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[1]Passed away on October 20, 2007
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World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 14 Number 28
July 28, 2008

Contents

EDITORIAL

4429 New insights into calcium, dairy and colon cancer
Holt PR

4434 Molecular basis of the potential of mesalazine to prevent colorectal cancer
Stolfi C, Pellegrini R, Franzè E, Pallone F, Monteleone G

4440 Thrombosis and inflammatory bowel disease-the role of genetic risk factors
Tsiolakidou G, Koutroubakis IE

TOPIC HIGHLIGHT

4445 Hepatocellular carcinoma: Defining the place of surgery in an era of organ shortage
Bartlett A, Heaton N

CLINICAL RESEARCH

4454 Clinical significance of NOD2/CARD15 and Toll-like receptor 4 gene single nucleotide polymorphisms in inflammatory bowel disease
Rigoli L, Romano C, Caruso RA, Lo Presti MA, Di Bella C, Procopio V, Lo Giudice G, Amorini M, Costantino G, Sergi MD, Cuppari C, Calabrò GE, Gallizzi R, Salpietro CD, Fries W

BASIC RESEARCH

4462 Metabolomic changes in fatty liver can be modified by dietary protein and calcium during energy restriction
Pilvi TK, Seppänen-Laakso T, Simolin H, Finckenberg P, Huotari A, Herzig KH, Korpela R, Orešič M, Mervaala EM

4473 Lysophosphatidic acid induced nuclear translocation of nuclear factor-κB in Panc-1 cells by mobilizing cytosolic free calcium
Arita Y, Ito T, Oono T, Kawabe K, Hisano T, Takayanagi R

RAPID COMMUNICATION

4480 Comparison of esophageal capsule endoscopy and esophagogastroduodenoscopy for diagnosis of esophageal varices
Frenette CT, Kuldau JG, Hillebrand DJ, Lane J, Pockros PJ

4486 Association between *calcium sensing receptor* gene polymorphisms and chronic pancreatitis in a US population: Role of *serine protease inhibitor Kazal 1type* and alcohol
Muddana V, Lamb J, Greer JB, Elinoff B, Hawes RH, Cotton PB, Anderson MA, Brand RE, Slivka A, Whitcomb DC

4492 Folic acid supplementation inhibits recurrence of colorectal adenomas: A randomized chemoprevention trial
Jaszewski R, Misra S, Tobi M, Ullah N, Naumoff JA, Kucuk O, Levi E, Axelrod BN, Patel BB, Majumdar APN

4499 CT colonography after incomplete colonoscopy in subjects with positive faecal occult blood test
Sali L, Falchini M, Bonanomi AG, Castiglione G, Ciatto S, Mantellini P, Mungai F, Menchi I, Villari N, Mascalchi M

Contents

World Journal of Gastroenterology
Volume 14 **Number** 28 **July** 28, 2008

4505 Development of hepatorenal syndrome in bile duct ligated rats
Pereira RM, dos Santos RAS, Oliveira EA, Leite VHR, Dias FLC, Rezende AS, Costa LP, Barcelos LS, Teixeira MM, Simões e Silva AC

4512 Effects of microalgae chlorella species crude extracts on intestinal adaptation in experimental short bowel syndrome
Kerem M, Salman B, Pasaoglu H, Bedirli A, Alper M, Katircioglu H, Atici T, Perçin EF, Ofluoglu E

4518 Risk factors of thrombosis in abdominal veins
Dutta AK, Chacko A, George B, Joseph JA, Nair SC, Mathews V

4523 Laryngopharyngeal reflux in patients with reflux esophagitis
Lai YC, Wang PC, Lin JC

4529 Macro-regenerative nodules in biliary atresia: CT/MRI findings and their pathological relations
Liang JL, Cheng YF, Concejero AM, Huang TL, Chen TY, Tsang LLC, Ou HY

4535 Celecoxib-related gastroduodenal ulcer and cardiovascular events in a randomized trial for gastric cancer prevention
Feng GS, Ma JL, Wong BCY, Zhang L, Liu WD, Pan KF, Shen L, Zhang XD, Li J, Xia HHX, Li JY, Lam SK, You WC

4540 Radiofrequency ablation as a treatment for hilar cholangiocarcinoma
Fan WJ, Wu PH, Zhang L, Huang JH, Zhang FJ, Gu YK, Zhao M, Huang XL, Guo CY

4546 Delayed ethyl pyruvate therapy attenuates experimental severe acute pancreatitis *via* reduced serum high mobility group box 1 levels in rats
Yang ZY, Ling Y, Yin T, Tao J, Xiong JX, Wu HS, Wang CY.

4551 Programmed death-1 expression is associated with the disease status in hepatitis B virus infection
Ye P, Weng ZH, Zhang SL, Zhang JA, Zhao L, Dong JH, Jie SH, Pang R, Wei RH

4558 Effect of admission hypertriglyceridemia on the episodes of severe acute pancreatitis
Deng LH, Xue P, Xia Q, Yang XN, Wan MH

4562 Correlation between expression and differentiation of endocan in colorectal cancer
Zuo L, Zhang SM, Hu RL, Zhu HQ, Zhou Q, Gui SY, Wu Q, Wang Y

4569 Diagnosis and treatment of spontaneous colonic perforation: Analysis of 10 cases
Yang B, Ni HK

CASE REPORT

4573 Anabolic steroid abuse causing recurrent hepatic adenomas and hemorrhage
Martin NM, Abu Dayyeh BK, Chung RT

4576 Cerebral venous thrombosis and heparin-induced thrombocytopenia in an 18-year old male with severe ulcerative colitis
Thorsteinsson GS, Magnussson M, Hallberg LM, Wahlgren NG, Lindgren F, Malmborg P, Casswall TH

4580 Septic thrombophlebitis of the porto-mesenteric veins as a complication of acute appendicitis
Chang YS, Min SY, Joo SH, Lee SH

World Journal of Gastroenterology
Volume 14 **Number** 28 **July** 28, 2008

Contents

	4583	Direct invasion to the colon by hepatocellular carcinoma: Report of two cases *Hirashita T, Ohta M, Iwaki K, Kai S, Shibata K, Sasaki A, Nakashima K, Seigo K*
LETTERS TO THE EDITOR	**4586**	Ten mg dexrabeprazole daily is as effective as 20 mg dexrabeprazole daily *Kanakia R, Jain S*
ACKNOWLEDGMENTS	**4588**	Acknowledgments to Reviewers of *World Journal of Gastroenterology*
APPENDIX	**4589**	Meetings
	4590	Instructions to authors
FLYLEAF	I-VII	Editorial Board
INSIDE BACK COVER		Online Submissions
INSIDE FRONT COVER		Online Submissions

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EDITORIAL

New insights into calcium, dairy and colon cancer

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Abstract

This paper is to review recent information about the relationship of calcium and dairy foods to colon cancer. The review focuses on primary prevention, discusses the potential components in dairy foods that might be anti-neoplastic, reviews the epidemiologic information and describes intervention studies demonstrating efficacy of calcium and vitamin D in reducing colorectal polyp recurrence. Since vitamin D is important in cancer prevention, pertinent data is discussed and potential mechanisms of actions presented. Calcium and vitamin D are important agents for the primary prevention of colorectal neoplasia.

