per LYS.

Interestingly, in our study of Korea, COL10, COL5 and COL3 had lower total medical costs than no screening of male cancer survivors. In other countries, screening for CRC usually leads to more costs than no screening. Cost estimates for the medical care of CRC treatment in the US range from $25 000 to $70 000 and the cost of COL is about $1000[9,16]. However, in Korea, the cost estimate of CRC treatment in the first year ranges from $7000 to $14 000 while the cost of COL was about $60[24,25]. The ratio of treatment cost to COL cost ranges from 25:1 to 70:1 in the US and 120:1 to 230:1 in Korea. Due to the difference in cost structures, screening with colonoscopy might be more cost-effective in Korea than in other countries. However, CRC screenings are not covered by the Korean NHI scheme in either the general population or cancer survivors. Instead, the Korean government started the national cancer screening program (NCSP) in 1999, which was extended to include CRC screening in 2004[11]. The government covers 50% of the screening cost for the insured and 100% for low-income people. However, the primary method for CRC screening in the Korean NCSP is FOBT annually. Our study shows that the strategy of "FOBT annually" is always the dominant strategy in male cancer survivors, and costs more than the strategy of no screening.

The major barrier to promotion of colonoscopy as a primary CRC screening tool is the lack of manpower to deliver colonoscopy to the public in Korea. In these human-resource limited setting, it is important to identify the more vulnerable population who has a greater potential to receive benefits. Our study suggests that cancer survivors who are at increased risk of second primary CRC have a favorable result of CE of CRC screening compared with the general population. Even in younger cancer survivors aged 40 years old, COL5 might be economically feasible, while COL10 is usually recommended for the Korean general population aged 50 years old. Therefore, at least for cancer survivors, CRC screening should be covered by the Korean NHI scheme and screening methods using colonoscopy are needed to be recommended as a primary screening strategy for CRC in this population.

Limitations

Our analysis has several limitations. In the design of the model, we tried to reduce the complex natural history of CRC to a few essential states and to avoid assumptions on treatments for which little or no published data existed. For instance, we assumed that 90% of second primary CRC arose from polyps. We used several sets of data from the general population, such as prevalence of colorectal polyp by age, polyp recurrence rate, and treatment costs of second primary CRC, if there were no published data available in cancer survivors. There were possible differences between these two populations. However, when we performed sensitivity analyses, the CE results were usually insensitive to the plausible range of these uncertain parameters. Second, we calculated only the direct costs and did not take into account the impact of CRC and screening on indirect costs. Third, in our study, recently developed CRC screening strategies such as CT colonoscopy were not included. However, the cost of CT colonoscopy is about four times higher than that of colonoscopy in Korea, while the sensitivity and specificity of CT colonoscopy is not superior to that of colonoscopy[46], and this new method does not seem to be an economically-efficient strategy.

In conclusion, with an increased population of long-term cancer survivors, effective systems for their health promotion are needed. Implementation of the economic SPC screening program might be one of the important interventions to improve their health. Our study showed that COL3 or COL5 might be suggested as a primary strategy for second primary CRC screening in cancer survivors who have a higher risk of CRC than the general population. This study supports the evidence

and rationale for second primary CRC screening in male cancer survivors.

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COMMENTS

Background

Recent improvement in cancer survival due to early diagnosis and advances in treatment has raised the issue of second primary cancers (SPCs) in cancer survivors after their primary treatment. The age-standardized incidence rate is about four times higher for second primary colorectal cancer (CRC) than for first primary CRC in Korea. However, until now, there have been few recommendations and economic evaluations of CRC screening for cancer survivors.

Research frontiers

To suggest a feasible economic strategy of second CRC screening for cancer survivors in Korea, the authors constructed a decision-analytic model, and compared the cost-effectiveness results of cancer screening between in male cancer survivors.

Innovations and breakthroughs

Non-dominated strategies were those using colonoscopy in both cancer survivors and the general population, and more strict and frequent recommendations for colonoscopy (COL) such as COL5 (screening every 5 years) and COL3 (screening every 3 years) could be considered as economic strategies for male cancer survivors.

Applications

The major barrier to promoting colonoscopy as a primary CRC screening tool is the lack of manpower to deliver colonoscopy to the public in Korea. In these human-resource limited settings, it is important to identify the most vulnerable population who has more potential to receive the benefits. In younger cancer survivors aged 40 years old, COL5 might be economically feasible, while COL10 is usually recommended for the Korean general population aged 50 years old. Therefore, at least for cancer survivors, CRC screening should be covered by the Korean national health insurance scheme and screening methods using colonoscopy should be recommended as a primary screening strategy for CRC in this population.

Terminology

SPC: A SPC is a new primary cancer developing in a person with a history of cancer.

Peer review

The authors investigated the cost-effective strategy of CRC screening for cancer survivors in Korea. The article is well written and the contents are reliable.

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Characterization of clarithromycin resistance in Malaysian isolates of *Helicobacter pylori*

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Abstract

AIM: To characterize the types of mutations present in the 23S rRNA genes of Malaysian isolates of clarithromycin-resistant *Helicobacter pylori* (*H pylori*).

METHODS: Clarithromycin susceptibility of *H pylori* isolates was determined by E test. Analyses for point mutations in the domain V of 23S rRNA genes in clarithromycin-resistant and -sensitive strains were performed by sequence analysis of amplified polymerase chain reaction products. Restriction fragment length polymorphism was performed using *Bsa* I and *Mbo* II enzymes to detect restriction sites that correspond to the mutations in the clarithromycin-resistant strains.

RESULTS: Of 187 isolates from 120 patients, four were resistant to clarithromycin, while 183 were sensitive. The MIC of the resistant strains ranged from 1.5 to 24 μg/mL. Two isolates had an A2142G mutation and another two had A2143G mutations. A T2182C mutation was detected in two out of four clarithromycin-resistant isolates and in 13 of 14 clarithromycin-sensitive isolates. Restriction enzyme analyses with *Bsa* I and *Mbo* II were able to detect the mutations.

CONCLUSION: Clarithromycin resistance is an uncommon occurrence among Malaysian isolates of *H pylori* strains and the mutations A2142G and A2143G detected were associated with low-level resistance.

Key words: Clarithromycin resistance; *Helicobacter pylori*; 23S rRNA mutation; Restriction fragment length polymorphism

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a microaerophilic, Gram-negative bacterium, and is implicated often as the causative agent of chronic gastritis, duodenal and gastric ulcers, and gastric carcinoma[1,2]. *H pylori*-associated disorders usually regress or heal completely after treatment with antibiotics[3]. Clarithromycin is a macrolide that has been used frequently in combination with other antimicrobial agents for the treatment of *H pylori* infection[4-6]. However, resistance to clarithromycin has become one of the major reasons for treatment failure[7]. The prevalence of *H pylori* resistance to clarithromycin varies among different countries, such as 12% in Japan, 1.7%-23.4% in Europe and 10.6%-25% in North America[8,9].

Clarithromycin acts by binding to the peptidyl transferase region of 23S rRNA and inhibits bacterial

protein synthesis. Resistance to clarithromycin results from structural changes in the 23S rRNA molecule caused by mutation of the 23S rRNA gene[10,11]. Adenine to guanine transitions at positions 2142 and 2143 are the main 23S rRNA mutations in clarithromycin-resistant isolates[12-14]. All these mutations have been shown to confer resistance to this macrolide by mutagenesis study[15]. Other mutations that have been observed in clarithromycin-resistant *H pylori* isolates are A2515G and T2717C, A2116G, G2141A, A2144T, T2182C, G2224A, C2245T, and T2289C[16]. The A2142C/G and A2143G mutation also generate specific restriction sites, namely *Mbo* Ⅱ and *Bsa* Ⅰ, and these may be used for the rapid screening of clarithromycin resistance.

In the present study, we determined the prevalence of clarithromycin resistance in our local *H pylori* strains and characterized the types of mutations that occurred in the resistant strains. In addition, we determined whether the Restriction fragment length polymorphism (RFLP) technique was suitable for rapid detection of the mutations among our local isolates.

MATERIALS AND METHODS

Patients

The patients enrolled in this study were the patients who underwent endoscopy for gastrointestinal symptoms at the Gastroenterology Unit, National University of Malaysia Hospital between 2005 and 2007. Written informed consent was obtained from all patients before biopsies were taken from the antrum and corpus of each patient.

H pylori culture and antimicrobial susceptibility determination

Biopsy samples were cultured on Columbia agar supplemented with 10% ox blood. Plates were incubated at 37℃ under microaerophilic condition for 5-7 d. Bacterial isolates were identified according to colony morphology, Gram-staining, urease, catalase and oxidase. The cultures were stored at -80℃ in Brucella broth supplemented with 15% glycerol and fetal calf serum (Invitrogen, USA).

The MIC of clarithromycin was determined by the E-test method. E tests (AB Biodisk, Sweden) were performed on Columbia agar supplemented with 10% ox blood. The plates were incubated under microaerophilic condition for 3-5 d. Isolates were classified as clarithromycin-resistant if MIC was > 1 μg/mL. *H pylori* strain ATCC43504 was included as a control clarithromycin-sensitive strain.

DNA extraction and polymerase chain reaction (PCR) amplification of 23S rRNA gene

DNA extraction was carried out using the Nucleospin® Tissue Kit (Macherey-Nagel, BD Biosciences, USA) and stored at -20℃ until use. The primers used for PCR amplification were Hp5 forward, 5'-GTCGTGCCAA GAAAAGCGTCT-3' (positions 1672-1693; GenBank accession number U27270), and Hp2 reverse, 5'-TGTG TGCTACCCAGCGATGCTC-3' (positions 2811-2790; GenBank accession number U27270). PCR was carried out on 10 mmol/L dNTP (Promega, USA), 10 pmol of each primer, 1.25 U *Taq* DNA polymerase (Promega), and genomic DNA of *H pylori*. The PCR was performed in a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer, USA) for 30 cycles with the following cycling conditions: 94℃ for 1 min, 58℃ for 30 s and 72℃ for 30 s. The PCR products were then analyzed by electrophoresis using 1% agarose and stained with ethidium bromide.

DNA sequencing and sequence analysis

PCR products were purified using a QIAquick PCR purification kit (Qiagen). Sequencing was performed using BigDye® Terminator v3.1 sequencing kit and analyzed on ABI PRISM® 377 Genetic Analyzer. The sequences were analyzed using free ClustalW software (http://www.ebi.ac.uk/clustalw).

RFLP analysis

To detect the A-G point mutations at positions 2142 and 2143, the amplified PCR products were digested with the *Mbo* Ⅱ and *Bsa* Ⅰ (MBI Fermentas, Lithuania) restriction enzymes, respectively, and analyzed on 1.6% agarose gels. *Mbo* Ⅱ-digested PCR products that contained the A2142G mutation were expected to yield two fragments (476 and 662 bp) when digested with *Mbo* Ⅱ, whereas the PCR products that contained the mutation A2143G were expected to yield three fragments (60, 418 and 666 bp) when digested with *Bsa* Ⅰ.

RESULTS

Antimicrobial susceptibility

A total of 187 *H pylori* were isolated from gastric biopsies of 120 patients. Four of these isolates (2.1%) from four patients were resistant to clarithromycin, while 183 isolates from 116 patients were sensitive to clarithromycin. The MIC values of all the clarithromycin-resistant isolates ranged from 1.5 to 24 μg/mL (Table 1). The patients with clarithromycin-resistant isolates had never been treated for *H pylori* infection.

PCR amplification of 23S rRNA and sequence analysis

All the clarithromycin-resistant strains and 14 randomly selected clarithromycin-sensitive strains from 14 different patients were subjected to DNA extraction and PCR amplification of domain V of the 23S rRNA gene. The mutations in domain V of 23S rRNA were determined by comparing the sequences of clarithromycin-resistant and -sensitive isolates with the sequences of the reference strain ATCC43504. All of the clarithromycin-resistant isolates were shown to have point mutations of either A2142G or A2143G, while none of the 14 clarithromycin-sensitive isolates had these types of mutations. T2182C mutation was detected in both clarithromycin-resistant and -sensitive isolates.

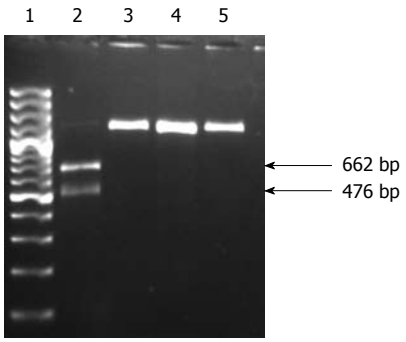

Figure 1 RFLP analysis of 23S rRNA to detect the A2142G mutation using *Mbo* Ⅱ restriction enzyme. Lane 1: 100 bp DNA ladder; Lane 2: Digestion product of resistant isolate with A2142G mutation, showing two fragments of 662 and 476 bp; Lanes 3 and 4: Digestion products of two clarithromycin-resistant isolates with A2143G mutation; Lane 5: Digestion product of a clarithromycin-sensitive isolate with T2182C mutation.

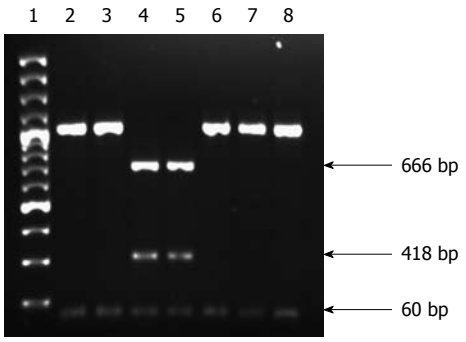

Figure 2 RFLP analysis of 23S rRNA to detect the A2143G using *Bsa* Ⅰ restriction enzyme. Lane 1: 100 bp DNA ladder; Lanes 2 and 3: Digestion products of two clarithromycin-resistant isolates with A2142G mutation; Lanes 4 and 5: Restriction analysis of resistant isolates with A2143G mutation, showing three fragments of sizes 666, 418 and 60 bp; Lanes 6-8: Digestion products of clarithromycin-sensitive isolates.

Table 1 MIC value of clarithromycin and mutations in the domain V 23S rRNA of *H pylori* isolates

Clarithromycin MIC (μg/mL) and phenotype	Mutation site	Number of isolate(s)
24 (R)	A2142G	1
24 (R)	A2143G, T2182C	1
3 (R)	A2143G	1
1.5 (R)	A2142G, T2182C	1
< 0.016 (S)	T2182C	13
< 0.016 (S)	No mutation	1

R: Resistant; S: Sensitive.

RFLP analysis of clarithromycin-resistant and -sensitive isolates

Sequence analysis of restriction sites of the amplified V domain of the 23S rRNA gene of the resistant strains showed restriction sites that could be digested with *Bsa* Ⅰ and *Mbo* Ⅱ. Digestion of the amplified PCR product with *Mbo* Ⅱ produced two fragments of 662 and 476 bp sizes in strains with the A2142G mutation. No fragment was produced with the A2143G or T2182C mutation (Figure 1). Resistant isolates with the A2143G mutation produced three fragments of 662, 476 and 60 bp when digested with *Bsa* Ⅰ. No restriction fragment was produced in strains with A2142G and T2182C mutations (Figure 2). None of the clarithromycin-sensitive isolates and the negative control (ATCC43504) produced restriction fragments when digested with *Mbo* Ⅱ and *Bsa* Ⅰ, which indicated the absence of A2142G and A2143G mutations.

DISCUSSION

The prevalence of clarithromycin resistance was found to be low among the isolates in the present study. To the best of our knowledge, this was the first study that reported and characterized clarithromycin-resistant strains in Malaysia.

Clarithromycin is used worldwide for *H pylori* eradication therapy. *H pylori* strains that are resistant to clarithromycin have been reported increasingly in several studies[9,17]. As resistance will often lead to failure of eradication therapy, knowledge of the current susceptibility patterns of local *H pylori* isolates could help in determining the choice of appropriate treatment for the patient. In the present study, the prevalence of clarithromycin resistance was still low; however, physicians should bear in mind the possibility of clarithromycin resistance if their patient does not respond to this antibiotic.

Versalovic *et al*[18] have shown that A2142G and 2143G mutations of 23S rRNA genes in *H pylori* are associated with resistance to clarithromycin. A mutagenesis study performed by Taylor *et al*[15] has confirmed that the A2142G and A2143G mutations are associated with clarithromycin resistance of *H pylori*. A review by Mégraud[8] of several studies worldwide has shown that 81.5% of the mutations in chlarithromycin-resistant isolates were the A2142G or A2143C mutations. In our study, either A2142G or A2143G mutations were found in the clarithromycin-resistant isolates, or they were absent in the clarithromycin-sensitive isolates. The mutations found in our local strains were in accordance with the findings in other countries. The mutations were also associated with low-level resistance.

Another type of mutation detected in our isolates was T2182C. This mutation was detected in most of the strains studied, which suggests a common occurrence among our local strains. This mutation is not new and has been described in previous reports[19,20]. This mutation is not associated with clarithromycin resistance[21]. However, it has not been discussed whether this type of mutation occurs commonly in *H pylori*. Mutations other than A2142G and A2143G have been reported in other studies[16,22], but among our strains, sequence analysis showed that the mutations were limited to these types only. Although the low number of resistant isolates in our study limit the scope of detection, the results suggest that clarithromycin resistance of *H pylori* in Malaysia can be predicted by detection of mutations at positions 2142 and 2143 of the 23S rRNA gene. The resistance to clarithromycin among the four patients is considered primary resistance

because they were never treated previously for *H pylori* eradication.

Knowledge about the molecular mechanism of resistance is important as it can be used to facilitate the development of other molecular methods to detect resistance. It has been shown that PCR-RFLP can be used to detect mutations in clarithromycin-resistant isolates[23,24]. Using this technique in our study, it was demonstrated that only strains with the A2142G and A2143G mutations produced restriction fragments when digested with *Mbo* Ⅱ or *Bsa* Ⅰ. This alleviated the need to use the more expensive and time-consuming processes of sequencing and analysis to look for mutation sites.

In conclusion, we showed that there was a low prevalence of clarithromycin resistance in our local strains of *H pylori*. The A2142G or A2143G mutations detected were in accordance with the findings in other countries. These mutations were not found among the analyzed clarithromycin-sensitive strains. T2182C mutation was a common occurrence in our local isolates. Other types of mutations associated with clarithromycin resistance were not observed in our resistant strains. The A2142G or A2143G mutations were detected also by RFLP, thus making it a rapid method for the detection of clarithromycin-resistant strains in our local isolates.

COMMENTS

Background

The incidence of clarithromycin-resistant *Helicobacter* is increasing worldwide. This limits the therapeutic options available for eradicating the bacterium. Clarithromycin resistance has been attributed to mutations in the 23S rRNA gene.

Research frontiers

Clarithromycin acts by binding to the peptidyl transferase region of 23S rRNA and inhibits bacterial protein synthesis. There are many types of mutations observed in the 23S rRNA genes of clarithromycin-resistant *Helicobacter pylori* (*H pylori*). There is little information on the prevalence and characteristics of clarithromycin resistance in *H pylori* strains isolated from Malaysian patients. In the present study, the authors determined the prevalence of resistance and characterized the types of mutations present in their resistant strains.

Innovations and breakthroughs

The study gave an insight into the low prevalence of clarithromycin resistance among the *H pylori* strains studied. The A2142G and A2143G mutation detected were in accordance with the findings in our other countries. However, other types of mutations associated with clarithromycin resistance were not observed among our resistant strains.

Applications

The A2142G and 2143G mutations in the 23S rRNA genes in *H pylori* can be detected easily by restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR) product of the genes, using *Mbo* Ⅱ or *Bsa* Ⅰ. This eliminates the need to detect these mutations by sequencing.

Peer review

This is a straightforward study that determined the prevalence of clarithromycin resistance in *H pylori* strains isolated from the authors' institute in Malaysia. The types of mutations that occurred in the resistant strains were further characterized by PCR and sequencing. Finally, the authors evaluated whether the RFLP technique was suitable for rapid detection of the mutations among these isolates. Although the sample size of clarithromycin-resistant isolates was relatively small, the techniques and methodology used were appropriate and standard. The information is potentially useful for the clinical management of *H pylori* infection in Malaysia, and also for our overall appreciation of the global distribution and prevalence of antibiotic resistance of this important pathogen.

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BRIEF ARTICLES

Cardiopulmonary bypass induced microcirculatory injury of the small bowel in rats

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Abstract

AIM: To investigate microvascular injury quantitatively in the small bowel with respect to cardiopulmonary bypass (CPB) and related mechanisms.

METHODS: In 10 male SD rats, normothermic CPB was established and continued with a flow rate of 100-150 mL/kg per minute for 60 min, while another 10 sham-operated animals served as controls. An approximate 10-cm loop of the terminal ileum was exteriorized for observation by means of intravital fluorescence microscopy. The small bowel microcirculatory network including arterioles, capillaries, and collecting venules was observed prior to CPB, CPB 30 min, CPB 60 min, post-CPB 60 min and post-CPB 120 min. The intestinal capillary perfusion, microvascular permeability and leukocyte adherence were also measured.

RESULTS: The systemic hemodynamics remained stable throughout the experiment in both groups. In CPB animals, significant arteriolar vasoconstriction, blood velocity reduction and functional capillary density diminution were found. As concomitances, exaggerated albumin extravasation and increased leukocyte accumulation were also noted. These changes were more pronounced and there were no signs of restitution at the end of the observation period.

CONCLUSION: CPB induces significant microcirculatory injury of the small bowel in rats. The major underlying mechanisms are blood flow redistribution and generalized inflammatory response associated with CPB.

Key words: Cardiopulmonary bypass; Functional capillary density; Intestinal microcirculation; Intravital microscopy

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INTRODUCTION

Despite excellent improvements, there is increasing evidence to show that cardiopulmonary bypass (CPB) is related to a generalized inflammatory response and splanchnic edema formation in both clinical and experimental investigations[1-5]. Impairment of gastrointestinal perfusion during CPB may lead to loss of mucosal vascular integrity and increased permeability. The translocation of microorganisms and endotoxin into the systemic circulation can result in a continuing toxic insult and systemic tissue injury. This is thought to be one of the essential mechanisms in the development of sepsis, shock, post-perfusion complications, and multiple organ failure after CPB[5-7]. Identification of rational clinical therapeutic approaches requires a precise elucidation of the underlying pathophysiological processes of the intestinal microvasculature induced by CPB.

However, the exact regional hemodynamic and functional changes of the small intestine in terms of the response to CPB remain unclear. Indirect methods may not be sufficient to delineate the pathomechanisms of intestinal microcirculatory conditions within the

different tissue compartments such as muscle, submucosa, and mucosa[8-10]. An appropriate experimental model is needed to investigate the variety of microvascular alterations including capillary perfusion injury, microvascular endothelial integrity loss, and leukocyte activation under both physiological and pathological conditions. In this study, using a rat model of CPB, we directly observed and quantitatively analyzed the microvascular alterations in small bowel by means of fluorescent intravital microscopy, aiming to demonstrate the influence of CPB in the different functional units of the microcirculatory network.

MATERIALS AND METHODS

Animal preparation

Adult male Sprague-Dawley rats (450-550 g) served as experimental animals. All animals received humane care in compliance with the principles of laboratory animal care formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996). Rats were anaesthetized with intraperitoneal administration of ketamine (50 mg/kg) and chlorpromazine (2 mg/kg). After tracheostomy and intubation, the animals were mechanically ventilated with about 10 mL/kg tidal volume, 60 breaths/min respiratory rate, and 100% inspiratory concentration of O_2. Anesthesia was maintained throughout the experiment with additional doses of intravenous ketamine.

CPB techniques

A rat CPB model previously developed by our group, with consistent excellent survival, was used in the current study[11]. Briefly, the right femoral artery was cannulated with a 24-gauge heparinized catheter for arterial pressure monitoring and blood gas analysis. The homolateral femoral vein was cannulated with a 20-gauge catheter for blood and fluid infusion. Following heparinization (500 U/kg), a 16-gauge catheter, modified to a multi-side-orifices cannula in the forepart, was inserted into the right jugular vein and advanced to the right atrium for blood drainage by gravity and siphon. A 22-gauge catheter was cannulated to the right carotid artery as the arterial infusion line for the CPB circuit. The membrane oxygenator was specially designed with a surface area for gas exchange of 0.05 m^2 and the total assembly dynamic priming volume of 4 mL. A rolling pump was used to drive the blood through silicone arterial inflow tubing and then returned to the right carotid artery. Priming was composed of 8 mL heparinized fresh homologous blood obtained from a donor and 8 mL synthetic colloid (HAES-steril®). The CPB flow-rate was gradually adjusted, stabilized and performed at 100-150 mL/kg per minute throughout the experiment. To ensure the mean arterial pressure (MAP) above 60 mmHg and the hematocrit about 30%-40%, additional whole blood and Ringer's solution was given during the experiment to supply the blood and fluid loss caused by surgery, sampling, leakage and evaporation. Body central temperature was monitored with a rectal probe and maintained at 36.5-38.0℃ by a heating lamp placed above.

Intravital microscopy

A modified intravital microscope (Shanghai 2XC4TV, China) with a 100 W mercury arc lamp was used to observe the intestinal microcirculation. With $I_{2/3}$ and N_2 filter blocks interposed into the light path, the green (530-560 nm) and the blue (450-490 nm) light was selected, respectively for epi-illumination. For *in vivo* microscopy, fluorescein isothiocyanate conjugated to bovine serum albumin (FITC-BSA), which fluoresces under blue light, allowed visualization of the intestinal microvessels. Acridine orange, which fluoresces under green light, stains leukocytes and allows their adhesion in the microvasculature to be monitored. The microscopic images were acquired by a charge-coupled device digital camera (Olympus C-5050, Japan), recorded by a digital video system (Cannon MVX-150i, Japan), and displayed on a high-resolution monitor (Patriot 798HD, China) for off-line analysis. The final magnification of the images can be approximately achieved by 250 × and 650 × with 10 × and 25 × long-distance objectives.

Experimental protocol

The animals were randomly divided into two groups. In the CPB group, 10 rats were subjected to the CPB circuit and received extracorporeal perfusion for 60 min. After the bypass was terminated, the remaining priming solution was infused gradually to maintain the main arterial pressure around 60 mmHg. Ten sham operated animals which were cannulated, heparinized and subjected to the CPB circuit but without CPB served as controls, and the CPB circuit was weaned 1 h later. The dosage of heparin for anticoagulation was the same in both groups.

In all animals, *via* a small transverse abdominal incision, an approximate 10 cm loop of ileum was exteriorized and placed on a movable stage attached to the microscope. A 10 mm incision along the antimesenteric border was made with an electric microcautery device for visualization of the mucosal surface. The exposed segment was gently extended, held in place by stay sutures, and covered with a glass microscopical cover slide. The tissues were constantly superfused with warm Ringer's lactate at 37℃ to avoid drying and local hypothermia.

The intestinal microcirculation was visualized after intravenous administration of FITC-BSA (0.2 mL/100 g body weight, 5%) and acridine orange (0.1 mL/100 g body weight, 0.2%). To avoid damage to the microcirculation due to long periods of light exposure, the recording time varied 30-60 s. The microvasculature of smooth muscle and the submucosa was assessed from the serosal side in the oral closed part of the segment by adjusting the focus level of the microscope. The villous microcirculation was visualized from the mucosal surface

Table 1 Macrohemodynamic parameters in CPB and sham animals (mean ± SD)

Variables	Groups	Prior to CPB	CPB 30 min	CPB 60 min	Post CPB 60 min	Post CPB 120 min
MAP (mmHg)	CPB	74 ± 8	77 ± 7	75 ± 6	75 ± 5	72 ± 5
	Sham	73 ± 5	75 ± 6	77 ± 6	75 ± 7	77 ± 5
pH	CPB	7.40 ± 0.02	7.36 ± 0.02[a,c]	7.31 ± 0.04[b,c]	7.27 ± 0.04[b,c]	7.22 ± 0.03[b,c]
	Sham	7.40 ± 0.02	7.39 ± 0.01	7.40 ± 0.02	7.39 ± 0.02	7.39 ± 0.02
PaO_2 (mmHg)	CPB	414 ± 16	279 ± 13[b,c]	280 ± 14[b,c]	370 ± 21[b,c]	372 ± 21[b,c]
	Sham	404 ± 16	401 ± 16	394 ± 15	406 ± 14	402 ± 19
$PaCO_2$ (mmHg)	CPB	38.8 ± 4.2	39.3 ± 2.8	38.9 ± 2.1	38.8 ± 3.8	40.2 ± 4.9
	Sham	39.1 ± 2.2	40.9 ± 1.6	38.3 ± 2.8	38.6 ± 2.5	40.3 ± 3.9
Hematocrit (%)	CPB	40.8 ± 2.2	33.0 ± 0.9[b,c]	33.4 ± 1.0[b,c]	32.3 ± 0.9[b,c]	32.8 ± 0.8[b,c]
	Sham	39.7 ± 1.4	40.8 ± 2.1	40.2 ± 1.7	39.3 ± 1.9	38.5 ± 1.6

[a]$P < 0.05$, [b]$P < 0.001$ *vs* sham group; [c]$P < 0.001$ *vs* baseline.

through the longitudinal incision in the aboral part of the segment.

After stabilization for about 15 min, four non-overlapping regions of interest were defined and measured to obtain representative values for every parameter. The quantitative analysis of intestinal microcirculation was performed by means of the standard technique usually used in intravital microscopic studies in small animals[12,13]. A computer-controlled image analysis system (ImageJ 1.30V, National Institutes of Health, USA) was applied in those processes. Microcirculatory parameters were recorded prior to CPB, CPB 30 min, CPB 60 min, post-CPB 60 min, and post-CPB 120 min. In addition, the systemic arterial pressure was monitored continually and the arterial blood gas analyses were performed repeatedly throughout the experiments.

Statistical analysis

SPSS for Windows 11.0.1 (SPSS Inc. 2001, USA) was used for statistics. All data were expressed as means ± SD. Following analysis of variance, the Student's *t* test was used as a test for statistical significance of normal distributed values between the groups. To analyze differences within each group for time effects, paired Student's *t* tests including a correction of the error according to Bonferroni probabilities for repeated measurements were used. $P < 0.05$ was considered statistically significant.

RESULTS

Macrohemodynamic parameters

The MAP remained constant and the results of blood gas analysis were within the acceptable range in both groups using this experimental design and CPB technique (Table 1). However, during the entire bypass period, the heart kept beating and lung perfusion persisted. Due to the existence of the shunt, this could result in lower blood oxygenation in the CPB group *vs* the sham. As is the nature of CPB, hemodilution and a significant decrease in pH were observed in our model similar to that seen in clinical practice.

Capillary perfusion

In the CPB group, a significant pathological segmental vasoconstriction not ascribed to undulating vasomotion was detected in small vessel segments. Mean arteriolar diameters of single arterioles in the mucosal layer were found to be diminished from 30.82 ± 3.57 prior to CPB to 20.59 ± 1.97 μm ($P < 0.001$) at the end of the experiment (Figure 1). Blood flow was apparently reduced in the collecting venules in the submucosa, with the red blood cell flow velocities reduced from 1.38 ± 0.08 mm/s prior to initiation of CPB to 0.62 ± 0.04 mm/s ($P < 0.001$) at 2 h after bypass (Figure 2). As a consequence, a concomitant hemoconcentration, typical sludge phenomena and cell aggregates within

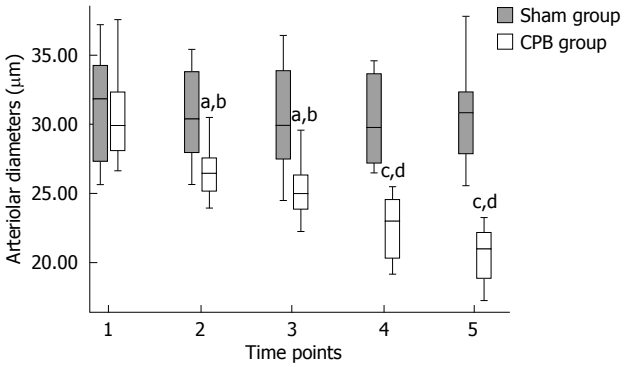

Figure 1 Diameter of arterioles in mucosa. Values are expressed as mean ± SD, [a]$P < 0.01$, [c]$P < 0.001$ *vs* baseline; [b]$P < 0.01$, [d]$P < 0.001$ *vs* sham group. Time points: 1: Baseline; 2: 30 min of CPB; 3: 60 min of CPB; 4: 60 min after weaning off CPB; 5: 120 min after weaning off CPB.

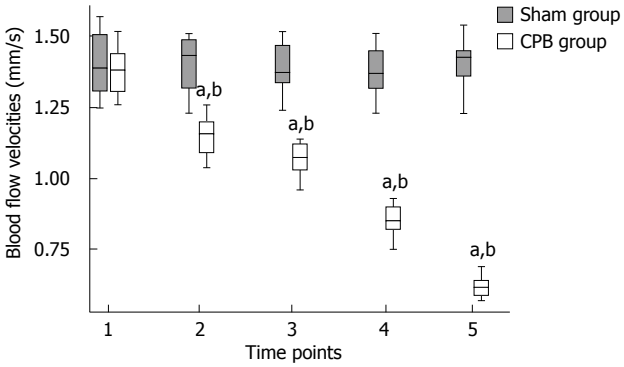

Figure 2 Blood cell velocities in collecting venules in submucosa. Values are expressed as means ± SD, [a]$P < 0.001$ *vs* baseline; [b]$P < 0.001$ *vs* sham group (Time points: see Figure 1).

Table 2 Changes in functional capillary density in CPB and sham animals (mean ± SD)

Variables	Groups	Prior to CPB	CPB 30 min	CPB 60 min	Post-CPB 60 min	Post-CPB 120 min
FCD in mucosa (cm^{-1})	CPB	511 ± 17	406 ± 12[a,b]	328 ± 11[a,b]	219 ± 13[a,b]	150 ± 10[a,b]
	Sham	504 ± 17	510 ± 20	499 ± 18	513 ± 14	507 ± 16
FCD in muscle (cm^{-1})	CPB	221 ± 13	152 ± 10[a,b]	125 ± 9[a,b]	102 ± 8[a,b]	64 ± 7[a,b]
	Sham	218 ± 14	221 ± 12	212 ± 17	217 ± 13	211 ± 14

[a]$P < 0.001$ *vs* sham group; [b]$P < 0.001$ *vs* baseline.