Key words: Colorectal cancer; Dairy foods; Calcium; Vitamin D; Colorectal polyp recurrence; Colon cancer prevention

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INTRODUCTION

Colorectal cancer is a common and lethal disease in the Western World and the incidence and mortality is increasing dramatically in the rest of the world. In the United States, colorectal cancer is the second most common cause of cancer deaths, with an incidence of around 130 000 cases a year and a mortality rate of approximately 55-60 000[1]. The World Health Organization statistics for colorectal cancer incidences worldwide in 1996 showed about 875 000 cases with a mortality of over 500 000. The incidence of this tumor in the less well developed countries of the world is increasing dramatically so that the death rate for this tumor far exceeds the 7.2% of all cancer deaths reported by the WHO. Incidence and mortality rates differ markedly across countries with the highest rates reported from Australia and Northern Great Britain and the lowest rates in Southern Africa. This 30-fold difference in incidence underscores the importance of environmental factors in inducing this cancer.

Although the treatment of established colorectal cancer has improved remarkably over the last half century, mortality rates still are high, particularly in our aging population. Therefore, a major focus of the management of this tumor has been in the area of cancer prevention which is better termed "cancer risk reduction". There are three modalities of cancer prevention; tertiary prevention is when efforts are made to lower the risk of a second cancer once a primary tumor has been diagnosed. A good example of this is the use of tamoxifen for risk reduction of breast cancer in women who have had one breast cancer and the use of retinoid acting agents to lower the risk of second squamous cancers of the aero-digestive tract. Secondary prevention involves the abolition of pre-cancerous neoplastic lesions, thus lowering the risk of the later development of cancer. For the colorectum, secondary prevention by detection of neoplastic colorectal adenomas and adenoma polypectomy has become an established preventive technique and has been demonstrated to be effective in lowering the incidence of this tumor[2]. Primary prevention aims to reduce the development of a cancer before tissue pre-neoplastic changes occur. This is the approach where calcium and dairy products appear to have an important role in lowering the risk of colorectal cancer. It must be emphasized that in order to advocate a primary cancer prevention modality which is likely to be applied to large numbers of the population, it must have an excellent benefit to risk ratio. The term "chemoprevention" which is better called "risk reduction" has been applied to these approaches. Chemoprevention involves the use

of an agent to slow the progress, to reverse or inhibit the process of carcinogenesis. Such agents may act at different levels modulating cancer production at the level of the cell, at the molecular level, at the whole tissue level or potentially at the whole patient clinical level.

There are many components of dairy foods that have been shown experimentally to have protective effects against colon cancer. These components include calcium and vitamin D for which there is most evidence and (which will be discussed below), conjugated linoleic acid[3], sphingolipids[4] and the potential of butyric acid formed by colonic lactobacilli from milk products. Clearly if one includes bacterial cultures, i.e. probiotics added to dairy products, there is an increasingly important literature that suggests that such agents may be beneficial in reducing the risk of colorectal neoplasia[5].

EPIDEMIOLOGY

There have been numerous epidemiologic studies that have suggested that calcium or dairy products may lower the risk of colorectal neoplasia. The data for many of these have been reviewed recently[6]. An important prospective epidemiologic study was performed in over 45 000 Swedish men, aged 45 to 79 years[7]. In this study, calcium intake was determined from food frequency questionnaires and there was a mean follow up of between 6 and 7 years. The data on colorectal cancer incidence, when analyzed for the highest versus the lowest quartile for calcium intake, showed a statistically significant reduction in colon cancer development with a mean odds ratio of 0.68 and for dairy intake a mean odds ratio of 0.46. Using multivariate analysis, the data from this study suggested that there might be a threshold effect at about 1200 mg to 1400 mg of calcium per day[7].

CALCIUM

At the present time, the gold standard for measuring risk reduction by an intervention in colorectal cancer uses determination of the incidence of recurrence of adenomatous polyps following removal of all colonic polyps by polypectomy. This approach was originally developed by Baron JA (Baron JA, Beach M, Mandel JS, van Stolk RU, Haile RW, Sandler RS, Rothstein R, Summers RW, Snover DC, Beck GJ, Bond JH, Greenberg ER. Calcium supplements for the prevention of colorectal adenomas. Calcium Polyp Prevention Study Group. *N Engl J Med* 1999; 340: 101-107) and his co-workers to analyze the beneficial effects of calcium for adenomatous polyp reduction[8]. Subsequent analyses have evaluated reduction of total adenomatous polyps and reduction of advanced polyps as defined by size and the presence of severe dysplasia. Calcium supplementation of 1200 mg per day reduced total adenomas by approximately 20%[8], but advanced adenomas by about 45%[9]. If one translates these data into numbers of adenomas that would be reduced in the United States by increased calcium intake this would total approximately 26 000 cases of adenomas with a more important impact on the advanced lesions. Subsequent analyses by Baron's group showed that most of the effect of calcium in lowering the incidence of recurrent adenomas occurred in individuals who had baseline levels of serum 25 hydroxy vitamin D above the median (about 29 ng per mL) with little effect in individuals with lower levels[10]. These data strongly suggest that it is the combination of calcium and vitamin D which is important in altering adenoma recurrence. A prospective US national study is ongoing to examine the relative effects of calcium, vitamin D or the combination of calcium plus vitamin D on colorectal adenoma recurrence. In a further recent publication, Baron JA and coworkers have followed calcium supplemented subjects for ten years after completing the ongoing study[11]. These data suggested that the beneficial effect of calcium upon adenoma recurrence persisted for five years even in subjects who were not taking supplemental calcium after stopping the formal study and showed 40% less adenoma recurrence when compared to control placebo treated subjects. After 5 years after no further beneficial effect of calcium administration was demonstrated[11].

The classical hypothesis for the beneficial effects of calcium derived from an original physiochemical hypothesis by Newmark and colleagues in which they suggested that fatty acids and bile acids in the colon may be detrimental to the epithelium and important in the initial steps of colorectal carcinogenesis and that calcium could bring bile acids and fatty acids out of solution in the colonic lumen and, thus, reduce the cytotoxicity of these agents[12]. A number of studies by Van der Meer's group subsequently were consistent with this hypothesis even in *in-vivo* studies[13,14].

However, calcium is known to have manifold cellular effects in colonic epithelial cells suggesting that these must be important in the action of this compound upon colorectal carcinogenesis. Two recent areas of research suggest that other mechanisms may well be crucial in the cellular effects of calcium. The human parathyroid calcium sensing receptor which senses minor changes in extra-cellular calcium concentrations is expressed in differentiated cells of the human colonic crypt. The receptor is partially or completely lost during colon carcinogenesis[15]. The calcium sensing receptor has two promoters with vitamin D response elements. *In vitro*, calcium and vitamin D stimulate the calcium sensing receptor promoter activity in colonic cells to reduce E-cadherin expression and inhibit TCF4. Thus, this receptor may function to regulate epithelial differentiation and be anticarcinogenic[15]. In addition, evidence suggests that the cardiac L-type calcium channel is present in colon tissue and may play a role in determining calcium influx into colonic epithelial cells[16].

Vitamin D stores of the body derive from photolysis of 7 dehydrocholesterol in the skin to form pre-vitamin D_3 which rapidly isomerizes at body temperature to form vitamin D_3 and then passes into the circulation.

Dietary vitamin D_2 and vitamin D_3 is absorbed from the intestinal lumen in micellar form and after transfer into intestinal lymph passes into the circulation where it is bound to a vitamin D binding protein. Vitamin D from both cutaneous and intestinal sources is taken up by the liver and converted to 25 hydroxy vitamin D (25 OHD_3). The circulating levels of serum 25 OHD_3 reflect the body stores of this vitamin. 25 OHD_3 is transported to the periphery and converted to calcitriol, (1,25 dihydroxy vitamin D3 1,25 $(OH)_2D_3$), mainly in the kidney but also in many peripheral tissues by the action of the enzyme 1 alpha hydroxylase (CYP 27 B2). 1,25 $(OH)_2D_3$ is bound to vitamin D receptors present in many tissues and has pleiomorphic actions in bone, the gastrointestinal tract and uterus, etc.

Epidemiologic studies of vitamin D effects upon human health have included evaluation of effects of sunlight (ultraviolet) exposure, analysis of dietary and supplemental vitamin D intake as well as measurement of serum 25 hydroxy vitamin D levels.

There is abundant epidemiologic data to show that exposure to sunlight results in a reduction in the incidence of many cancers, but most clearly reduced colorectal cancer. The original observations of Cedric Garland[17] which followed upon a forgotten report in 1941[18] showed a distinct North to South latitude difference in colorectal cancer development. More recently, several studies have shown not only a lowering of colorectal cancer incidence, but also that for breast, ovary and prostate[19] accompanied by the expected increased non-melanoma skin cancer formation. Other studies have also pointed to beneficial changes of sunlight in esophageal, renal and bladder cancer as well as non-Hodgkins lymphoma[20].

Many investigators have analyzed the relationship of colorectal cancer prevalence with vitamin D body stores, i.e. measurement of circulating 25 OHD_3. A metanalysis of vitamin D intake studies using dose response gradient analysis showed a reduction in colorectal cancer of approximately 20%[21]. In 2006, Garland analyzed positive and negative studies comparing serum 25 OHD levels and the development of colorectal, breast and prostate cancer. Six of seven studies of colorectal cancers showed a significant reduction of cancer and one was borderline, whereas for breast cancer only one was significant and one showed no effect with similar negative results for prostate cancer[22]. Further evidence for the beneficial effect of higher levels of 25 OHD were shown in the distal colon of older women (OR = 0.45)[23] and in black men[24], because of lower action of sunlight in pigmented skin to form this vitamin. A prospective 7.75 year study of serum levels of vitamin D showed a 55% reduction in cancer development in the highest compared to the lowest quartile[25].

MECHANISMS OF ACTION OF VITAMIN D

The cellular activity of vitamin D is dependent on the action of calcitriol principally through interaction with a high affinity binding protein (VDR). VDR is a member of the steroid receptor superfamily ligand dependent transcription factor and the binding of calcitriol to VDR induces a configurational change in the receptor. The receptor then heterodimerizes with the retinol receptor (RxR) and the VDR-RxR complex binds to vitamin D response elements in the nucleus. This interaction induces gene transcription which results in cell cycle arrest through the regulation of CDK2, p21, p27, p53, KI67 and E-cadherin. In addition there are effects on differentiation and apoptosis, the latter through changes in BcL-2, BcL-x_L, Mcl-1, etc. Furthermore, there are non-receptor dependent actions of vitamin D upon the cell which include activation of calcium channels at least in the small intestine and colon[26].

The clinical effects of vitamin D are also dependent on polymorphisms in the vitamin D receptor. Such polymorphisms can occur at the 3' end of the receptor and includes Bsm1, Taq1 and at the 5' end FoK1. It is known that such polymorphisms are functionally associated with differences in bone density and in serum calcitriol levels, but whether they affect the action of vitamin D upon the colon is unclear[27]. However, high dairy intake effects upon colon adenoma recurrence has been restricted to individuals with the Apal aA/AA genotype[28].

One small, but unique study has described the effects of 6 mo supplemental calcium (1200 mg) plus vitamin D (400 IU) to subjects with adenomatous polyps which were transected with one half tattooed and left *in situ*. Calcium plus vitamin D reduced proliferation indices in the remaining polyps and flat mucosa, but also dramatically down-regulated polyp mucin 5AC (MUC5AC)[29]. These data suggest that the combination alters important cellular processes both in the adenoma and the flat colorectal mucosa.