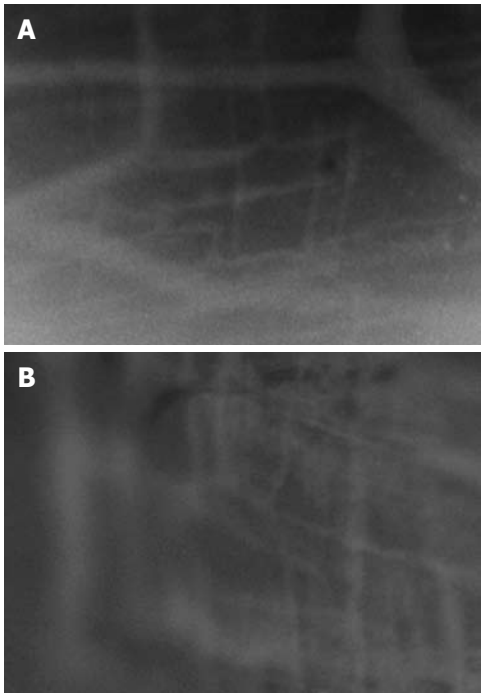

Figure 3 Interstitial fluorescence after administration of FITC-BSA in muscular layer. A: Control animals. FITC-BSA was contained within the vessels and the capillaries are easily identified; B: CPB animals. FITC-BSA extravasated from the capillaries into the interstitium, and a brighter flare surrounded the microvasculature (Magnification, × 650).

these vessels could be observed. In the control animals, the arterioles showed no changes in diameter and the blood cell velocities remained constant throughout the observation period. Functional capillary density (FCD) was expressed as the length of FITC-BAS perfused capillaries per observational area. Both the intestinal mucosa and the smooth muscle layer were analyzed. In the sham group, the perfusion was homogeneous in both compartment capillary networks (Table 2). However, in the CPB group, the FCD values decreased significantly during and after bypass and reached only 30% of initial values at the end of the observation period.

Microvascular permeability

A significant increase in macromolecular leakage induced by CPB was observed in this study. Under circumstances resulting in increased microvascular permeability to macromolecules, FITC-BSA was seen to leak from the vasculature, appearing as a flare in the perivascular interstitium. The interstitial fluorescent intensity was proportional to the degree of FITC-BSA leakage from the vessels. In control animals, the vascular

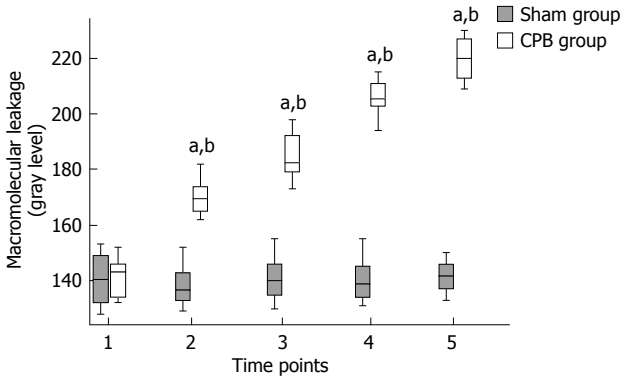

Figure 4 Macromolecular leakage of intestinal microcirculation in muscular layer. FITC-BSA leakage was represented as the gray level of Intestinal fluorescent intensity. Values are expressed as means ± SD, [a]$P < 0.001$ *vs* baseline; [b]$P < 0.001$ *vs* sham group (Time points: see Figure 1).

integrity was maintained with no observed increases in interstitial fluorescence during the experimental period (Figures 3 and 4). In the CPB group, the interstitial fluorescence increased at all time points from a gray level of 141.8 ± 7.3 at the beginning of CPB to 219.9 ± 7.1 at 2 h after weaning off bypass ($P < 0.001$).

Leukocyte adhesion

Leukocyte adherence was analyzed within postcapillary venules of the intestinal submucosa. A leukocyte was considered to be adherent in each vessel segment if it did not move or detach from the endothelial lining for at least 30 s. Stationary leukocytes that had migrated to the interstitium but remained in close proximity to the capillaries were also regarded as adherent. Data was expressed as the number of adherent leukocytes per 100 μm length of venular endothelial surface. In the sham group, only occasional adherent leukocytes (2.7 ± 0.9 cells/100 μm) were found firmly adhered to the microvascular endothelium throughout the experimental period (Figures 5 and 6). In the CPB group, a rapid, sustained, and significant increase in leukocyte adherence (about 10-fold at the end of experiments) was induced within the submucosal capillaries ($P < 0.001$) during and after CPB.

DISCUSSION

Nowadays, due to various improvements, CPB has made cardiovascular surgery more and more practical and safe even in high-risk patients. However, there are still many complications which are thought to result from

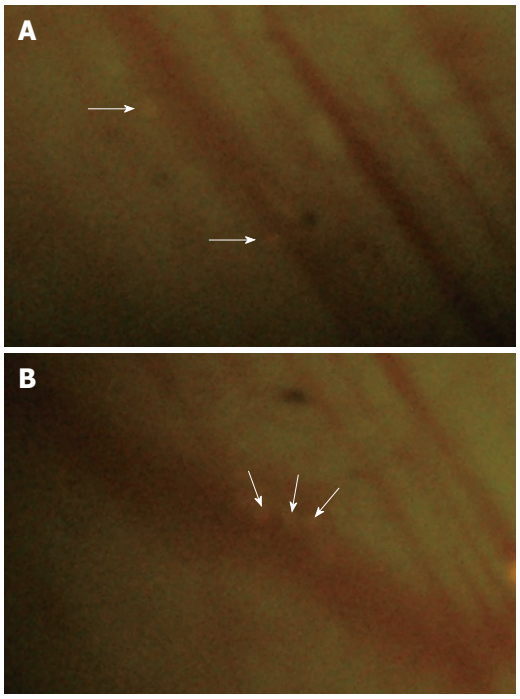

Figure 5 Leukocyte adhesion in intestinal submucosa. A: Control animals. Few adherent leukocytes (arrows) were observed within the microcirculation; B: CPB animals. Numerous adherent leukocytes (arrows) were observed within the capillaries.

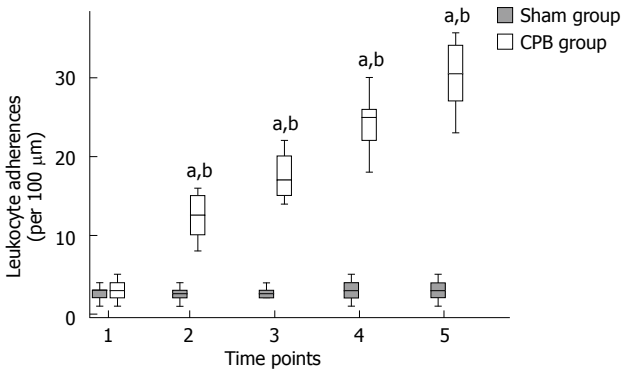

Figure 6 Quantitative analysis of leukocyte adhesion in intestinal submucosa. Adherent leukocytes per 100 μm length of venule. Values are expressed as means ± SD, $^{a}P < 0.001$ *vs* baseline; $^{b}P < 0.001$ *vs* sham group (Time points: see Figure 1).

a systemic inflammatory response and non-occlusive visceral ischemia[5,7,14,15]. Recent research has suggested that the intestinal mucosa is extremely sensitive to ischemia-reperfusion (I/R) injury and the gut is both the source and the target of inflammatory mediators[16,17]. These changes might contribute to a severe loss of intestinal function with significant complications or even multiorgan failure and systemic shock as a pathological consequence. In clinical practice, the incidence of gastrointestinal complications is low (0.58%-2.0%), however, they are associated with an unacceptable high mortality (15%-63%), especially in patients with pre-existing impairment of bowel perfusion[15,18,19]. With the aim of identifying effective preventive and therapeutic approaches, studies on the underlying pathomechanisms of CPB in small bowel capillary perfusion seem to be imperative.

Methods such as laser Doppler flow measurements and tonometry have been described in this area, but they are both indirect measures which do not allow quantitative analysis in different segments of the microvascular bed[9,20,21]. In contrast, intravital microscopy allows direct observation and quantitative analysis of the tissues and organs at the level of microcirculation and acquires more information on the pathologic role of CPB. The mesentery has been used frequently in studies of the intestinal microvasculature, but it has very little in common with intestinal microvascular networks[22,23]. In our experiments, the use of intravital microscopy employing different fluorescent dyes, allowed the simultaneous quantitative investigation of the microcirculation within all layers of the small intestine, i.e. subserosa, smooth muscle, submucosa and mucosa. For the first time, we directly investigated the intestinal microcirculatory changes within different layers relating to CPB by intravital microscopy.

The majority of studies on CPB relating to pathophysiological processes have been performed in large animal models[24]. These models have substantial limitations, mainly in that their costs are extremely high, instruments are very complex and they are too labor intensive. Rats are one of the most standard models for intravital microscopy and have been extensively used for the experimental investigation of multiple organ injury in various areas[12,13,22]. In our experiments, a rat CPB model with full flow perfusion was established. This model also reduced the cost of animals and equipment.

In the CPB group, the blood pressures were kept constant at a physiological level throughout the experiments without vasoactive drugs and the systemic temperatures were stable at around 37℃. Despite the consistent condition of systemic hemodynamics, microvascular perfusion impairments of the small bowel were clearly demonstrated. Arteriolar vasoconstriction, the reduction in blood cell velocities, together with the consequence of intravascular hemoconcentration and sludge formation, reflected hypoperfusion at the microvascular level. The reduction in the FCD values at the end of the observation period to approximately 30% of the pre-bypass values revealed that the capillary bed had been severely injured. The extravasation of FITC-BSA showed a distinct increase in the permeability of the vascular wall which might result in a loss of the natural barrier function against toxins and microorganisms. This might aggravate the injury to capillary perfusion further. Enhanced leukocyte adherence to the venular wall within submucosal vessels was also demonstrated in this study, which indicated that activated leukocytes may be the primary mediators and may have a causative role in the following pathophysiological sequelae.

The potential underlying pathogenesis of the intestinal injury induced by CPB includes several pathophysiological mechanisms[1,14,15,25], such as a transient reduction in systemic blood pressure and low cardiac outflow (LCO), hypotemperature in the bypass process,

redistribution of local organ blood flow, reactions of inflammatory mediators or vasoconstrictive agents, and obstruction of arteriolae with fragments of cells or tissues. With our model, due to the fact that the flow rate of the bypass circuit was kept constant and the arterial blood pressure remained unchanged, the influences of macrohemodynamics could be avoided. This allowed the conclusion that the observed changes in microvascular perfusion in the small bowel were not only a result of systemic hypoperfusion or LCO syndrome. By keeping the systemic and tissue temperatures in the normal range, we could exclude the effects of temperature on the microvascular perfusion in our study. Redistribution of blood flow and induction of inflammatory response seemed to be the major mechanisms responsible for the observed results[2,26]. Non-physiological patterns of perfusion originating from the rolling pump results in maldistribution of blood flow away from visceral organs as a reaction to CPB which serves as the primary contributor to microvascular impairments. It is important to note that the degree of damage to the microcirculation reached a maximum even after CPB removal for 2 h, suggesting that the ensuing inflammatory process as a consequence of CPB could be the dominant mechanism. The contact of blood and blood cells with the bypass circuit surfaces, the trauma induced by mechanical stress from the pump, together with the I/R injury of the gastrointestinal tract resulting from the transient decrease in local blood flow can induce the release of inflammatory mediators (e.g. cytokines, thromboxane, and histamine, *etc*), secretion of proteases (e.g. collagenase and elastase), activation of complements, extravasation of leukocytes, and the generation of oxygen free radicals[6,12,25]. These substances may lead to vasoconstriction, hemoconcentration and sludge formation, endothelial cell swelling and the consequent occlusion of vessels, and an increase in vascular permeability.

In conclusion, using intravital microscopy, progressive microvascular injury in the small bowel was demonstrated in a rat CPB model. The ensuing injury response was associated with microvascular perfusion failure, vascular permeability increase, and promotion of circulating leukocyte adherence. These changes in microvessels were even more prominent after the bypass period and no signs of restitution could be observed. The observed phenomena may have resulted from the redistribution of blood flow in different organs and the generation of inflammatory metabolites with potential negative effects on microvascular perfusion during and after CPB. The information obtained from our experiments will facilitate further investigations of different therapeutic regimens aimed at ameliorating microvascular damage due to CPB.

COMMENTS

Background

Impairment of intestinal microvascular perfusion induced by cardiopulmonary bypass (CPB) is thought to be related to mucosal edema formation and microvascular barrier injury, which may result in postoperative gastrointestinal complications and multiple organ failure. A better understanding of the microvascular alterations during and after CPB will facilitate the identification of therapeutic strategies.

Research frontiers

In general, experimental investigations on the intestinal injury induced by CPB are scarce. The identification of rational clinical therapeutic approaches requires a precise elucidation of the underlying pathophysiological processes of intestinal microvasculature induced by CPB.

Innovations and breakthroughs

With a novel rat model of CPB, the authors quantitatively investigated the microvascular alterations post CPB in small bowel by means of fluorescent intravital microscopy, which allows the simultaneous quantitative investigation of the microcirculation of the small intestine.

Applications

By understanding pathomechanisms of CPB in small bowel capillary perfusion, this study may help to identify effective preventive and therapeutic approaches.

Terminology

Functional capillary density (FCD) is one of the parameters obtained by intravital microscopy using epi-illumination of the tissue surface or trans-illumination of thin tissue layers. FCD, defined as the length of red cell-perfused capillaries per observation area (cm^{-1}), has been used as an indicator of the quality of tissue perfusion in various animal models. Quantitative analysis of FCD in randomly selected regions of the tissue is performed by means of a computer-assisted video analysis system which allows calculation of the length of RBC-perfused capillaries.

Peer review

In this paper, the authors used intravital microscopy for assessment of intestinal microcirculation in rats during and after CPB. The authors confirmed arteriolar vasoconstriction, blood velocity reduction and FCD diminution, exaggerated albumin extravasation and increased leukocyte accumulation in intestine in rats during and after CPB.

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Translation and validation of the Nepean Dyspepsia Index for functional dyspepsia in China

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Abstract

AIM: To assess the reliability and validity of the translated version of Nepean Dyspepsia Index (NDI) in Chinese patients with documented functional dyspepsia (FD).

METHODS: The translation process included forward translation, back translation, pretest and cross-cultural adaptation. Reliability and validity of the translated version were examined by asking 300 subjects to complete the Chinese version of the NDI. The mean age of subjects was 39.24 years and 68.7% of the subjects were women. Internal consistency analysis with Cronbach's α was performed to test the reliability. Correlation analysis was used to assess the content validity. Factor analysis and structural equation models were used to assess the construct validity.

RESULTS: The Cronbach's α coefficients ranged 0.833-0.960, well above the acceptable level of 0.70. Correlation analysis showed that each item had a strong correlation with the corresponding domain, but a weak correlation with other domains. Confirmatory factor analysis indicated that the comparative fit index was 0.94, higher than the acceptable level of 0.90.

CONCLUSION: The Chinese version of the NDI is a reliable and valid scale for measuring health-related quality of life and disease severity in Chinese patients with FD.

Key words: Nepean Dyspepsia Index; Functional dyspepsia; Health-related quality of life; Validation studies; Questionaires

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INTRODUCTION

Functional dyspepsia (FD) is a common clinical condition characterized by chronic or recurrent upper abdominal pain associated with a variety of gastrointestinal symptoms in the absence of organic disease[1]. An epidemiological survey in Western countries showed that 19%-41% of the population have symptoms of FD[2]. The report on the incidence of FD in citizens of Tianjin, China, revealed that the number of patients with FD accounts for 23.29% of the total population[3]. Since FD greatly affects the quality of life (QOL) of patients[4-6], and contributes to a higher medical cost[7,8], it represents an important healthcare problem in modern society. However, treatment of FD is still controversial and no single therapy is uniformly effective, partially due to lack of a reliable evaluation instrument[9]. Many difficulties exist in the single treatment approach to FD[10]. A combined approach, which includes herbal medicine, acupuncture and massage therapy, may be more effective than any single treatment[11].

In addition to symptom evaluation, assessment of QOL has been recognized as a major factor for overall

health outcome assessment in FD clinical studies. Nepean Dyspepsia Index (NDI) was designed to measure both symptom scores and impairment of the dyspepsia-specific health-related QOL (H-QOL). Since the development of NDI in 1998[12], it has been used as an outcome assessment in many FD clinical researches[13,14] and translated into several languages including French, Dutch, Italian, German, Spanish, and American English[15,16]. NDI has proven vital in the diagnosis of FD[12], and its utility has been validated[4,17]. A number of studies about the effect of FD on QOL in Western patients are available[7,18,19]. However, the effect of FD on the QOL in Asian patients has not received great attention.