Turning to other studies of dairy products upon colon neoplasia formation, Cho *et al* (Cho E, Smith-Warner SA, Spiegelman D, Beeson WL, van den Brandt PA, Colditz GA, Folsom AR, Fraser GE, Freudenheim JL, Giovannucci E, Goldbohm RA, Graham S, Miller AB, Pietinen P, Potter JD, Rohan TE, Terry P, Toniolo P, Virtanen MJ, Willett WC, Wolk A, Wu K, Yaun SS, Zeleniuch-Jacquotte A, Hunter DJ. Dairy foods, calcium, and colorectal cancer: a pooled analysis of 10 cohort studies. *J Natl Cancer Inst* 2004; 96: 1015-1022) published pooled data of dairy product intake from 10 cohort studies and reported a 12% reduction in colon cancer risk with each 500 mL increase in milk intake. There also was a significant and 17% reduction in colorectal cancer incidence with the ingestion of ricotta cheese greater than 25 mg per day[30]. An important study that has received a large amount of attention was the Women's Health Initiative Dietary modification study (WHI study) in which women 50-79 years of age received supplemental 1100 mg calcium plus 400 IU vitamin D with meals. Some of these women also participated in a study of the effects of estrogen replacement therapy upon colon cancer development.

This prospective study had a mean follow up of 7.0 ± 1.4 years and had colorectal cancer as an end point[31]. There was no significant difference between the development of colorectal cancer in women taking the calcium plus vitamin D when compared to controls (OR = 1.08) (0.86-1.34). There clearly were a number of issues related to this study which have resulted in some considerable criticism. Such issues included the fact that the mean age of the women in the study was 62 and the increase in colon cancer in women occurs only after age 60, they had a high basal dietary intake of calcium of approximately 1100 mg to 1200 mg per day and relatively adequate vitamin D intake approaching 400 IU per day. There was low compliance with the intervention with only 70% of subjects consuming more than 50% of the pills and in addition the subjects were permitted to continue to take calcium and/or vitamin D supplements separately from study drugs. It also was felt that the duration of the study was too short for a cancer end point and the amount of vitamin D provided in the intervention was relatively low. Importantly however, women who showed a low serum 25 hydroxy vitamin D level at base line demonstrated a 2.5 fold increased risk of colorectal cancer compared to the top quartile of serum 25 hydroxy vitamin D levels ($P < 0.02$)[31].

VITAMIN D AND CANCER MORTALITY

A unique study by Lappe prospectively evaluated the development of any cancer in approximately 1200 postmenopausal women who received calcium 1500 mg/d with or without vitamin D 1100 IU per day. The unadjusted relative risk for the development of any cancer with calcium administration was 0.53 and for vitamin D plus calcium 0.40. Serum 25 hydroxy vitamin D also was a significant predictor of decreased cancer development[32]. A recent meta-analysis of 18 studies of effects of vitamin D on overall mortality by Philippe Autier showed an 8% reduction in overall mortality in subjects who received vitamin D[33].

ANIMAL STUDIES

Over the past decade Martin Lipkin's group have been evaluating the effects of a Western style diet (WD) relatively high in fat content (20%) and relatively low in vitamin D and calcium upon carcinogenic changes in the colon of mice. This represented the first demonstration of diet-induced colorectal tumor formation in the absence of a carcinogen[34]. When folic acid also was reduced in the WD over a period of 18-24 mo, adenomas and carcinomas developed with increases in both the percent of mice developing tumors as well as in tumor frequency. The addition of calcium and vitamin D to the diet dramatically reduced or eliminated most of these tumors[35]. It also is of interest that this Western style diet in mice produced hyperproliferation in mammary duct epithelial cells[36], pancreatic epithelial cells[37] and prostate cells[38] and that calcium and vitamin D suppressed such hyperproliferation. The molecular underpinnings of these observations are presently under study.

CONCLUSION

Epidemiologic intake and intervention studies have shown that calcium administration lowers colorectal adenomatous polyps as well as cancer rates and this effect may be prolonged. Most evidence suggests that the effect of calcium is dependent or partially related to simultaneous vitamin D intake. Vitamin D may also reduce colon cancer risk independent of the presence of increased amounts of calcium or dairy products in the diet. Few studies have been performed with dairy products alone, but the data generally supports the positive effects shown with calcium and vitamin supplementation as well. Understanding the cellular effects of calcium and vitamin D in humans' *in-vivo* is crucial to make further progress in this field.

REFERENCES

1 **Jemal A**, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. *CA Cancer J Clin* 2008; **58**: 71-96
2 **Winawer SJ**, Zauber AG. Colonoscopic polypectomy and the incidence of colorectal cancer. *Gut* 2001; **48**: 753-754
3 **Liew C**, Schut HA, Chin SF, Pariza MW, Dashwood RH. Protection of conjugated linoleic acids against 2-amino-3- methylimidazo[4,5-f]quinoline-induced colon carcinogenesis in the F344 rat: a study of inhibitory mechanisms. *Carcinogenesis* 1995; **16**: 3037-3043
4 **Duan RD**. Sphingomyelin hydrolysis in the gut and clinical implications in colorectal tumorigenesis and other gastrointestinal diseases. *Scand J Gastroenterol* 1998; **33**: 673-683
5 **Saikali J**, Picard C, Freitas M, Holt P. Fermented milks, probiotic cultures, and colon cancer. *Nutr Cancer* 2004; **49**: 14-24
6 **Shaukat A**, Scouras N, Schunemann HJ. Role of supplemental calcium in the recurrence of colorectal adenomas: a metaanalysis of randomized controlled trials. *Am J Gastroenterol* 2005; **100**: 390-394
7 **Larsson SC**, Bergkvist L, Rutegard J, Giovannucci E, Wolk A. Calcium and dairy food intakes are inversely associated with colorectal cancer risk in the Cohort of Swedish Men. *Am J Clin Nutr* 2006; **83**: 667-673; quiz 728-729
8 **Baron JA**, Beach M, Mandel JS, van Stolk RU, Haile RW, Sandler RS, Rothstein R, Summers RW, Snover DC, Beck GJ, Bond JH, Greenberg ER. Calcium supplements for the prevention of colorectal adenomas. Calcium Polyp Prevention Study Group. *N Engl J Med* 1999; **340**: 101-107
9 **Grau MV**, Baron JA, Sandler RS, Haile RW, Beach ML, Church TR, Heber D. Vitamin D, calcium supplementation, and colorectal adenomas: results of a randomized trial. *J Natl Cancer Inst* 2003; **95**: 1765-1771
10 **Grau MV**, Baron JA, Barry EL, Sandler RS, Haile RW, Mandel JS, Cole BF. Interaction of calcium supplementation and nonsteroidal anti-inflammatory drugs and the risk of colorectal adenomas. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 2353-2358
11 **Grau MV**, Baron JA, Sandler RS, Wallace K, Haile RW, Church TR, Beck GJ, Summers RW, Barry EL, Cole BF, Snover DC, Rothstein R, Mandel JS. Prolonged effect of calcium supplementation on risk of colorectal adenomas in a randomized trial. *J Natl Cancer Inst* 2007; **99**: 129-136
12 **Newmark HL**, Wargovich MJ, Bruce WR. Colon cancer and dietary fat, phosphate, and calcium: a hypothesis. *J Natl*