Since no validated disease-specific questionnaire is currently available for assessing the impact of FD on the QOL, we translated the original version of NDI (25 items) into Chinese. We also assessed the reliability and validity of this translated version in Chinese patients with documented FD in order to provide researchers, clinicians, and patients with a suitable questionnaire to assess FD.

MATERIALS AND METHODS

Ethics

This study was approved by the Ethics Committee of Chengdu University of Traditional Chinese Medicine. All subjects provided their written consent before participation in this study.

Demographics

Four hundred and six Chinese outpatients with a suspected diagnosis of FD were screened between March 20, 2007 and June 1, 2007. One hundred and six patients were excluded and the symptom scores for the remaining 300 patients enrolled in our study were 2.970 ± 3.515 for burping/belching, 4.690 ± 3.770 for fullness after eating or slow digestion, 1.800 ± 2.886 for nausea, 2.367 ± 3.421 for inability to finish a regular meal, 1.540 ± 3.038 for bad breath, 3.050 ± 3.575 for bitter/sour tasting fluid coming up into mouth or throat, 0.670 ± 1.855 for vomiting, 5.790 ± 2.939 for discomfort in upper abdomen, 0.786 ± 2.185 for cramps in upper abdomen, 1.306 ± 2.803 for burning sensation in upper abdomen, 5.130 ± 3.567 for pain or ache in upper abdomen, 1.206 ± 2.601 for pressure in upper abdomen, 5.300 ± 3.516 for bloating in upper abdomen, 0.903 ± 2.380 for burning sensation in chest (heartburn), and 0.640 ± 2.045 for pain or ache in chest. The mean age of subjects was 39.24 years and 68.7% of the subjects were women.

The study was performed at Chengdu, Wuhan and Changsha medical centers, which comprise five hospitals in total. Before inclusion in the trial, all patients underwent physical examination, abdominal ultrasonography and upper gastrointestinal endoscopy to exclude organic gastrointestinal cause for their symptoms. Patients at the age of 18 years and older were considered eligible in the study. If their endoscopy was either normal or showed only chronic superficial gastritis, they were diagnosed with FD according to Rome-Ⅲ criteria[20] with no cholecystitis or cholelithiasis on their sonogram. Patients with a history of upper gastrointestinal surgery, or who were pregnant or lactating, were excluded. Mental deficiency, psychiatric, cardiovascular, hepatic, and renal diseases were the additional exclusion criteria. The investigation was carried out in a quiet environment. Specialists explained each item to the patients so that the patients could understand and complete the questionnaire correctly.

Chinese version of NDI

The original 25-item NDI[17] consists of three parts: a symptom checklist that measures the frequency, intensity and level of discomfort of 15 upper gastrointestinal symptoms over the prior 14 d, 25 items designed to assess QOL, and an 11-item questionnaire designed to measure the relevance or importance of the above items.

The QOL includes four domains, namely interference (13 items), know/control (seven items), eat/drink (three items), and sleep/disturb (two items). The scores were determined with a five-point Likert scale which ranges "not at all"-"extremely". The NDI for QOL score ranges 0-99, higher scores indicate poorer QOL. Symptoms were scored separately from questions representing the QOL. The higher the score of each individual symptom is, the severer the symptom is.

After permission, the original version of NDI was translated into Chinese according to the WHO-QOL methodology of cross-culture adaptation for QOL[21]. Forward translation from the original English version was performed independently by two gastroenterologists with good English. Both forward versions were then reconciled and incorporated into the Chinese version. Back translation was carried out by an English teacher understanding the Chinese language with no knowledge of NDI. The semifinal version was derived from reconciliation of the original, back and forward translations. A pretest, NDI for five patients with FD was performed. The final version was obtained after cross-cultural adaptation.

Statistical analysis

The reliability of NDI was evaluated for internal consistency using Cronbach's α. Correlation analyses were used to assess the content validity. Factor analysis and structural equation models were used to assess the construct validity.

All data were analyzed with SPSS 13.0 version. Data derived from descriptive statistical analysis were presented in the form of percentages including categorical variables and calculation of the mean. $P < 0.05$ was considered statistically significant.

RESULTS

Checklist of symptoms

The total score for each symptom on the checklist was calculated by adding its corresponding frequency, severity and level of discomfort. As stated above, higher scores were elicited for symptoms of discomfort, bloating, and

Table 1 Internal consistency of items in each domain

Factor	Domain	Cronbach's α
1	Interference	0.944
2	Know/control	0.906
3	Eat/drink	0.833
4	Sleep disturb	0.960

Table 2 Pearson item-dimension correlation coefficients

Item	Domains			
	Interference	Know/control	Eat/drink	Sleep disturb
01	0.626	0.531	0.461	0.354
02	0.701	0.817	0.450	0.376
03	0.666	0.812	0.492	0.396
04	0.522	0.493	0.906	0.457
05	0.457	0.476	0.840	0.454
06	0.589	0.484	0.852	0.389
07	0.473	0.476	0.472	0.982
08	0.511	0.536	0.509	0.980
09	0.822	0.640	0.521	0.464
10	0.828	0.638	0.520	0.399
11	0.826	0.677	0.455	0.437
12	0.818	0.635	0.446	0.403
13	0.815	0.568	0.482	0.342
14	0.800	0.575	0.432	0.397
15	0.794	0.564	0.471	0.289
16	0.771	0.715	0.467	0.442
17	0.753	0.832	0.424	0.430
18	0.742	0.843	0.505	0.528
19	0.746	0.774	0.482	0.374
20	0.824	0.827	0.554	0.458
21	0.751	0.624	0.477	0.370
22	0.557	0.771	0.368	0.347
23	0.506	0.710	0.362	0.321
24	0.709	0.814	0.527	0.487
25	0.631	0.577	0.327	0.294

pain or ache in upper abdomen, and fullness after eating or slow digestion.

Reliability

The scores were evaluated using Cronbach's α coefficient. An α score > 0.7 was considered internally consistent as previously described[22]. The Cronbach's α coefficient ranged 0.833-0.960 (Table 1).

Validity

The content validity of 25 items and four-field scores were regarded as 29 independent variables. Pearson item-dimension correlation coefficient was employed to evaluate the content validity. Most of the coefficients were higher than 0.6 ($P < 0.01$, Table 2).

Construct validity

The values of the four preceding factors were above 1.0, and their cumulative factor loading rate was 69.287%. The rotated component matrix showed that component 1 had more loadings on items 1, 9-16, 20, 21 and 25; component 2 had more loadings on items 2, 3, 17-20, 22-24; component 3 had more loadings on items 4-6; component 4 had more loadings on items 7 and 8 (Table 3). The confirmatory factor analysis indicated that

Table 3 Rotated component matrix

Item	Component			
	1	2	3	4
22	0.777	0.101	0.154	
23	0.735		0.145	
19	0.725	0.352	0.201	
17	0.715	0.417		0.223
18	0.685	0.367	0.178	0.312
20	0.684	0.448	0.261	0.184
24	0.640	0.332	0.266	0.257
03	0.634	0.312	0.336	
02	0.612	0.413	0.263	
16	0.598	0.445	0.156	0.225
25	0.536	0.374		
13	0.206	0.853	0.200	
14	0.216	0.835	0.117	0.164
15	0.225	0.828	0.204	
10	0.354	0.695	0.270	0.122
12	0.395	0.689	0.107	0.202
09	0.341	0.659	0.284	0.215
11	0.449	0.630	0.132	0.248
21	0.458	0.501	0.258	0.129
01	0.335	0.378	0.377	0.124
04	0.182	0.235	0.834	0.188
05	0.277		0.762	0.248
06	0.171	0.399	0.713	
07	0.197	0.180	0.225	0.908
08	0.255	0.192	0.256	0.878

degrees of freedom = 269, minimum fit function chi-square = 1703.32 ($P < 0.0001$), normal theory weighted least chi-square = 1809.13 ($P < 0.0001$), comparative fit index (CFI) = 0.94, non-normal fit index = 0.94. A structural equation model of construct validity is illustrated in Figure 1.

DISCUSSION

QOL has received increasing attention as more foci are placed on individual satisfaction as an important health outcome in clinical studies. In addition, QOL is particularly significant in diseases lacking of obvious biological or clinical markers, such as FD[23,24]. FD greatly affects the QOL of patients. However, treatment of FD is still controversial and no single therapy is uniformly effective, in part, due to the absence of a reliable evaluation instrument.

From our symptom checklist scores, symptoms with the highest scores were discomfort, bloating, and pain or ache in upper abdomen and fullness after eating or slow digestion, all of which are congruent with the main symptoms of FD according to Rome-Ⅲ criteria[25]. However, heartburn, a major symptom in the Rome-Ⅲ criteria, had a lower score in our study, possibly due to lack of patient comprehension or lack of adequate explanation by the investigator.

In clinical trials, Leeds dyspepsia questionnaire (LDQ) and MOS 36-item short-form health survey (SF-36) have been applied as an evaluation instrument for FD[26-28]. LDQ is a valid, reliable and responsive instrument for measuring the presence and severity of dyspepsia, but it lacks of QOL assessment[29]. As it is

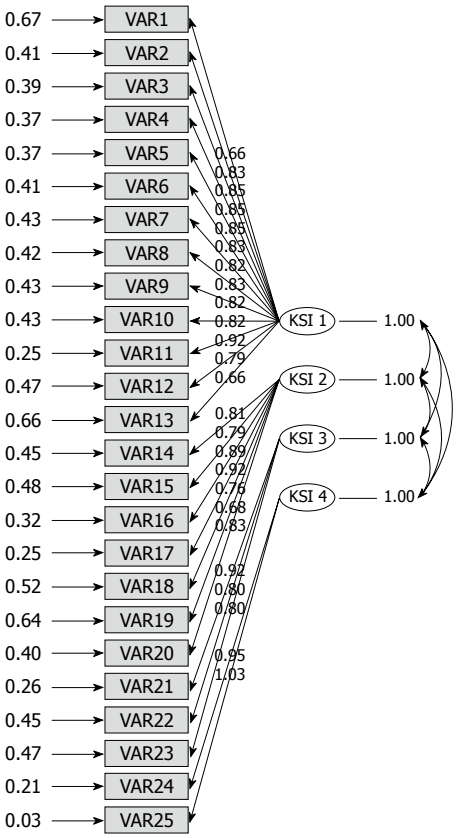

Figure 1 Structural equation model of construct validity.

known that, in addition to symptoms, QOL assessment represents a major part of health outcome assessment in clinical studies of FD. However, as different diseases cause different symptoms which necessitate disease-specific H-QOL instruments. SF-36, a generic QOL measurement, contains a large number of questions, the majority of which are often irrelevant to a particular disease. As a result, it may be insensitive to changes in the relevant items due to interference from the irrelevant items. Thus, the evaluation of FD should contain two aspects, namely symptom measure and disease-specific H-QOL assessment. The NDI addresses both aspects.

The original NDI consists of 42 questions, and is shortened to 25 items, excluding those items with a negative response rate of over 60%. The remaining 25 items represent the clinically relevant QOL concepts in subjects with FD[17]. Our study was based on the revised version of 25 items.

In order to maintain the sensitivity of the original version, our questionnaire considered differences in language, cultural diversities, and life style. After our version of the NDI was established, its reliability and validity were evaluated using Cronbach's α. We found that all domains were above 0.80, demonstrating that our scale has an excellent reliability. Correlation analysis demonstrated that each item had a strong correlation with the corresponding domain, but a weak correlation with other domains, supporting that our questionnaire is valid. In addition, our results are in accordance with the original NDI design, representing superior construct validity. Confirmatory factor analysis also indicated that the CFI was higher than the acceptable level of 0.90[25,30], demonstrating that our scale has a good construct validity.

In the past, FD research in China was limited by the absence of a valid assessment instrument for the disease. Our results suggest that the Chinese version of NDI with excellent psychometrics would promote the clinical study of FD and align its medical course in China with international practice in future.

Our study has some limitations, including lack of extensive demographic characteristics, analysis of multi-dimensional factors (such as education, career, financial and social status), "test-retest" in reliability assessment, and discriminating validity in validity assessment, all of which need to be further studied.

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COMMENTS

Background

Functional dyspepsia (FD) is a common non-organic disease in the world. However, treatment of FD is still controversial, partially due to lack of a reliable evaluation instrument. Since 1998, the Nepean Dyspepsia Index (NDI) has been designed to diagnose FD and proven to be valid in measuring both symptom scores and impairment of dyspepsia-specific health-related quality of life (H-QOL) in FD patients.

Research frontiers

In addition to symptom evaluation, assessment of QOL has been recognized to be more and more important in overall health outcome assessment in FD clinical studies. In this study, the reliability and validity of the Chinese NDI translated from it English version which reflects the dyspepsia-specific H-QOL in Chinese FD patients, were assessed.

Innovations and breakthroughs

Studies on validation of NDI for FD in Western countries are widely available. However, NDI has not been translated and validated in China, since much attention is not paid to the effect of FD on the QOL of patients. This is the first study to translate the original version of NDI (25 items) into Chinese and to assess its reliability and validity in Chinese FD patients.

Applications

By validating the translated version of NDI, this study may provide researchers, clinicians, and patients with a suitable questionnaire for the assessment of FD.

Terminology

Reliability is the extent to which the measurements of a test remain consistent over repeated tests of the same subject under identical conditions. Validity, containing construct, content and convergent validity, refers to the degree to which evidence and theory support the interpretations of test scores entailed by proposed tests. Assessment of the validity of a scale involves evaluation of the scale in relation to the desired conclusion on the basis of prevailing standards.

Peer review

The authors have made a detail description of how to use the Chinese version of NDI to diagnose FD patients in China. The study is clinically important, because a Chinese version of NDI is needed to diagnose and compare FD patients in China and other countries.

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BRIEF ARTICLES

Specific activation of 2'-5'oligoadenylate synthetase gene promoter by hepatitis C virus-core protein: A potential for developing hepatitis C virus targeting gene therapy

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Abstract

AIM: To examine whether 2'-5'oligoadenylate synthetase (*OAS*) gene promoter can be specifically activated by hepatitis C virus (HCV)-core protein.

METHODS: Human embryo hepatic cell line L02 was transfected with pcDNA3.1-core plasmid and selected by G418. Expression of HCV-core was detected by reverse transcription polymerase chain reaction and Western blotting. The *OAS* promoter sequence was amplified from the genomic DNA and inserted into pGL3-basic vector. The resultant pGL3-OAS-Luci plasmid was transiently transfected into L02/core cells and luciferase activity was assayed.

RESULTS: L02/core cell line stably expressing HCV-core protein was established. The pGL3-OAS-Luci construct exhibited significant transcriptional activity in the L02/core cells but not in the L02 cells.

CONCLUSION: HCV-core protein activates the *OAS* gene promoter specifically and effectively. Utilization of *OAS* gene promoter would be an ideal strategy for developing HCV-specific gene therapy.

Key words: Hepatitis C virus; Gene promoter; Gene therapy; Core; 2'-5'oligoadenylate synthetase

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Wang Y, Mao SS, He QQ, Zi Y, Wen JF, Feng DY. Specific activation of 2'-5'oligoadenylate synthetase gene promoter by hepatitis C virus-core protein: A potential for developing hepatitis C virus targeting gene therapy. *World J Gastroenterol* 2009; 15(25): 3178-3182 Available from: URL: http://www.wjgnet.com/1007-9327/15/3178.asp DOI: http://dx.doi.org/10.3748/wjg.15.3178

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide[1]. Currently, there is no vaccine to prevent the infection and no specific antiviral drug directed against the disease. Gene therapy has emerged as a novel approach to combat HCV infection in the last few years[2,3]. However, one of the most important obstacles to overcome is "targeting": the appropriate genes must be delivered to and expressed in HCV infected hepatocytes without harming normal tissues. This problem could be addressed by using a promoter that has a higher level of activity in HCV infected liver cells *vs* normal cells.

HCV is a member of the Flaviviridae family, containing approximately 9.5 kb of positive strand RNA. The viral genome encodes a large precursor polyprotein which is cleaved into functional proteins such as core, envelope (E1, E2) and non-structural proteins (NS2-NS5)[4,5]. The viral core protein consists of 191 amino acids and has an apparent molecular mass of 21 kDa. In addition to being the viral capsid protein, it also functions as a transcriptional regulator of various viral and cellular promoters. Some groups have reported that HCV-core protein activates the human

c-myc, Erk, IL-2, and SV40 early promoters, and trans-suppresses some cellular promoters such as p53 and p21[6-12]. Though the mechanisms of these regulations are still unclear, it is possible that these properties of HCV-core protein influence host cell growth, survival, and carcinogenesis. Therefore, utilization of a promoter that is predominantly active in HCV-core positive hepatocytes would be an ideal strategy for developing HCV-specific gene therapy.

During the investigation of interferon (IFN)-inducible JAK-STAT signaling affected by HCV, Naganuma *et al*[13] found that HCV-core protein specifically activated the 2'-5'oligoadenylate synthetase (*OAS*) gene promoter in human hepatocyte cells, while the E1, E2, and NS5A proteins did not activate the *OAS* gene promoter. These data indicate that *OAS* promoter might be used to restrict gene expression in HCV-core positive liver cells. However, the role of HCV-core protein in modulating *OAS* gene expression is much controversial, some other groups have reported that HCV-core protein does not affect the activation of IFN-responsive genes (including *OAS* gene), although it modulates the JAK/STAT signaling pathway[14-16]. So whether this viral core protein interferes with the *OAS* promoter and whether the promoter could be used to target gene therapy remain to be demonstrated. For these reasons, in the present study, we cloned the *OAS* promoter and examined its activity in the HCV-core expressing liver cells *in vitro*.

MATERIALS AND METHODS

Cell culture

Human embryo hepatocyte derived L02 cell line was obtained from Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences (Shanghai, China). The cells were routinely grown in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg /mL streptomycin.

Stable transfection and establishment of stable cell line

The pcDNA3.1-core, which contains the complete coding region of HCV-core protein (1b genotype) under the control of cytomegalovirus (CMV) immediate early promoter, was kindly provided by Professor Jun Cheng (Institute for Epidemic Disease Research, Beijing Ditan Hospital, China). The L02 cells were seeded in 6-well culture plates at 70% confluence 24 h before transfection. Cells were transfected with 2 μg of pcDNA3.1-core plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For establishment of stable cell line, the transfected cells were subjected to selection medium containing 400 mg/L G418 until several G418-resistant clones (L02/core) were obtained, and then the cells were maintained in G418 at a concentration of 200 mg/L. Non-transfected L02 cells were used as parallel control.

Reverse transcription polymerase chain reaction (RT-PCR) analysis for core-specific gene expression

Total RNA was isolated from L02/core cells with TRIzol reagent (Invitrogen) and reversely transcribed using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas). PCR was subsequently performed with HCV core-specific primers: 5'-ATGAGCACCAATCCTAAA C-3' (forward), and 5'-GGCTGAAGCGGGCACA-3' (reverse). The cycle parameters were as follows: 95℃ for 1 min, 52℃ for 50 s, and 72℃ for 1 min; 30 cycles. The PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide and visualized by UV illumination. The expected size of the PCR product for HCV-core mRNA was 572 base-pairs.

Western blotting analysis for core protein

The L02/core cells were collected, washed with PBS and lyzed in ice-cold lysis buffer (20 mmol/L Tris HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Nonidet P-40, 1 mg/L Aprotinin, 100 mg/L PMSF). The protein concentration of the whole cell extract was measured using Bio-Rad DC protein assay kit. Equal amounts of cell lysates (60 μg protein) were separated by 12% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked for more than 1 h in PBS containing 2% nonfat milk and 0.25% Tween-20 and then immunoblotted using anti-HCV core monoclonal antibody (1:500, Santa Cruz). Immunoreactive bands were detected using horseradish-peroxidase-conjugated secondary antibodies (1:1000) in conjunction with an enhanced chemiluminescent system.

Promoter cloning and luciferase assay

Template genomic DNA was extracted from human whole blood using DNAzol BD reagent (Invitrogen). The *OAS1* promoter sequence (-157 to +82) was amplified by PCR from the genomic DNA. The primers incorporating *Sac* I and *Hin*dⅢ restriction sites were: 5' -CCGAGCTCGGGATCAGGGGAGTGT-3' (forward) and 5'-CCCAAGCTTGCATGCGGAAACACG-3' (reverse). The PCR fragment was digested and cloned into the *Sac* I /*Hin*dⅢ sites of pGL3-basic vector (Promega) to generate pGL3-OAS-Luci. Negative and positive control constructs were pGL3-basic lacking any promoter sequence, and pGL3-promoter containing the SV40 promoter sequence.

These luciferase reporter plasmids were transiently transfected into L02/core and L02 cells using Lipofectamine 2000 (Invitrogen). The cells were harvested 48 h after the transfection. Luciferase assays were performed according to the manufacturer's protocols (Promega). Briefly, cells were lysed with reporter lysis buffer, and the luciferase activity was determined using a luminometer. A β-galactosidase expression plasmid (pSV-β-galactosidase; Promega) was co-transfected to allow normalization for transfection efficiency. All experiments were performed at least three times in each plasmid and represent the relative luciferase activity as average.

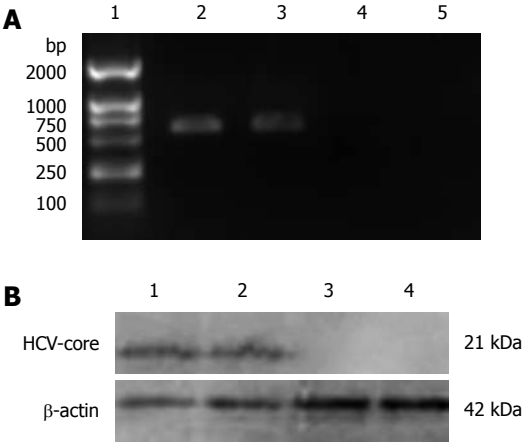

Figure 1 Expression of HCV-core in the stable cell line L02/core. A: Detection of HCV-core mRNA by RT-PCR. 572 bp visible fragments consistent with the predicted size of HCV-core mRNA were detected in L02/core cell clones. 1: DL2000 marker; 2, 3: L02 cells transfected with pcDNA3.1-core (L02/core); 4: L02 cells transfected with pcDNA3.1 (empty vector); 5: Untransfected L02 cells; B: Detection of HCV-core protein by Western blotting. 1, 2: L02/core cells; 3: L02 cells transfected with empty vector; 4: Untransfected L02 cells.

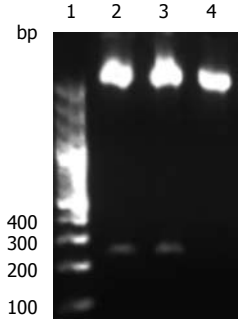

Figure 3 The OAS-luciferase construct (pGL3-OAS-Luci) was confirmed by double enzyme digestion with *Sac* I and *Hind* III. 1: GeneRuler™ 100 bp DNA Ladder; 2-3: Double digestion of pGL3-OAS-Luci; 4: Double digestion of pGL3-Basic.

Statistical analysis

All data are shown as the mean ± SD. Statistical analysis was performed using the *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

HCV-core expression in stably transfected L02 cells

In order to investigate the effect of HCV-core on the *OAS* promoter, L02 cells were transfected with pcDNA3.1-core plasmid and were selected on the basis of their resistance to G418 for 2 wk. Expression of HCV-core was detected by RT-PCR and Western blotting. As shown in Figure 1A, 572 bp visible fragments, consistent with the predicted size of HCV-core mRNA, were detected in L02/core cell clones. No PCR-amplification product was found in the pcDNA3.1 transfected L02 cells, neither in the nontransfected L02 cells. The expression of HCV-core protein was confirmed by immunoblotting using anti-HCV core monoclonal antibody. As shown in Figure 1B, HCV-core protein of the expected molecular mass of 21 kDa was observed in L02/core cells, whereas no expression was detected in pcDNA3.1 transfected or nontransfected L02 cells. These data demonstrate that hepatocyte line stably expressing HCV-core protein has been established.

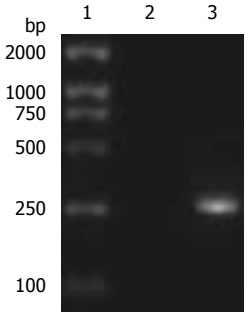

Figure 2 PCR product of *OAS* promoter. A 240 bp visible fragment consistent with the predicted size was observed by electrophoresis. 1: DL2000 marker; 2: Negative control; 3: PCR product.

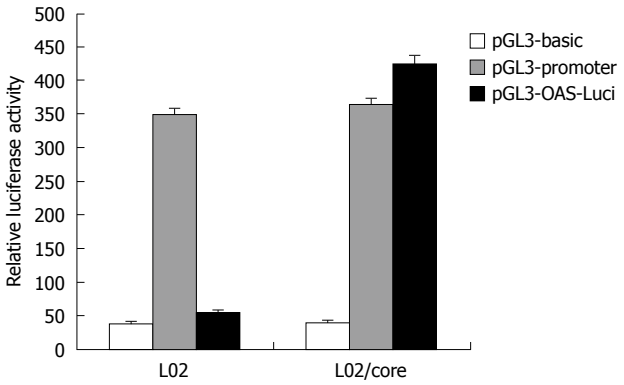

Figure 4 Transcriptional activity of the *OAS* promoter in L02 and L02/core cells. The *OAS* promoter showed significant transcriptional activity in the presence of HCV-core protein (L02/core), but not in the normal liver cells (L02). The data shown are the mean ± SD from three independent experiments.

Transcriptional activity of OAS promoter in HCV-core positive or negative cells

The *OAS* promoter sequence was amplified by PCR from human genomic DNA. As shown in Figure 2, a 240 bp visible fragment consistent with the predicted size was obtained by electrophoresis. Sequencing analysis confirmed that the cloned gene was identical with the original sequence in GenBank (accession number NW_925395). The *OAS* promoter was put upstream of the firefly luciferase gene in the pGL3-basic vector which lacks eukaryotic promoter and enhancer sequences. The resultant pGL3-OAS-Luci plasmid was confirmed by double enzyme digestion with *Sac* I and *Hind* III (Figure 3).

To examine the transcriptional activity of the *OAS* gene promoter, the pGL3-OAS-Luci was transiently transfected into L02/core and L02 cells, and the firefly luciferase activity obtained was compared with those from the pGL3-basic and pGL3-promoter plasmid. As shown in Figure 4, the pGL3-OAS-Luci construct exhibited significantly higher transcriptional activity in the presence of HCV-core protein (L02/core). The relative luciferase activity was almost 1.2 fold of pGL3-Promoter, and 10-15 fold of the baseline activity of the pGL3-basic vector. In contrast, this *OAS* promoter construct resulted in no or little luciferase activity in the normal liver cell line L02. These findings suggest that HCV-core protein strongly activates the *OAS* gene promoter.

DISCUSSION

To increase the specificity and safety of gene therapy, the expression of the therapeutic gene need to be tightly controlled within the target tissue. This is particularly important for toxic gene strategies because inappropriate transgene expression may lead to severe toxicity. Targeted expression of therapeutic genes can be accomplished at several levels. The first approach for targeting specificity is at the level of vector delivery[17]. The strategies include exploiting natural viral tropisms, and incorporating tissue-specific ligands or monoclonal antibodies onto the surface of viral vectors or liposomes. However, there will undoubtedly be some genes delivered to local and distant normal tissues. Therefore, further safeguards must be put in place to ensure that gene delivery to these tissues does not result in significant expression and toxicity. One attractive approach to this problem is to use promoter elements to control gene expression tightly at the transcriptional level.

Many tissue and tumor specific promoters have been developed in target gene therapy. For example, the α-fetoprotein promoter has been used to drive gene expression in hepatic carcinoma cells, the tyrosinase promoter in melanoma cells, the prostate specific antigen promoter in prostate cancer cells, and the carcinoembryonic antigen promoter in adenocarcinomas[18]. The results of these studies have demonstrated the feasibility of using specific promoters for targeting gene therapy in various cancer cell types.

Although several promoters have been identified more active in HCV-core positive hepatocytes, most of these promoters are much weaker than commonly used viral promoters such as the CMV early promoter, the Rous sarcoma virus long terminal repeat, and the SV40 early promoter. Consequently, their applications in gene therapy are hampered by the low expression. In the present study, we cloned the human *OAS* promoter and examined its transcriptional ability in the HCV-core positive hepatocytes and normal liver cells. We found that the luciferase expression driven by *OAS* promoter was markedly increased in the presence of core protein, but not in the normal liver cells. These data strongly suggest that HCV-core protein can activate *OAS* promoter. Since HCV-core protein plays an important role in persistent infection and hepatocellular carcinogenesis, and amino acid sequence of core protein is relatively conserved, utilization of the OAS promoter to drive therapeutic gene expression would be an ideal strategy for developing HCV-specific gene therapy.

COMMENTS

Background

The current standard therapy for chronic hepatitis C is inadequate for the majority of patients. Gene therapy has emerged as a novel approach to combat hepatitis C virus (HCV) infection in the last few years. However, a specific promoter is required to restrict transgene expression only in HCV infected cells. Since HCV-core protein plays an important role in persistent infection and hepatocellular carcinogenesis, and amino acid sequence of core protein is relatively conserved, utilization of a promoter that is predominantly active in HCV-core positive hepatocytes would be an ideal strategy for HCV targeting gene therapy.

Research frontiers

To increase the specificity and safety of gene therapy, the expression of the therapeutic gene need to be tightly controlled within the target tissue. This is particularly important for toxic gene strategies because inappropriate transgene expression may lead to severe toxicity. Targeted expression of therapeutic genes can be accomplished at several levels, including vector targeting and tissue-specific gene expression. However, no HCV-specific gene delivery system has yet been developed. One attractive approach to this problem is to use promoter elements to control gene expression tightly at the transcriptional level.

Innovations and breakthroughs

In the present study, the authors cloned the 2'-5'oligoadenylate synthetase (OAS) promoter and examined its activity in the human embryo hepatocytes expressing HCV-core *in vitro*. They demonstrated that HCV-core protein can activate *OAS* gene promoter specifically and effectively.

Applications

Utilization of *OAS* gene promoter to drive therapeutic gene expression would be an ideal strategy for developing HCV-specific gene therapy.

Terminology

L02/core is the human embryo hepatocyte that stably expresses HCV-core protein. OAS is a metabolic enzyme originally identified as a regulator of the ribonuclease L (RNase L) pathway during viral infection.

Peer review

This is a well written and interesting paper.

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Improved quality of life in patients with gastric cancer after esophagogastrostomy reconstruction

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Abstract

AIM: To compare postoperative quality of life (QOL) in patients with gastric cancer treated by esophagogastrostomy reconstruction after proximal gastrectomy.

METHODS: QOL assessments that included functional outcomes (a 24-item survey about treatment-specific symptoms) and health perception (Spitzer QOL Index) were performed in 149 patients with gastric cancer in the upper third of the stomach, who had received proximal gastrectomy with additional esophagogastrostomy.

RESULTS: Fifty-four patients underwent reconstruction by esophagogastric anterior wall end-to-side anastomosis combined with pyloroplasty (EA group); 45 patients had reconstruction by esophagogastric posterior wall end-to-side anastomosis (EP group); and 50 patients had reconstruction by esophagogastric end-to-end anastomosis (EE group). The EA group showed the best postoperative QOL, such as recovery of body weight, less discomfort after meals, and less heart burn or belching at 6 and 24 mo postoperatively. However, the survival rates, surgical results and Spitzer QOL index were similar among the three groups.

CONCLUSION: Postoperative QOL was better in the EA than EP or EE group. To improve QOL after proximal gastrectomy for upper third gastric cancer, the EA procedure using a stapler is safe and feasible for esophagogastrostomy.

Key words: Gastric cancer; Proximal gastrectomy; Esophagogastrostomy; Quality of life

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INTRODUCTION

Although the incidence of gastric carcinoma has been decreasing continuously during the past decade, gastric cancer remains the second most common cause of cancer-related deaths worldwide. Given equivalent results with regards to survival, the impact of anastomotic methods on quality of life (QOL) becomes even more important. It has been reported that QOL is the main outcome for judging the efficacy of treatment modalities when no overall survival differences are demonstrated[1]. There is still no consensus on how to choose a reconstruction method for proximal gastrectomy in patients with upper third gastric cancer[2]. This study was designed to compare in detail different types of esophagogastrostomy.

Proximal gastrectomy impacts severely on physical and mental health, and has highly negative consequences for QOL at 6 and 24 mo. Although postoperative QOL has been shown to be important in the surgical literature[3], there have only been a few studies on QOL in patients after proximal gastrectomy. For patients undergoing oncological surgery, QOL is generally accepted as an important outcome parameter, in addition to long-term survival, mortality, and complication rates. In order to provide patients with improved postoperative

QOL, many surgeons seek the optimal surgical methods. Compared with total gastrectomy, the advantages of proximal gastrectomy were shorter operation times, less blood loss and convenience of procedure[4]. No previous studies have assessed different types of esophagogastrostomy in terms of QOL.

The aim of this study was to assess outcome in terms of QOL in patients after proximal gastrectomy, by comparing three types of esophagogastrostomy, in order to find the optimal reconstruction method that offers the optimal postoperative QOL at 6 and 24 mo.

MATERIALS AND METHODS

Patients

Between January 2002 and December 2005, 195 patients with proximal gastric cancer were treated at the Department of Surgical Oncology, First Affiliated Hospital of China Medical University, Shenyang, China. Only 149 patients who were in the present prospective randomized study met the following study criteria: (1) underwent curative resection with lymph node dissection; (2) had no history of other organ malignancies; and (3) had more than 15 lymph nodes retrieved and confirmed by a specialist pathologist. The exclusion criteria included: (1) age > 80 years; (2) renal, pulmonary, or heart failure; (3) undergoing palliative surgery; (4) tumor recurrence during the survey; (5) preoperative or postoperative adjuvant chemotherapy or radiotherapy; and (6) albumin level < 3.5 g/dL and a lymphocyte count lower than 1000 lymphocytes/mm^3 in peripheral blood.