Cancer Inst 1984; **72**: 1323-1325
13 **Govers MJ**, Van der Meet R. Effects of dietary calcium and phosphate on the intestinal interactions between calcium, phosphate, fatty acids, and bile acids. *Gut* 1993; **34**: 365-370
14 **Govers MJ**, Termont DS, Lapre JA, Kleibeuker JH, Vonk RJ, Van der Meer R. Calcium in milk products precipitates intestinal fatty acids and secondary bile acids and thus inhibits colonic cytotoxicity in humans. *Cancer Res* 1996; **56**: 3270-3275
15 **Chakrabarty S**, Wang H, Canaff L, Hendy GN, Appelman H, Varani J. Calcium sensing receptor in human colon carcinoma: interaction with Ca(2+) and 1,25-dihydroxyvitamin D(3). *Cancer Res* 2005; **65**: 493-498
16 **Wang XT**, Nagaba Y, Cross HS, Wrba F, Zhang L, Guggino SE. The mRNA of L-type calcium channel elevated in colon cancer: protein distribution in normal and cancerous colon. *Am J Pathol* 2000; **157**: 1549-1562
17 **Garland CF**, Garland FC. Do sunlight and vitamin D reduce the likelihood of colon cancer? *Int J Epidemiol* 1980; **9**: 227-231
18 **Apperly FL**. The relation of solar radiation to cancer mortality in North America. *Cancer Res* 1941; **1**: 191-195
19 **Freedman DM**, Dosemeci M, McGlynn K. Sunlight and mortality from breast, ovarian, colon, prostate, and non-melanoma skin cancer: a composite death certificate based case-control study. *Occup Environ Med* 2002; **59**: 257-262
20 **Grant WB**. An estimate of premature cancer mortality in the U.S. due to inadequate doses of solar ultraviolet-B radiation. *Cancer* 2002; **94**: 1867-1875
21 **Gorham ED**, Garland CF, Garland FC, Grant WB, Mohr SB, Lipkin M, Newmark HL, Giovannucci E, Wei M, Holick MF. Vitamin D and prevention of colorectal cancer. *J Steroid Biochem Mol Biol* 2005; **97**: 179-194
22 **Garland CF**, Garland FC, Gorham ED, Lipkin M, Newmark H, Mohr SB, Holick MF. The role of vitamin D in cancer prevention. *Am J Public Health* 2006; **96**: 252-261
23 **Feskanich D**, Ma J, Fuchs CS, Kirkner GJ, Hankinson SE, Hollis BW, Giovannucci EL. Plasma vitamin D metabolites and risk of colorectal cancer in women. *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 1502-1508
24 **Giovannucci E**, Liu Y, Willett WC. Cancer incidence and mortality and vitamin D in black and white male health professionals. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 2467-2472
25 **Pilz S**, Dobnig H, Winklhofer-Roob B, Riedmuller G, Fischer JE, Seelhorst U, Wellnitz B, Boehm BO, Marz W. Low serum levels of 25-hydroxyvitamin D predict fatal cancer in patients referred to coronary angiography. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 1228-1233
26 **Lamprecht SA**, Lipkin M. Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. *Nat Rev Cancer* 2003; **3**: 601-614
27 **Slatter ML**, Yakumo K, Hoffman M, Neuhausen S. Variants of the VDR gene and risk of colon cancer (United States). *Cancer Causes Control* 2001; **12**: 359-364
28 **Hubner RA**, Muir KR, Liu JF, Logan RF, Grainge MJ, Houlston RS. Dairy products, polymorphisms in the vitamin D receptor gene and colorectal adenoma recurrence. *Int J Cancer* 2008; **123**: 586-593
29 **Holt PR**, Bresalier RS, Ma CK, Liu KF, Lipkin M, Byrd JC, Yang K. Calcium plus vitamin D alters preneoplastic features of colorectal adenomas and rectal mucosa. *Cancer* 2006; **106**: 287-296
30 **Cho E**, Smith-Warner SA, Spiegelman D, Beeson WL, van den Brandt PA, Colditz GA, Folsom AR, Fraser GE, Freudenheim JL, Giovannucci E, Goldbohm RA, Graham S, Miller AB, Pietinen P, Potter JD, Rohan TE, Terry P, Toniolo P, Virtanen MJ, Willett WC, Wolk A, Wu K, Yaun SS, Zeleniuch-Jacquotte A, Hunter DJ. Dairy foods, calcium, and colorectal cancer: a pooled analysis of 10 cohort studies. *J Natl Cancer Inst* 2004; **96**: 1015-1022
31 **Wactawski-Wende J**, Kotchen JM, Anderson GL, Assaf AR, Brunner RL, O'Sullivan MJ, Margolis KL, Ockene JK, Phillips L, Pottern L, Prentice RL, Robbins J, Rohan TE, Sarto GE, Sharma S, Stefanick ML, Van Horn L, Wallace RB, Whitlock E, Bassford T, Beresford SA, Black HR, Bonds DE, Brzyski RG, Caan B, Chlebowski RT, Cochrane B, Garland C, Gass M, Hays J, Heiss G, Hendrix SL, Howard BV, Hsia J, Hubbell FA, Jackson RD, Johnson KC, Judd H, Kooperberg CL, Kuller LH, LaCroix AZ, Lane DS, Langer RD, Lasser NL, Lewis CE, Limacher MC, Manson JE. Calcium plus vitamin D supplementation and the risk of colorectal cancer. *N Engl J Med* 2006; **354**: 684-696
32 **Lappe JM**, Travers-Gustafson D, Davies KM, Recker RR, Heaney RP. Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. *Am J Clin Nutr* 2007; **85**: 1586-1591
33 **Autier P**, Gandini S. Vitamin D supplementation and total mortality: a meta-analysis of randomized controlled trials. *Arch Intern Med* 2007; **167**: 1730-1737
34 **Newmark HL**, Yang K, Lipkin M, Kopelovich L, Liu Y, Fan K, Shinozaki H. A Western-style diet induces benign and malignant neoplasms in the colon of normal C57Bl/6 mice. *Carcinogenesis* 2001; **22**: 1871-1875
35 **Yang K**, Yang W, Mariadason J, Velcich A, Lipkin M, Augenlicht L. Dietary components modify gene expression: implications for carcinogenesis. *J Nutr* 2005; **135**: 2710-2714
36 **Xue L**, Newmark H, Yang K, Lipkin M. Model of mouse mammary gland hyperproliferation and hyperplasia induced by a western-style diet. *Nutr Cancer* 1996; **26**: 281-287
37 **Xue L**, Yang K, Newmark H, Leung D, Lipkin M. Epithelial cell hyperproliferation induced in the exocrine pancreas of mice by a western-style diet. *J Natl Cancer Inst* 1996; **88**: 1586-1590
38 **Xue L**, Yang K, Newmark H, Lipkin M. Induced hyperproliferation in epithelial cells of mouse prostate by a Western-style diet. *Carcinogenesis* 1997; **18**: 995-999