Surgical procedure

The standard surgical procedure and extent of lymph node dissection were defined according to the recommendations of the Japanese Research Society for the Study of Gastric Cancer[5]. All patients were treated with stapler suture for digestive tract reconstruction after malignancy removal during the primary surgical procedure. Esophagogastrostomy was performed by using a mechanical stapler (Ethicon Endo-Surgery, USA). In addition, a row of external seromuscular sutures with interrupted absorbable stitches was also performed.

Esophagogastrostomy procedure and randomization

All patients underwent esophagogastrostomy with curative intent and were randomized using a table of random numbers into three groups. To perform esophagogastric anterior wall end-to-side anastomosis combined with pyloroplasty (EA procedure), the anastomosis should be 2-3 cm under the gastric incision line, to guarantee the blood supply of the anastomosis. The stapler inserted into the remnant stomach through the gastric antrum and the center rod of the circular stapler was pierced through the center of the anterior wall, and during this stapling, an ischemic area is not created at all. The esophagus was then anastomosed to the anterior wall in the center of the remnant stomach. A 3-4 mm wide serosal surface of the anterior wall of the remnant stomach strapped the esophagus circularly. After completion of esophagogastrostomy, the left end of the gastric stump was fixed to the diaphragm. Furthermore, the pyloroplasty was done in the standard manner with interrupted sutures[6-8]. To perform esophagogastric posterior wall end-to-side anastomosis (EP procedure), the stapler that was inserted into the remnant stomach through the anterior wall, and the center rod of the circular stapler was pierced through the center of the posterior wall. To perform esophagogastric end-to-end anastomosis (EE procedure), the center rod of the circular stapler was pierced through the left end of the staple line of the stomach.

Evaluation of QOL and follow-up study

Functional outcome was assessed using a 24-item survey designed to assess treatment-specific symptoms, largely gastrointestinal function[9]. The questions were scaled according to the Eastern Cooperative Oncology Group[10]. The Spitzer QOL index, which reflects the patient's postoperative health perception[11], is a global health assessment with a valid questionnaire that includes five items rated on a three-point scale: activity, daily living, health, support of family and friends, and outlook. The answers were analyzed in a quantitative fashion using a scoring system: the scores ranged from 0 (unsatisfactory result with severe symptoms) to 2 (excellent result with no symptoms); low scores reflected more symptoms. Questionnaires were administered at 6 and 24 mo postoperatively and annually thereafter until tumor recurrence. Average scores were calculated for each question. The Ethics Committee approved the study protocol and informed consent was obtained from each patient. Patients were closely followed after surgery until December 2007. The median follow-up duration was 42.5 mo (range, 9-67 mo). At the time of the last follow-up, 115 patients (77.2%) were alive, no patient was lost to follow-up, and 34 (22.8%) had died from recurrence or other causes.

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Science software for Windows version 13.0 (SPSS, Chicago, IL, USA). Data were expressed as mean ± SD. Categorical data were compared using the Kruskal-Wallis test, Mann-Whitney *U* test, analysis of variance and χ^2 test. $P < 0.05$ was considered as statistically significant. For calculation of survival rate, the Kaplan-Meier method was used, and compared using the log-rank test.

RESULTS

Patient characteristics

Table 1 summarizes clinicopathological factors in relation to the three groups. There were 95 (65%) male and 54 (35%) female patients, with a mean age of 61.5 ± 6.5 years. There were no significant differences in the

Table 1 Clinicopathological factors of the three groups

Clinicopathological factors	EA group (54)	EP group (45)	EE group (50)	*P*
Age (yr)	62.5 ± 5.4	56.6 ± 7.6	65.1 ± 8.6	0.668
Gender				0.775
Male	36	29	30	
Female	18	16	20	
Tumor size (cm)	4.6 ± 1.6	5.0 ± 1.7	4.8 ± 1.3	0.963
Histological type				0.993
Well-differentiated	18	15	17	
Moderately differentiated	20	17	19	
Poorly differentiated	10	9	8	
Signet ring cell type	6	4	6	
Lauren's classification				0.978
Intestinal	30	24	27	
Diffuse	18	16	17	
Mixed	6	5	6	
Lymph node status				0.989
Negative	38	34	36	
Positive	16	11	14	
Lymphovascular invasion				0.989
Negative	45	38	42	
Positive	9	7	8	
Stage				0.788
Ⅰ	17	16	18	
Ⅱ	21	17	20	
Ⅲa	11	7	9	
Ⅲb	5	5	3	

clinicopathological features such as sex, age, tumor size, histological type, Lauren's classification, lymph node status, or lymphovascular invasion among the three groups.

Overall survival rate and surgical results

The survival time was defined as the time from diagnosis until last contact, date of death, or the date used as a cutoff for the follow-up database, in which case the survival information was censored. The 2-year survival rate was 79.6%, 73.3% and 78.0% for the EA, EP and the EE group, respectively. The survival rates were similar in all treatment groups ($P = 0.713$, Figure 1). The surgical results did not differ among the three groups (Table 2).

Six months postoperative QOL evaluation

The evaluation scores for eating time were 1.83, 1.58 and 1.42 in EA, EP and the EE groups, respectively. The EA group had a significantly shorter eating time than the EP group (1.83 *vs* 1.58, $P = 0.005$) and the EE group (1.83 *vs* 1.42, $P = 0.000$).

The evaluation scores for dietary volume were 1.20, 0.60 and 0.58 in the EA, EP and the EE groups, respectively. The EA group had a significantly better dietary volume than the EP group (1.20 *vs* 0.60, $P = 0.000$) and the EE group (1.20 *vs* 0.58, $P = 0.000$).

The evaluation scores for heartburn or belching were 1.37, 0.73 and 0.80 in the EA, EP and the EE groups, respectively. The EA group had significantly less heartburn or belching than the EP group (1.37 *vs* 0.73, $P = 0.000$) and the EE group (1.37 *vs* 0.80, $P = 0.000$) (Table 3).

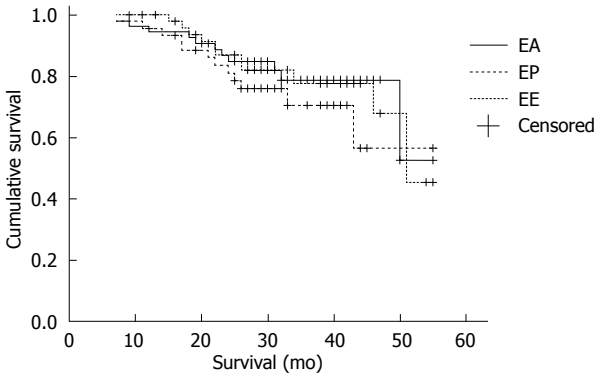

Figure 1 Kaplan-Meier survival curves for EA, EP and EE patients ($P = 0.713$) (Censored indicate patients who were still alive at follow up).

Most parameters tended to be normalized sooner after surgery in the EA group.

Twenty-four months postoperative QOL evaluation

The evaluation scores for body weight were 1.48, 1.13 and 1.14 in the EA, EP and the EE groups, respectively. The EA group had significantly better body weight recovery than the EP group (1.48 *vs* 1.13, $P = 0.005$) and the EE group (1.48 *vs* 1.14, $P = 0.030$).

The evaluation scores for heartburn or belching were 1.78, 1.73 and 1.38 in the EA, EP and the EE groups, respectively. The EA group (1.78 *vs* 1.38, $P = 0.005$) and the EP group (1.73 *vs* 1.38, $P = 0.023$) had significantly less heartburn or belching than the EE group.

The evaluation scores for postprandial discomfort were 1.69, 1.07 and 1.22 in the EA, EP and the EE groups, respectively. The EA group showed less postprandial discomfort than the EP group (1.69 *vs* 1.07, $P = 0.000$) and the EE group (1.69 *vs* 1.22, $P = 0.001$) (Table 4).

Comparison of evaluation scores of postoperative QOL between 6 and 24 mo in the same group

When the QOL scores between 6 and 24 mo were compared in the same group, the frequency of eating, food consistency, food volume, body weight, appetite, and heartburn or belching at 24 mo were all improved significantly in each group compared with at 6 mo. The eating time at 24 mo was significantly shorter than at 6 mo in the EP and EE group (EP, $P = 0.031$ and EE, $P = 0.000$). The postprandial discomfort at 6 mo was significantly less than at 24 mo in the EE group ($P = 0.048$). Wound pain at 24 mo was significantly less than at 6 mo in the EA group ($P = 0.031$).

It can be seen that, as postoperative time progresses, most symptoms improved, especially in terms eating frequency, food consistency, food volume, body weight, appetite, and heartburn or belching, and the total scores at 24 mo were all improved significantly compared with at 6 mo in the EA ($P = 0.015$), EP ($P = 0.007$) and EE ($P = 0.011$) groups (Table 5).

Spitzer index evaluation

The data from the Spitzer index (Tables 6 and 7) showed

Table 2 Surgical results comparison among the three groups

Surgical factors	EA group	EP group	*P*	EA group	EE group	*P*	EP group	EE group	*P*
Additional organ resection			0.609			0.831			0.766
Splenectomy	8	7	0.919	8	7	0.906	7	7	0.832
Splenopancreatectomy	1	2	0.456	1	2	0.515	2	2	0.915
Transverse mesocolectomy	2	2	0.853	2	3	0.586	2	3	0.736
Wedge liver resection	1	1	0.897	1	2	0.515	1	2	0.623
Margin status			0.670			0.938			0.623
Negative	52	44		52	48		44	48	
Positive	2	1		2	2		1	2	
Lymph node dissection			0.902			0.804			0.721
D1	15	12		15	14		12	14	
⩾ D2	39	33		39	36		33	36	
No. of lymph nodes	29.6 ± 7.6	28.5 ± 6.8	0.524	29.6 ± 7.6	30.1 ± 7.2	0.857	28.5 ± 6.8	30.1 ± 7.2	0.657
Operating time (min)	166.3 ± 47.5	156.8 ± 53.1	0.758	166.3 ± 47.5	149.7 ± 61.2	0.564	156.8 ± 53.1	149.7 ± 61.2	0.265
Blood loss (mL)	263.4 ± 112.6	267.3 ± 121.6	0.273	263.4 ± 112.6	276.9 ± 135.5	0.647	267.3 ± 121.6	276.9 ± 135.5	0.798
Postoperative stay (d)	18.0 ± 5.7	20.9 ± 7.8	0.874	18.0 ± 5.7	19.0 ± 5.7	0.953	20.9 ± 7.8	19.0 ± 5.7	0.849
Postoperative complications	12	11	0.795	12	13	0.654	11	13	0.862
Mortality	0	1	0.273	0	1	0.299	1	1	0.940

Table 3 Comparison of evaluation scores for 6 mo postoperative QOL among the three groups (mean ± SD)

Question	EA group	EP group	*P*	EA group	EE group	*P*	EP group	EE group	*P*
Frequency of eating	0.76 ± 0.799	0.80 ± 0.968	0.957	0.76 ± 0.799	0.78 ± 0.616	0.664	0.80 ± 0.968	0.78 ± 0.616	0.698
Eating time	1.83 ± 0.376	1.58 ± 0.499	0.005[a]	1.83 ± 0.376	1.42 ± 0.642	0.000[a]	1.58 ± 0.499	1.42 ± 0.642	0.287
Consistency of food	0.76 ± 0.845	0.71 ± 0.815	0.803	0.76 ± 0.845	0.84 ± 0.792	0.543	0.71 ± 0.815	0.84 ± 0.792	0.391
Volume of food	1.20 ± 0.810	0.60 ± 0.780	0.000[a]	1.20 ± 0.810	0.58 ± 0.702	0.000[a]	0.60 ± 0.780	0.58 ± 0.702	0.950
Body weight	0.80 ± 0.711	0.78 ± 0.636	0.969	0.80 ± 0.711	0.66 ± 0.848	0.221	0.78 ± 0.636	0.66 ± 0.848	0.225
Appetite	1.13 ± 0.674	0.87 ± 0.968	0.120	1.13 ± 0.674	1.16 ± 0.817	0.724	0.87 ± 0.968	1.16 ± 0.817	0.118
Difficulty swallowing	1.56 ± 0.664	1.42 ± 0.723	0.333	1.56 ± 0.664	1.54 ± 0.734	0.898	1.42 ± 0.723	1.54 ± 0.734	0.308
Diarrhea	1.83 ± 0.376	1.69 ± 0.733	0.843	1.83 ± 0.376	1.78 ± 0.582	0.852	1.69 ± 0.733	1.78 ± 0.582	0.734
Heartburn or belch	1.37 ± 0.708	0.73 ± 0.863	0.000[a]	1.37 ± 0.708	0.80 ± 0.756	0.000[a]	0.73 ± 0.863	0.80 ± 0.756	0.547
Postprandial discomfort	1.46 ± 0.693	1.24 ± 0.957	0.433	1.46 ± 0.693	1.52 ± 0.677	0.646	1.24 ± 0.957	1.52 ± 0.677	0.267
Abdominal pain	1.54 ± 0.770	1.49 ± 0.787	0.721	1.54 ± 0.770	1.60 ± 0.495	0.698	1.49 ± 0.787	1.60 ± 0.495	0.965
Vomiting	1.48 ± 0.771	1.49 ± 0.757	0.993	1.48 ± 0.771	1.68 ± 0.471	0.368	1.49 ± 0.757	1.68 ± 0.471	0.389
General fatigue	1.80 ± 0.528	1.82 ± 0.387	0.792	1.80 ± 0.528	1.92 ± 0.274	0.250	1.82 ± 0.387	1.92 ± 0.274	0.154
Dizziness	1.80 ± 0.562	1.89 ± 0.318	0.687	1.80 ± 0.562	1.74 ± 0.600	0.507	1.89 ± 0.318	1.74 ± 0.600	0.289
Intestinal obstruction	1.65 ± 0.555	1.56 ± 0.693	0.669	1.65 ± 0.555	1.66 ± 0.519	0.997	1.56 ± 0.693	1.66 ± 0.519	0.667
Performance status	1.54 ± 0.719	1.64 ± 0.743	0.240	1.54 ± 0.719	1.58 ± 0.642	0.910	1.64 ± 0.743	1.58 ± 0.642	0.269
Early dumping syndrome	1.54 ± 0.665	1.40 ± 0.720	0.319	1.54 ± 0.665	1.42 ± 0.673	0.307	1.40 ± 0.720	1.42 ± 0.673	0.970
Late dumping syndrome	1.78 ± 0.502	1.73 ± 0.688	0.669	1.78 ± 0.502	1.86 ± 0.452	0.241	1.73 ± 0.688	1.86 ± 0.452	0.532
Physical condition	1.67 ± 0.673	1.80 ± 0.548	0.258	1.67 ± 0.673	1.76 ± 0.517	0.661	1.80 ± 0.548	1.76 ± 0.517	0.450
Wound pain, present	1.57 ± 0.690	1.53 ± 0.815	0.874	1.57 ± 0.690	1.62 ± 0.490	0.809	1.53 ± 0.815	1.62 ± 0.490	0.704
Wound pain, postoperative	1.07 ± 0.866	1.18 ± 0.716	0.600	1.07 ± 0.866	1.22 ± 0.764	0.413	1.18 ± 0.716	1.22 ± 0.764	0.727
Satisfaction with operation	1.57 ± 0.690	1.69 ± 0.557	0.495	1.57 ± 0.690	1.66 ± 0.688	0.357	1.69 ± 0.557	1.66 ± 0.688	0.784
Recommendation to others	1.41 ± 0.714	1.29 ± 0.787	0.484	1.41 ± 0.714	1.46 ± 0.706	0.680	1.29 ± 0.787	1.46 ± 0.706	0.290
Mood or feeling	1.67 ± 0.673	1.67 ± 0.707	0.859	1.67 ± 0.673	1.70 ± 0.463	0.610	1.67 ± 0.707	1.70 ± 0.463	0.507
Total	1.45 ± 0.748	1.36 ± 0.825	0.059	1.45 ± 0.748	1.42 ± 0.743	0.147	1.36 ± 0.825	1.42 ± 0.743	0.389

[a]$P < 0.05$.

that after all the postoperative and oncological problems were solved, proximal gastrectomy was quite compatible with normal life. From the results of Spitzer index evaluation, we also concluded that patients after proximal gastrectomy may have a nearly normal life with activity and daily living at 6 and 24 mo.

DISCUSSION

The choice of reconstruction method after proximal gastrectomy remains a controversial issue. Previously, several studies have found no difference in the 5-year survival among patients with proximal gastrectomy and those with total gastrectomy[6,7]. Proximal gastrectomy for early gastric cancer in the upper third of the stomach is an appropriate operation in terms of radical treatment and safety. Proximal gastrectomy was introduced to improve performance status of patients and minimize late postoperative complications such as reflux esophagitis[8,12-15].

It is well known that moderate malnutrition is often associated with gastric cancer surgery[16], and that esophagogastrostomy reconstruction is more favorable to the natural food passage of the duodenum

Table 4 Comparison of evaluation scores of 24 mo postoperative QOL among the three groups (mean ± SD)

Question	EA group	EP group	*P*	EA group	EE group	*P*	EP group	EE group	*P*
Frequency of eating	1.67 ± 0.583	1.62 ± 0.614	0.711	1.67 ± 0.583	1.70 ± 0.580	0.693	1.62 ± 0.614	1.70 ± 0.580	0.461
Eating time	1.85 ± 0.359	1.78 ± 0.471	0.472	1.85 ± 0.359	1.88 ± 0.328	0.676	1.78 ± 0.471	1.88 ± 0.328	0.273
Consistency of food	1.65 ± 0.619	1.73 ± 0.618	0.306	1.65 ± 0.619	1.80 ± 0.535	0.105	1.73 ± 0.618	1.80 ± 0.535	0.601
Volume of food	1.69 ± 0.639	1.62 ± 0.684	0.613	1.69 ± 0.639	1.70 ± 0.580	0.915	1.62 ± 0.684	1.70 ± 0.580	0.680
Body weight	1.48 ± 0.637	1.13 ± 0.625	0.005[a]	1.48 ± 0.637	1.14 ± 0.047	0.030[a]	1.13 ± 0.625	1.14 ± 0.047	0.837
Appetite	1.57 ± 0.662	1.78 ± 0.560	0.056	1.57 ± 0.662	1.70 ± 0.580	0.290	1.78 ± 0.560	1.70 ± 0.580	0.349
Difficulty swallowing	1.52 ± 0.720	1.49 ± 0.787	0.116	1.52 ± 0.720	1.74 ± 0.600	0.059	1.49 ± 0.787	1.74 ± 0.600	0.136
Diarrhea	1.87 ± 0.339	1.80 ± 0.505	0.654	1.87 ± 0.339	1.82 ± 0.523	0.977	1.80 ± 0.505	1.82 ± 0.523	0.658
Heartburn or belch	1.78 ± 0.502	1.73 ± 0.539	0.651	1.78 ± 0.502	1.38 ± 0.805	0.005[a]	1.73 ± 0.539	1.38 ± 0.805	0.023[a]
Postprandial discomfort	1.69 ± 0.609	1.07 ± 0.915	0.000[a]	1.69 ± 0.609	1.22 ± 0.790	0.001[a]	1.07 ± 0.915	1.22 ± 0.790	0.445
Abdominal pain	1.65 ± 0.649	1.60 ± 0.720	0.848	1.65 ± 0.649	1.70 ± 0.505	0.968	1.60 ± 0.720	1.70 ± 0.505	0.841
Vomiting	1.69 ± 0.577	1.67 ± 0.674	0.814	1.69 ± 0.577	1.70 ± 0.463	0.786	1.67 ± 0.674	1.70 ± 0.463	0.628
General fatigue	1.76 ± 0.581	1.78 ± 0.420	0.626	1.76 ± 0.581	1.84 ± 0.370	0.800	1.78 ± 0.420	1.84 ± 0.370	0.442
Dizziness	1.83 ± 0.505	1.93 ± 0.252	0.410	1.83 ± 0.505	1.80 ± 0.535	0.671	1.93 ± 0.252	1.80 ± 0.535	0.223
Intestinal obstruction	1.78 ± 0.502	1.64 ± 0.679	0.396	1.78 ± 0.502	1.68 ± 0.513	0.204	1.64 ± 0.679	1.68 ± 0.513	0.771
Performance status	1.63 ± 0.653	1.71 ± 0.661	0.322	1.63 ± 0.653	1.70 ± 0.463	0.951	1.71 ± 0.661	1.70 ± 0.463	0.318
Early dumping syndrome	1.70 ± 0.603	1.62 ± 0.650	0.465	1.70 ± 0.603	1.60 ± 0.728	0.560	1.62 ± 0.650	1.60 ± 0.728	0.901
Late dumping syndrome	1.70 ± 0.537	1.73 ± 0.580	0.567	1.70 ± 0.537	1.72 ± 0.479	0.966	1.73 ± 0.580	1.72 ± 0.479	0.594
Physical condition	1.72 ± 0.627	1.64 ± 0.712	0.616	1.72 ± 0.627	1.76 ± 0.476	0.820	1.64 ± 0.712	1.76 ± 0.476	0.753
Wound pain, present	1.83 ± 0.423	1.73 ± 0.580	0.450	1.83 ± 0.423	1.78 ± 0.465	0.494	1.73 ± 0.580	1.78 ± 0.465	0.910
Wound pain, postoperative	1.07 ± 0.866	1.18 ± 0.716	0.600	1.07 ± 0.866	1.22 ± 0.764	0.413	1.18 ± 0.716	1.22 ± 0.764	0.727
Satisfaction with operation	1.72 ± 0.596	1.76 ± 0.484	0.937	1.72 ± 0.596	1.72 ± 0536	0.746	1.76 ± 0.484	1.72 ± 0.536	0.807
Recommendation to others	1.59 ± 0.567	1.42 ± 0.690	0.239	1.59 ± 0.567	1.54 ± 0.646	0.795	1.42 ± 0.690	1.54 ± 0.646	0.382
Mood or feeling	1.63 ± 0.708	1.71 ± 0.695	0.371	1.63 ± 0.708	1.66 ± 0.519	0.634	1.71 ± 0.695	1.66 ± 0.519	0.162
Total	1.67 ± 0.612	1.61 ± 0.663	0.116	1.67 ± 0.612	1.65 ± 0.609	0.150	1.61 ± 0.663	1.65 ± 0.609	0.532

[a]$P < 0.05$.

Table 5 Comparison of evaluation scores of QOL at 6 and 24 mo postoperatively between the same group (mean ± SD)

Question	EA group		*P*	EP group		*P*	EE group		*P*
	6 mo	24 mo		6 mo	24 mo		6 mo	24 mo	
Frequency of eating	0.76 ± 0.799	1.67 ± 0.583	0.000[a]	0.80 ± 0.968	1.62 ± 0.614	0.000[a]	0.78 ± 0.616	1.70 ± 0.580	0.000[a]
Eating time	1.83 ± 0.376	1.85 ± 0.359	0.793	1.58 ± 0.499	1.78 ± 0.471	0.031[a]	1.42 ± 0.642	1.88 ± 0.328	0.000[a]
Consistency of food	0.76 ± 0.845	1.65 ± 0.619	0.000[a]	0.71 ± 0.815	1.73 ± 0.618	0.000[a]	0.84 ± 0.792	1.80 ± 0.535	0.000[a]
Volume of food	1.20 ± 0.810	1.69 ± 0.639	0.001[a]	0.60 ± 0.780	1.62 ± 0.684	0.000[a]	0.58 ± 0.702	1.70 ± 0.580	0.000[a]
Body weight	0.80 ± 0.711	1.48 ± 0.637	0.000[a]	0.78 ± 0.636	1.13 ± 0.625	0.010[a]	0.66 ± 0.848	1.14 ± 0.047	0.004[a]
Appetite	1.13 ± 0.674	1.57 ± 0.662	0.000[a]	0.87 ± 0.968	1.78 ± 0.560	0.000[a]	1.16 ± 0.817	1.70 ± 0.580	0.000[a]
Difficulty swallowing	1.56 ± 0.664	1.52 ± 0.720	0.890	1.42 ± 0.723	1.49 ± 0.787	0.431	1.54 ± 0.734	1.74 ± 0.600	0.112
Diarrhea	1.83 ± 0.376	1.87 ± 0.339	0.590	1.69 ± 0.733	1.80 ± 0.505	0.823	1.78 ± 0.582	1.82 ± 0.523	0.754
Heartburn or belch	1.37 ± 0.708	1.78 ± 0.502	0.001[a]	0.73 ± 0.863	1.73 ± 0.539	0.000[a]	0.80 ± 0.756	1.38 ± 0.805	0.000[a]
Postprandial discomfort	1.46 ± 0.693	1.69 ± 0.609	0.052	1.24 ± 0.957	1.07 ± 0.915	0.321	1.52 ± 0.677	1.22 ± 0.790	0.048[a]
Abdominal pain	1.54 ± 0.770	1.65 ± 0.649	0.547	1.49 ± 0.787	1.60 ± 0.720	0.481	1.60 ± 0.495	1.70 ± 0.505	0.241
Vomiting	1.48 ± 0.771	1.69 ± 0.577	0.199	1.49 ± 0.757	1.67 ± 0.674	0.188	1.68 ± 0.471	1.70 ± 0.463	0.830
General fatigue	1.80 ± 0.528	1.76 ± 0.581	0.775	1.82 ± 0.387	1.78 ± 0.420	0.600	1.92 ± 0.274	1.84 ± 0.370	0.221
Dizziness	1.80 ± 0.562	1.83 ± 0.505	0.757	1.89 ± 0.318	1.93 ± 0.252	0.461	1.74 ± 0.600	1.80 ± 0.535	0.585
Intestinal obstruction	1.65 ± 0.555	1.78 ± 0.502	0.138	1.56 ± 0.693	1.64 ± 0.679	0.417	1.66 ± 0.519	1.68 ± 0.513	0.834
Performance status	1.54 ± 0.719	1.63 ± 0.653	0.502	1.64 ± 0.743	1.71 ± 0.661	0.740	1.58 ± 0.642	1.70 ± 0.463	0.498
Early dumping syndrome	1.54 ± 0.665	1.70 ± 0.603	0.114	1.40 ± 0.720	1.62 ± 0.650	0.097	1.42 ± 0.673	1.60 ± 0.728	0.072
Late dumping syndrome	1.78 ± 0.502	1.70 ± 0.537	0.377	1.73 ± 0.688	1.73 ± 0.580	0.538	1.86 ± 0.452	1.72 ± 0.479	0.050
Physical condition	1.67 ± 0.673	1.72 ± 0.627	0.637	1.80 ± 0.548	1.64 ± 0.712	0.259	1.76 ± 0.517	1.76 ± 0.476	0.853
Wound pain, present	1.57 ± 0.690	1.83 ± 0.423	0.031[a]	1.53 ± 0.815	1.73 ± 0.580	0.326	1.62 ± 0.490	1.78 ± 0.465	0.059
Wound pain, postoperative	1.07 ± 0.866	1.07 ± 0.866	1.000	1.18 ± 0.716	1.18 ± 0.716	1.000	1.22 ± 0.764	1.22 ± 0.764	1.000
Satisfaction with operation	1.57 ± 0.690	1.72 ± 0.596	0.197	1.69 ± 0.557	1.76 ± 0.484	0.597	1.66 ± 0.688	1.72 ± 0536	0.599
Recommendation to others	1.41 ± 0.714	1.59 ± 0.567	0.206	1.29 ± 0.787	1.42 ± 0.690	0.467	1.46 ± 0.706	1.54 ± 0.646	0.606
Mood or feeling	1.67 ± 0.673	1.63 ± 0.708	0.802	1.67 ± 0.707	1.71 ± 0.695	0.636	1.70 ± 0.463	1.66 ± 0.519	0.780
Total	1.45 ± 0.748	1.67 ± 0.612	0.015[a]	1.36 ± 0.825	1.61 ± 0.663	0.007[a]	1.42 ± 0.743	1.65 ± 0.609	0.011[a]

[a]$P < 0.05$.

than esophagojejunostomy. It has been shown that independent prognostic factors such as ensuring radical cure, maintaining food passage in the duodenum, and less excision of the stomach, are very important for postoperative QOL[17,18]. A high incidence of reflux symptoms after simple esophagogastrostomy has prompted the development of several novel reconstructions to prevent reflux[12,19-21]. Previous reports have shown that proximal gastrectomy is more likely to produce complications such as heartburn and poor

Table 6 Comparison of evaluation scores of 6 and 24 mo postoperative Spitzer Index among the three groups (mean ± SD)

Factors	EA group	EP group	*P*	EA group	EE group	*P*	EP group	EE group	*P*
6 mo									
Activity	1.74 ± 0.556	1.80 ± 0.548	0.403	1.74 ± 0.556	1.76 ± 0.476	0.920	1.80 ± 0.548	1.76 ± 0.476	0.347
Daily living	1.85 ± 0.359	1.78 ± 0.420	0.344	1.85 ± 0.359	1.84 ± 0.422	0.940	1.78 ± 0.420	1.84 ± 0.422	0.326
Health	1.65 ± 0.588	1.62 ± 0.535	0.630	1.65 ± 0.588	1.76 ± 0.517	0.266	1.62 ± 0.535	1.76 ± 0.517	0.115
Support	1.78 ± 0.462	1.71 ± 0.506	0.467	1.78 ± 0.462	1.74 ± 0.487	0.661	1.71 ± 0.506	1.74 ± 0.487	0.767
Outlook	1.76 ± 0.473	1.73 ± 0.539	0.946	1.76 ± 0.473	1.78 ± 0.465	0.790	1.73 ± 0.539	1.78 ± 0.465	0.752
24 mo									
Activity	1.72 ± 0.492	1.71 ± 0.506	0.835	1.72 ± 0.492	1.82 ± 0.388	0.674	1.71 ± 0.506	1.82 ± 0.388	0.802
Daily living	1.87 ± 0.339	1.84 ± 0.367	0.714	1.87 ± 0.339	1.90 ± 0.364	0.724	1.84 ± 0.367	1.90 ± 0.364	0.273
Health	1.76 ± 0.473	1.76 ± 0.484	0.352	1.76 ± 0.473	1.90 ± 0.303	0.557	1.76 ± 0.484	1.90 ± 0.303	0.947
Support	1.80 ± 0.451	1.80 ± 0.457	0.367	1.80 ± 0.451	1.84 ± 0.370	0.839	1.80 ± 0.457	1.84 ± 0.370	0.583
Outlook	1.83 ± 0.376	1.76 ± 0.529	0.892	1.83 ± 0.376	1.70 ± 0.614	0.692	1.76 ± 0.529	1.70 ± 0.614	0.793

Table 7 Comparison of evaluation scores of Spitzer Index at 6 and 24 mo postoperatively between the same group (mean ± SD)

Factors	EA group		*P*	EP group		*P*	EE group		*P*
	6 mo	24 mo		6 mo	24 mo		6 mo	24 mo	
Activity	1.74 ± 0.556	1.72 ± 0.492	0.583	1.80 ± 0.548	1.71 ± 0.506	0.164	1.76 ± 0.476	1.82 ± 0.388	0.588
Daily living	1.85 ± 0.359	1.87 ± 0.339	0.782	1.78 ± 0.420	1.84 ± 0.367	0.422	1.84 ± 0.422	1.90 ± 0.364	0.350
Health	1.65 ± 0.588	1.76 ± 0.473	0.342	1.62 ± 0.535	1.76 ± 0.484	0.177	1.76 ± 0.517	1.90 ± 0.303	0.148
Support	1.78 ± 0.462	1.80 ± 0.451	0.813	1.71 ± 0.506	1.80 ± 0.457	0.325	1.74 ± 0.487	1.84 ± 0.370	0.301
Outlook	1.76 ± 0.473	1.83 ± 0.376	0.444	1.73 ± 0.539	1.76 ± 0.529	0.807	1.78 ± 0.465	1.70 ± 0.614	0.701

appetite, and worsen nutritional status compared to other types of gastrectomy[22]. To prevent or minimize postgastrectomy complications, proximal gastrectomy with an interposed jejunal pouch has been advocated as an organ-preserving strategy to improve QOL[13,23-25]. However, there some studies have shown that patients with an interposed jejunal pouch need a second operation because of food stasis or disordered gastric emptying, and the length of the jejunal pouch is still under discussion[26-28].

To avoid postoperative symptoms, there is no consensus on the need for pyloroplasty after proximal gastrectomy. Our study suggested that pyloroplasty improved gastric emptying and decreased stomach stasis. Other studies have shown that pyloroplasty might significantly relieve gastric distention and speed up gastric emptying[29,30]. Our data showed that most patients suffered from heartburn and postprandial abdominal fullness after proximal gastrectomy. However, patients in the EA group showed significantly less heartburn and postprandial abdominal fullness compared to the other groups. Pyloroplasty as a draining procedure helps the patient have fewer complications such as gastric distention, nausea and vomiting, and promotes faster gastric emptying[31]. Patients with pyloroplasty may recover 80% of the dietary volume in the short term after surgery. In addition, our study found that pyloroplasty also helped to recover or improve body weight in the long term, postoperatively. Our study suggested that patients treated with an EA procedure had a better QOL than the other groups, however, this was correlated with pyloroplasty and promoted gastric emptying to some extent.

The EA procedure decreased the postoperative reflux symptoms in the long term. This simple and safe technique does not result in postgastrectomy syndrome. The mortality rate was zero and the absence of early postoperative complications highlighted the safety of this procedure. Thus, proximal gastrectomy reconstruction by EA provided excellent clinical results in patients with proximal gastric cancer.

After proximal gastrectomy, body weight decreased (approximately 5-10 kg) below baseline after the first few postoperative months, and later, the weight stabilized if there were no tumor recurrence, and recovered slowly almost to baseline. Our study showed that even 2 years postoperatively, the EA group showed a significantly better recovered or improved body weight than the other groups. Seven patients in the EA group recovered 100%, and more than two-thirds of patients maintained at least 85% of baseline weight 2 years after surgery. In summary, the EA procedure benefited the patients with upper third gastric cancer in terms of body weight.