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EDITORIAL

Molecular basis of the potential of mesalazine to prevent colorectal cancer

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Abstract

Patients with ulcerative colitis (UC) and Crohn's disease (CD) are at increased risk for developing colorectal cancer (CRC), and this is believed to be a result of chronic inflammation. Although conclusive evidence is still missing, both epidemiological and experimental observations suggest that certain drugs used to treat inflammation, such as mesalazine, can reduce the incidence of colitis-associated CRC. Therefore, in recent years, several studies have been conducted to dissect the mechanisms by which mesalazine interferes with CRC cell growth and survival. This review summarizes the current information on the molecular mechanisms that underlie the antineoplastic action of mesalazine.

Key words: Chemoprevention; Colorectal cancer; Cyclooxygenase-2; Epidermal growth factor receptor; Inflammatory bowel disease; Mesalazine; Wnt/β-catenin

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INTRODUCTION

Patients with ulcerative colitis (UC) and Crohn's disease (CD), the major forms of inflammatory bowel disease (IBD) in humans, are at increased risk of developing colorectal cancer (CRC)[1,2]. Chronic inflammation is believed to be the driving force for neoplastic transformation, and clinical factors that increase the risk include disease duration > 10 years, extensive disease, severity of colitis, a positive family history of sporadic CRC, and the concomitant presence of primary sclerosing cholangitis[3-5].

While in sporadic CRC, the dysplastic precursor is usually the adenomatous polyp, dysplasia in IBD patients can be both polypoid and flat, localized or diffuse. Detection of dysplasia during programmed screening and surveillance colonoscopy is the goal of the current strategy for CRC prevention in IBD patients. When dysplasia or CRC is identified, proctocolectomy is performed to remove the at-risk organ[6]. However, no evidence exists that screening for colonic dysplasia and cancer with surveillance colonoscopy prolongs survival in IBD patients. Indirect evidence also suggests that this is a cost-effective approach[7,8]. Therefore, gastroenterologists have recently shifted their attention towards alternative strategies of chemoprevention, and particular interest has been given to the possibility of reducing the risk of IBD-related CRC by using mesalazine or 5-aminosalicylic acid (5-ASA). Mesalazine is widely used in the maintenance of remission and in the treatment of mild flare-ups of IBD, and recent epidemiological studies have suggested that this drug is chemopreventive for CRC development in UC patients[9-11], even though some studies have documented no benefit[12]. The mechanisms behind the antineoplastic effect of mesalazine are incompletely understood, but it is likely that they are mostly dependent on the ability of the drug to attenuate ongoing mucosal inflammation. Indeed, it is well known that mesalazine can modulate various inflammatory pathways (e.g. production of inflammatory cytokines, activity of inducible nitric oxide synthase, activation of nuclear factor-κB) that are

relevant to CRC initiation and progression[13-16]. There is also evidence that mesalazine inhibits the formation of reactive oxygen species (ROS) from polymorphonuclear leukocytes[17], which leads to a decrease or complete inhibition of DNA damage, a phenomenon that has been involved in colon carcinogenesis. More recently, it has also been shown that mesalazine can directly target CRC cells and interfere with biological pathways that control their growth and survival[18]. The object of this article is to summarize recent data that elucidate the basic mechanisms of the antineoplastic effect of mesalazine.

MESALAZINE HAS DIRECT INHIBITORY EFFECTS ON CRC CELL GROWTH AND SURVIVAL

Carcinogenesis is a complex and multistage process that involves interactions between genes and environmental insults which ultimately affect cell proliferation and apoptosis. Apoptosis progressively decreases and proliferation increases in the sequential stages from normal colonic mucosa to dysplastic and CRC tissue. Therefore, strategies that inhibit cell proliferation and/or restore cell susceptibility to apoptosis have been shown to be effective in interfering with CRC initiation and/or progression.

Accumulating evidence indicates that mesalazine can block growth and promote apoptosis of CRC cells. This was initially suggested by *ex vivo* studies in patients with colonic adenoma. In particular, Reinacker-Schick *et al* have analyzed the effect of orally administered mesalazine on apoptosis and proliferation of colorectal mucosa in 21 patients with sporadic polyps. An increase in the apoptotic rate and decrease in cell proliferation were seen 1 and 3 d, respectively, after the initiation of treatment with mesalazine[19]. Bus *et al* have demonstrated that 2 wk treatment with 4 g/d mesalazine enema in patient with sporadic CRC resulted in enhanced apoptosis of tumor cells, while no change was seen in the normal mucosa that surrounded the tumor lesion. Moreover, the cellular proliferation rate as assessed by means of Ki-67 expression was unchanged in both the tumor and normal tissue[20]. Studies in rodent models of CRC showed that mesalazine inhibits tumor growth and reduces the number of aberrant cryptic foci[21,22]. Moreover, in a mouse model of colitis-associated CRC, Ikeda *et al* have shown that mesalazine, given in the remission stage of colitis, markedly suppresses the number and size of neoplasms. Notably, mesalazine treatment reduces the rate of proliferation of tumor cells, which leaves the proliferation of normal epithelial cells unaltered[23]. These observations have been reinforced by *in vitro* studies that show that mesalazine inhibits the growth and enhances apoptosis of several cultured CRC cell lines, in a time- and dose-dependent manner[18,24]. Altogether, these later findings indicate that mesalazine has direct effects on CRC cells. This novel information has boosted new research aimed at dissecting the molecular mechanisms by which mesalazine interferes with CRC development/growth.