Some patients after proximal gastrectomy suffered from reduced meal size, due to microgastria. To avoid microgastria, the esophagogastrostomy performed by the EE procedure may help to maintain the longest distance from the pylorus to the anastomotic stoma. The center rod of the circular stapler is pierced through the left end of the staple line of the stomach. The longest distance from the pylorus to the esophagogastrostomy site can be found in this position. The longer distance will reduce microgastria and also decrease the tension of the stoma.

There were some limitations in our study. Firstly, the patients were not at the same stage in terms of their gastric cancer; 32.2% were early stage patients. Although QOL in the EA group was better than in the other two groups, whether QOL in the EA group is better than that for other reconstruction methods, or if it reaches

the threshold of normal QOL requires further study. Whether QOL in the EA group will remain improved after long-term follow-up (5-year or longer) requires further research.

The clinical implications of this study showed that the EA procedure seems to confer clinical benefit in terms of postoperative QOL, especially in the form of improved meal intake, reduced gastroesophageal reflux, and improved body weight, however, overall survival rate and surgical results are the same using all three procedures. Our data suggest that, to avoid gastroesophageal reflux and improve QOL in patients with upper third gastric cancer after proximal gastrectomy, the EA procedure for reconstruction using a stapler is safe and feasible for esophagogastrostomy. However, better reconstruction methods are still required to decrease postoperative symptoms in the future.

COMMENTS

Background

Given equivalent results with regards to survival, the impact of anastomotic methods on quality of life (QOL) becomes even more important. QOL is the main outcome for judging the efficacy of treatment modalities when no overall survival differences are demonstrated.

Research frontiers

There is still no consensus on how to choose a reconstruction method for proximal gastrectomy in patients with upper third gastric cancer. This study was designed to compare in detail different methods of esophagogastrostomy.

Innovations and breakthroughs

No previous studies have assessed different methods of esophagogastrostomy in terms of QOL. The aim of this study was to assess outcome in terms of QOL in patients after proximal gastrectomy, by comparing three methods of esophagogastrostomy, in order to find the optimal reconstruction method that offers the optimal postoperative QOL.

Applications

The clinical implications of this study showed that the anterior wall end-to-side anastomosis combined with pyloroplasty (EA) procedure seems to confer clinical benefit in terms of postoperative QOL, especially in the form of improved meal intake, reduced gastroesophageal reflux, and improved body weight.

Terminology

The Spitzer QOL Index, which reflects the patient's postoperative status of health perception, is a global health assessment with a valid questionnaire, which includes five items: activity, daily living, health, support of family and friends, and outlook.

Peer review

The authors evaluated postoperative QOL in patients with upper third gastric cancer, who received proximal gastrectomy with additional esophagogastrostomy. The results suggest that to avoid gastroesophageal reflux and improve QOL for patients with upper third gastric cancer after proximal gastrectomy, the EA procedure of reconstruction using a stapler is safe and feasible for esophagogastrostomy.

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CASE REPORT

Enigma of primary aortoduodenal fistula

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Abstract

A diagnosis of primary aortoenteric fistula is difficult to make despite a high level of clinical suspicion. It should be considered in any elderly patient who presents with upper gastrointestinal bleeding in the context of a known abdominal aortic aneurysm. We present the case of young man with no history of abdominal aortic aneurysm who presented with massive upper gastrointestinal bleeding. Initial misdiagnosis led to a delay in treatment and the patient succumbing to the illness. This case is unique in that the fistula formed as a result of complex atherosclerotic disease of the abdominal aorta, and not from an aneurysm.

Key words: Aortoduodenal fistula; Gastrointestinal hemorrhage; Computed tomography; Aortography

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INTRODUCTION

Primary aortoenteric fistula is an extremely rare but potentially fatal cause of gastrointestinal bleeding. We present the case of a 49-year-old man with no past history of abdominal aortic aneurysm, who presented with recurrent gastrointestinal bleeding.

CASE REPORT

A 49-year-old man was admitted to the emergency room with clinical signs of gastrointestinal bleeding (massive hematemesis, tachycardia of 140 bpm, and blood pressure of 70/40 mmHg). His hemoglobin was 5.5 g/dL. The patient was resuscitated with intravenous fluids and transfusion of 4 U of packed red blood cells. His medical history included multiple sclerosis with minimal neurological deficit, which was treated with Copaxone and Novitropan. He had had no previous surgery or peptic ulcer disease.

An initial evaluation with upper endoscopy and colonoscopy presented as normal. Twenty-four hours later, the patient had recurrent bleeding and required a transfusion of an additional 2 U of packed red blood cells. Repeated upper endoscopy showed blood in the stomach and duodenum, but no source of bleeding could be found. The patient was taken to the angiography suite, where flash aortography and visceral angiography showed no active extravasation of contrast material.

The left gastric artery was embolized empirically with fibered platinum coils, which was tolerated well by the patient. Blood pressure began to normalize immediately after embolization, and the patient's condition stabilized. To evaluate the source of bleeding, he underwent CT examination and capsule endoscopy, which appeared to be normal.

Technetium-99m-red blood cell scintigraphy was used to identify the site of bleeding during the next short episode of hematochezia, but it was inconclusive for bleeding. On a subsequent occasion, the patient displayed an episode of fresh blood in a nasogastric tube. Upper endoscopy (session four) revealed multiple gastric erosions. The patient became unstable with clinical

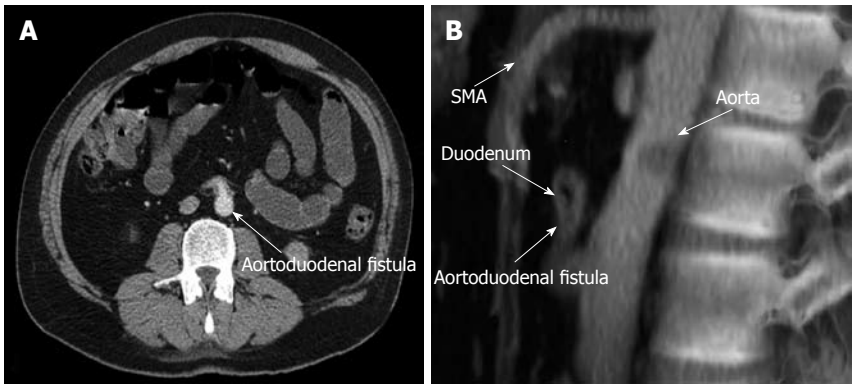

Figure 1 CT and lateral aortography. A: CT showing direct extravasation of contrast material from the aorta into the duodenal lumen (arrow); B: Lateral aortography showing aortoduodenal fistula. Note there is no evidence of abdominal aneurysm.

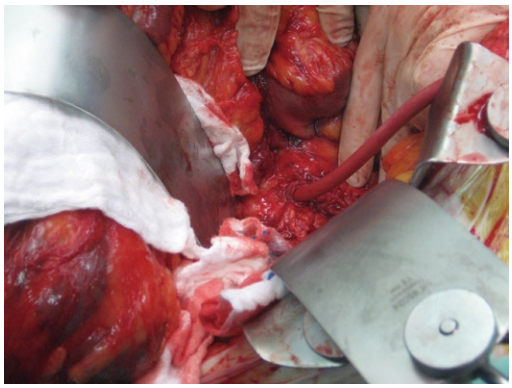

Figure 2 Perioperative findings of primary fistula between the third part of the duodenum and aorta. A Foley catheter was inserted to control the bleeding.

signs of ongoing bleeding. Exploratory laparotomy and gastrotomy were performed with undersuturing of acute gastric ulcers (most were located in the fundus). Intraoperative endoscopy showed no other site of bleeding throughout the stomach, duodenum and 1.5 m of proximal small intestine. Twenty-four hours perioperatively, the patient received 10 red packed cells and other blood supplements and high-dose omeprazole (Omeprazole infusion of 8 mg/h for 72 h, following 40 mg of omeprazole orally per day). The recovery was uneventful and 8 d after surgery, the patient was observed in the regular ward.

Ten days postoperatively, the patient had another episode of bleeding accompanied by syncope with hematochezia and hematemesis. Since he had been receiving fluid resuscitation, blood pressure was undetectable. The patient underwent full cardiopulmonary resuscitation (CPR) and was sent to the operating theater. Repeated angiography and CT revealed an aortoduodenal fistula that had not been detected 3 wk previously (Figure 1).

Exploratory laparotomy was performed and the aortoduodenal fistula was found between the anterior wall of the aorta and the third part of the duodenum. The bowel was full of fresh blood. The fistula was disconnected and a Foley catheter was inserted into the aorta to control the bleeding (Figure 2). This occurred whilst the patient was in prolonged CPR, with a clamp on the descending aorta through left thoracotomy. After 2 h resuscitation in the operating theater, the patient developed disseminated intravascular coagulation and developed bradycardia and acidosis. His blood pressure and urine output dropped drastically and he died on the operating table from irreversible hemorrhagic shock.

Pathological examination of periaortic tissue revealed non-specific inflammation in the connective tissue, fibrin and blood cells. No postmortem examination was performed.

DISCUSSION

The diagnosis and treatment of aortoenteric fistulas are difficult. Nevertheless, in a patient with hematemesis and melena who has undergone an aortobifemoral bypass or aortic interposition grafting without esophagogastroduodenal pathology, a diagnosis of aortoenteric fistula should not be overlooked[1]. Primary aortoduodenal fistulas are nearly always associated with abdominal aortic aneurysm, mostly atherosclerotic. In the present clinical case, the available clinical, instrumental and radiological supports made such a diagnosis feasible.

The penetrating aortic ulcer has been recognized recently as an independent pathological entity. It may penetrate through the aortic wall, which leads to fistulas into adjacent organs. Certik *et al*[2] have reported the case of a 78-year-old woman who was admitted to hospital with massive gastrointestinal hemorrhage. Endoscopy did not reveal the cause of hemorrhage. The diagnosis was made by CT that showed a primary aortoduodenal fistula without aortic aneurysm, and the patient underwent a successful operated. During urgent surgery, the penetrating atherosclerotic ulcer was found to be the cause of the aortoduodenal communication.

The classic presentation of an aortoduodenal fistula is that of a "herald bleed" (brief, with spontaneous resolution), followed anywhere from hours to weeks later by a massive upper gastrointestinal bleed. A high index of suspicion and rapid diagnosis is imperative as exsanguinating hemorrhage may occur. An aortoduodenal fistula or erosion is rarely seen endoscopically[3]. Endoscopy may disclose another cause of bleeding, as was discovered in our patient. Therefore, normal findings, a positive finding of gastritis or ulcers without active bleeding do not rule out aortoduodenal fistula. Careful inspection of the distal duodenum should be performed in the setting of unexplained torrential bleeding. More commonly, fresh blood or clots may be found in the third portion of the duodenum.

CT with contrast is the most suitable diagnostic test and provides a reported detection rate of 30%-61%[4-6]. CT may show the abnormal communication between the aorta and the intestine. Angiography is seldom a helpful procedure, unless a pseudoaneurysm is seen[7], and the diagnosis is usually made or confirmed by laparotomy. Surgical repair is the only therapeutic option.

In summary, a diagnosis of primary aortoenteric fistula should be considered in any patient known to have abdominal aortic aneurysm or lower abdominal pain associated with midline mass and upper gastrointestinal bleeding of unexplained etiology. A herald bleed is an indication for prompt intervention. Endoscopy is the first step in diagnosis, and CT may be used for confirmation. An emergency exploratory laparotomy should be performed as soon as the diagnosis is considered.

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LETTERS TO THE EDITOR

Pentoxifylline: A first line treatment option for severe alcoholic hepatitis and hepatorenal syndrome?

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Abstract

Although favourable results of pentoxifylline (PTX) used in treatment of severe alcoholic hepatitis patients with a Maddrey discriminant function score $\geqslant$ 32 have been previously reported, it is not currently recommended as a first line treatment for alcoholic hepatitis owing to lack of evidence for its efficacy as compared to the standard treatment with corticosteroids. In a very recent issue of *World Journal of Gastroenterology*, Dr. De BK and colleagues compared for the first time the two treatment modalities head to head in a randomized controlled study, demonstrating the advantage of PTX over corticosteroids in terms of patients' survival and risk-benefit profile. The advantage of PTX over corticosteroids in survival of patients with severe alcoholic hepatitis was found to be related to the prevention of hepatorenal syndrome in their study. This study raises the question of the use of PTX as a standard treatment for severe alcoholic hepatitis. Considering the fact that PTX presented a spectacular efficiency in prevention of hepatorenal syndrome in their study as well as that previous studies have shown that this effect is possibly related to a primary renoprotective action because it is irrelevant of tumor necrosis factor-α synthesis inhibition or improved liver function, we tempted to speculate that PXT might be an effective option for prevention and/or treatment of hepatorenal syndrome complicating other forms of advanced liver disease. This attractive theory remains to be elucidated by pressing future studies in view of the lack of effective treatment modalities for hepatorenal syndrome.

TO THE EDITOR

We read with great interest the article recently published by Dr. De and colleagues in *World Journal of Gastroenterology*[1], who evaluated in a randomized double-blind controlled study the advantage of pentoxifylline (PTX) over prednisolone in treatment of severe alcoholic hepatitis [Maddrey discriminant function (DF) score $\geqslant$ 32]. The most important observation was the significantly reduced mortality of patients after treatment with PTX (14.71%) as compared to those after treatment with prednisolone (35.29%, $P = 0.04$). Reduced mortality in patients after treatment with PTX was found to be related to a significant reduction in the development of hepatorenal syndrome. Among patients who died, hepatorenal syndrome developed in 50% of prednisolone-treated patients but in none of PTX-treated patients.

Current guidelines of the American College of Gastroenterology recommend the use of glucocorticosteroids in treatment of patients with severe alcoholic hepatitis as defined by the Maddrey score (DF $\geqslant$ 32)[2,3]. Primary use of PTX in treatment of severe alcoholic hepatitis patients is not recommended due to the lack of evidence for improvement in patient-oriented outcomes[2]. Also, the early switch of corticosteroids to PTX, if no improvement in bilirubin is seen after 7 d of treatment, has been proved to be an inefficient treatment strategy[4]. However, a number of French experts in the field consider PTX a reasonable alternative to corticosteroids for severe acute alcoholic hepatitis based on the

favourable results of previous studies comparing PTX with placebo[5,6].

Specifically, up to now, the use of PTX in treatment of severe alcoholic hepatitis has been supported by two clinical studies[5,6]. The first one was conducted in 1991 by McHutchison *et al*[5], in patients with severe alcoholic hepatitis (defined as DF score ⩾ 32), which showed that PTX could reduce the development of hepatorenal syndrome and the mortality, in comparison to those receiving placebo. These findings were confirmed in 2000 by Akriviadis *et al*[6] in a double-blind placebo-controlled trial, which showed that 24% of PTX-treated patients and 46.1% of control patients died during hospitalization. The survival benefit of PTX was found to be related to a significant reduction in the development of hepatorenal syndrome. Among the patients who died, hepatorenal syndrome developed in 50% of PTX-treated patients and in 91.7% of placebo-treated patients. The study by De *et al*[1] is the first to compare PTX and corticosteroids head to head in a randomized controlled manner. The results of this study, demonstrating the advantage of PTX over corticosteroids, raise the question of the use of PTX as a standard treatment modality for severe alcoholic hepatitis.

A second issue we would like to comment on is the fact that the advantage of PTX in survival of patients with severe alcoholic hepatitis is clearly related to the prevention of hepatorenal syndrome[1,5,6]. The available data do not show the evident mechanism underlying this beneficial effect of PTX and only speculations could be made on this matter. The use of PTX in treatment of alcoholic hepatitis is based on its ability to inhibit the synthesis of tumor necrosis factor (TNF)-α, which is considered a pivotal mediator of alcohol-induced liver injury[6,7]. Although the authors did not assess in this study the immunological and inflammatory status (e.g. TNF-α) of their patients, it has been previously reported that the prevention of hepatorenal syndrome and survival advantage in patients with severe alcoholic hepatitis after treatment with PTX are not associated with decreased circulating TNF-α levels or improved liver function[6]. These findings give an alternative explanation for the positive effect of PTX on renal function of alcoholic hepatitis patients, which is the beneficial action of PTX on renal microcirculation and hemodynamics. This potential primary protective effect of PTX on renal function and its repeatedly confirmed efficacy on prevention of hepatorenal syndrome in severe alcoholic hepatitis patients (in this study, PTX prevented the development of hepatorenal syndrome) tempt us to speculate that it could potentially be used in the prevention and/or treatment of hepatorenal syndrome complicating other forms of advanced liver disease. To the best of our knowledge, this possibility has not been investigated up to now and appears to be an attractive new field for experimental and clinical research, given the current difficulties in the management of patients with hepatorenal syndrome.

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
Marriott Wardman Park Hotel
Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
http://www.easl.ch/

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
http://www.ddw.org

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension,

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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

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

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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

Patent (list all authors)

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

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc*.
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc*.
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