EFFECTS OF MESALAZINE ON REPLICATION FIDELITY

Many of the molecular alterations that are believed to play a major role in the development of sporadic CRC are also seen in IBD-associated CRC tissue. For instance, both these types of CRC are characterized by a very similar frequency of the two main types of genomic instability, namely chromosomal instability (CIN, 85%) and microsatellite instability (MSI, 15%)[25]. CIN results in abnormal segregation of chromosomes and abnormal DNA content (aneuploidy). As a result, loss of chromosomal material often occurs, which contributes to the loss of function of important tumor suppressor genes [e.g. adenomatous polyposis coli (*APC*) and *p53*]. The MSI pathway involves the primary loss of function of genes that usually repair DNA base-pair mismatches that occur during the normal process of DNA replication in dividing cells. During this process, frameshift mutations, called microsatellites, tend to accumulate. Since microsatellites are mainly located in intronic DNA sequences, microsatellite mutations generally result in no gene function alteration. However, if microsatellites are located in exonic gene regions, the mutations can lead to a shift in the codon reading frame and changes in the amino acid sequence during mRNA translation. The introduction of an early stop codon can eventually cause protein truncation, and this is generally associated with a loss of protein function[26]. Recently, Gasche and colleagues, using an assay based on a stable transfection of a plasmid carrying a microsatellite sequence into HCT-116 cells, have shown that mesalazine improves replication fidelity in cultured CRC cell lines by reducing frameshift mutations at microsatellites. Since the effect of mesalazine is seen in mismatch repair-deficient CRC cells, it is highly likely that mesalazine acts on replication fidelity independently of post-replicational mismatch repair. The molecular mechanism by which mesalazine inhibits the generation of frameshift mutations has not yet been characterized. However, studies by the same group have shown that mesalazine can interact with cellular machinery involved in cell-cycle progression[28]. In particular, it has been shown that mesalazine can slow down DNA replication and cell division, thus allowing cells to either repair DNA damage or undergo apoptosis. Analysis of cell-cycle phases has revealed that CRC cells are arrested in S-phase when treated with mesalazine for 24-48 h. Such results are, however, somewhat different from those published by Reinacher-Schick *et al* that have shown that CRC cells are arrested in G2-M phase when exposed to mesalazine[18]. The

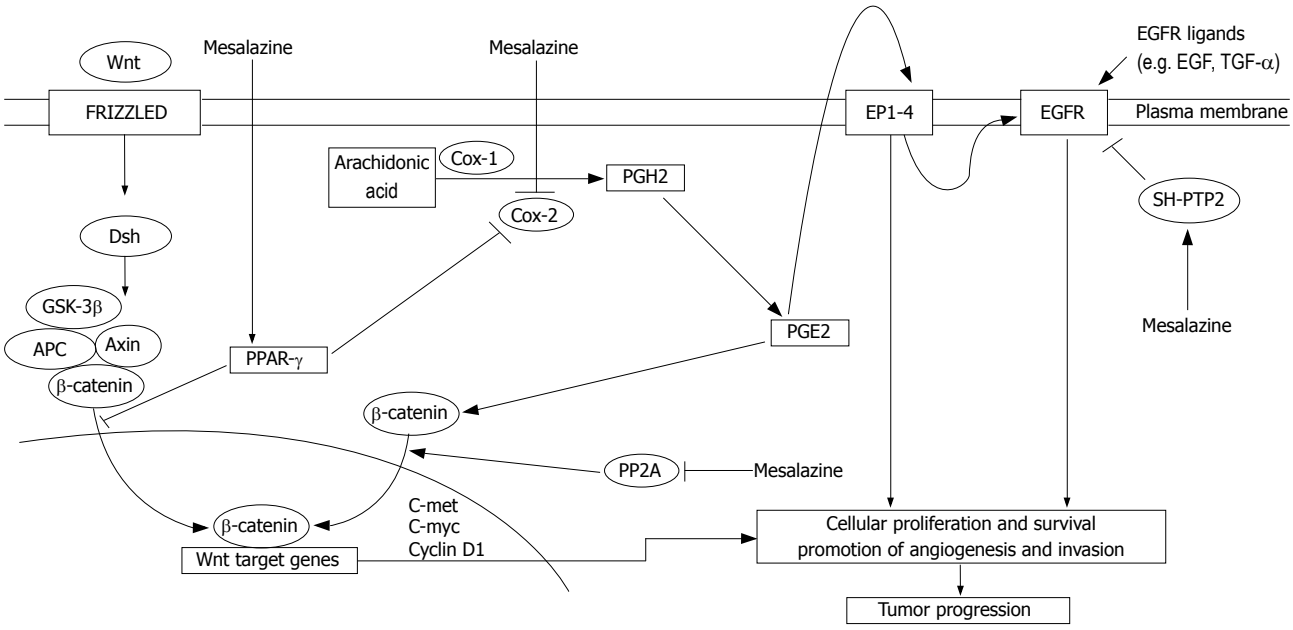

Figure 1 Some putative molecular mechanisms that underlie the antineoplastic activity of mesalazine. Mesalazine inhibits COX-2, thereby blocking synthesis of PGH2, an intermediate that is metabolized into various prostaglandins, including PGE2. PGE2 binds to its cell surface cognate receptors (EP1-4) and sustains various functions of tumor cells, including proliferation, survival, angiogenesis and invasion. These are mediated through the transactivation of EGFR or activation of other intracellular pathways. The antineoplastic effects of mesalazine rely also on its ability to inhibit Wnt/β-catenin, through inactivation of PP2A (and downstream down-regulation of β-catenin), and EGFR pathways. Moreover, mesalazine activates PPAR-γ, thus leading to inactivation of the Wnt/β-catenin pathway and down-regulation of COX-2.

reasons for these discrepancies remain unclear, but it is likely that they are due to differences in the culture systems used by these investigators.

THE ANTI-MITOGENIC EFFECT OF MESALAZINE IS NOT STRICTLY DEPENDENT ON CYCLOOXYGENASE (COX)-2 INHIBITION

COX-2 is a major target of CRC chemopreventive programs, as it is highly expressed in both sporadic and familial CRC, and activation of COX-2 is known to trigger and/or amplify biological pathways that sustain CRC growth[29-32]. COX-2 is also over-expressed in IBD-related CRC tissue[33]. Since mesalazine inhibits COX-2 in inflammatory cells, it is hypothesized that the antineoplastic effect of this drug is strictly dependent on the inhibition of COX-2 in CRC cells. In this context, we have recently shown that mesalazine inhibits the growth of HT-115, a CRC cell line that expresses a functionally active COX-2, and that the anti-mitogenic effect of mesalazine is associated with a marked down-regulation of COX-2 at the protein and mRNA level[34]. Consistently, treatment of HT-115 cells with mesalazine causes a significant reduction in secretion of prostaglandin (PG) E2, a product of COX-2 activity that positively regulates CRC cell growth (Figure 1). However, exogenously added PGE2 does not abrogate the inhibitory effect of mesalazine on HT-115 cell growth. Moreover, mesalazine blocks the growth of DLD-1, a CRC cell line that does not express COX-2, which suggests that the effect of this drug on CRC cell growth is partially independent of inhibition of the COX-2/PGE2 axis.

MESALAZINE INHIBITS BOTH THE WNT/β-CATENIN AND EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) SIGNALING PATHWAYS IN CRC CELLS

An intriguing hypothesis that has emerged from the available experimental data is that mesalazine may act on one or more pathways that are both early and common in colorectal carcinogenesis. A potential candidate is the wingless and integration site growth factor (Wnt)/β-catenin pathway, since it is constitutively activated in the majority of CRC[35-37]. In this pathway, Wnt binds to the transmembrane Frizzled receptor, which leads to activation of the cytoplasmic disheveled (Dsh) protein. Dsh forms a complex with the β-catenin degradation complex, which consists of the APC gene product, glycogen synthase kinase-3β (GSK-3β), axin and β-catenin. The current model for the Wnt signaling pathway proposes that, in the absence of Wnt, GSK-3β phosphorylates β-catenin, thereby promoting ubiquitination and degradation of β-catenin[38]. In response to Wnt signals, β-catenin is no longer targeted for degradation and accumulates to high levels in the cytoplasm[39]. The accumulated β-catenin translocates to the nucleus, associates with the transcriptional enhancers of the lymphoid enhancer-binding factor/Tcf family, and stimulates the expression of genes, such as *Myc*, that

play important roles in tumor progression[40,41].

Elegant studies by Bos *et al* have shown that mesalazine inhibits the Wnt/β-catenin pathway in APC-mutated CRC cells with intact β-catenin (Figure 1)[42]. Consistent with this, mesalazine treatment reduces expression of nuclear β-catenin and Wnt/β-catenin target genes (e.g. *cyclin D1, c-met and c-Myc*), and increased β-catenin phosphorylation. Mesalazine fails to inhibit the expression of Wnt/β-catenin target genes in β-catenin mutant CRC cell lines, in which the Wnt/β-catenin pathway is not regulated by β-catenin phosphorylation. These observations suggest that inhibition of the Wnt/β-catenin pathway by mesalazine is dependent on increased phosphorylation of β-catenin. In line with this, pre-incubation of CRC cells with okadaic acid, a specific phospho-serine/phospho-threonine phosphatase inhibitor, prevents mesalazine-induced β-catenin phosphorylation. The precise mechanism by which mesalazine enhances β-catenin phosphorylation remains to be ascertained, even though Bos *et al* have demonstrated that mesalazine reduces the activity of protein phosphatase 2A (PP2A), a known regulator of the β-catenin phosphorylation status and Wnt/β-catenin pathway in CRC cells.

Another target of mesalazine is EGFR (Figure 1). This receptor is highly activated in CRC cells, in which it is supposed to trigger mitogenic and pro-survival signals[43,44]. Consistent, EGFR inhibitors, such as monoclonal antibodies or small molecules that inhibit tyrosine kinase activity, have been shown to be effective in patients with advanced CRC[45]. A very high percentage of IBD-associated CRC displays immunohistochemical positivity for EGFR. Expression of EGFR is frequent in IBD-associated intestinal cancer[46]. Expression occurs at an early stage (i.e. premalignant lesions), as described in sporadic CRC[47], which suggests that blocking EGFR activity is useful for reducing the occurrence of IBD-related CRC[48,49]. We have shown that exposure of CRC cell lines to mesalazine results in a marked suppression of EGFR phosphorylation/activation, and that mesalazine-induced EGFR dephosphorylation is dependent on neither CRC cell death induction nor shedding of the receptor[50]. Moreover, mesalazine suppresses EGFR activation induced by exogenous EGF or TGF-α, thus excluding the possibility that dephosphorylation of EGFR in mesalazine-treated cells is secondary to inhibition of EGFR ligand synthesis. EGFR phosphorylation is a tightly controlled phenomenon, which is the net result of the action of tyrosine kinase and phosphatases (PTPs). Therefore, we have examined whether mesalazine-mediated inhibition of EGFR activation reflects changes in the expression/activity of PTPs, which have been reported to control the extent of EGFR activation[51]. Notably, treatment of CRC cells with mesalazine causes a significant increase in the activity, but not expression, of phosphorylated-EGFR-targeting PTPs, and pre-incubation of cells with PTP inhibitors largely reduces the inhibitory effect of mesalazine on EGFR activation. Among these PTPs, both SH-PTP1 and SH-PTP2 interact with EGFR upon mesalazine treatment. However, targeted silencing of SH-PTP2, but not SH-PTP1 prevents mesalazine-induced EGFR dephosphorylation. Consistent with these data, mesalazine also attenuates EGFR phosphorylation in *ex vivo* organ cultures of human sporadic CRC explants.

MESALAZINE ACTIVATES PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-γ (PPAR-γ) IN CRC CELLS

PPAR-γ is a nuclear receptor that is highly expressed in the colon, and plays a key role in bacteria-induced inflammation. Many factors can modulate the activity of PPAR-γ, but the most important activating factor in colon epithelial cells appears to be the luminal flora[52]. Activation of PPAR-γ also has anti-tumorigenic effects that are manifested as both anti-proliferative and pro-apoptotic activities[53, 54], inhibition of the formation of aberrant cryptic foci[55], and inhibition of CRC development[56]. It has also been shown that PPAR-γ suppresses tumor formation by interfering with the Wnt/β-catenin signaling pathway[57,58]. Recent *in vitro* and *in vivo* studies have shown that mesalazine can activate PPAR-γ (Figure 1). In particular, using HT-29 CRC cells, Rousseaux *et al* have shown that mesalazine enhances PPAR-γ expression, stimulates translocation of PPAR-γ to the nucleus, induces conformational changes in the PPAR-γ molecule, and increases the interaction between PPAR-γ and vitamin D3 receptor-interacting protein 205[59]. In competitive binding studies, mesalazine displaces rosiglitazone and the selective PPAR-γ ligand GW1929 from their binding sites on the PPAR-γ molecule[59]. In line with these *in vitro* findings, it has been shown that the antineoplastic effects of mesalazine are mediated by PPAR-γ in a model of CRC, in which SCID mice were engrafted with human CRC cells. In particular, in this model, locally administered mesalazine significantly reduced the growth of xenografts, and this effect was blocked by the selective PPAR-γ antagonist GW9662[60].

CONCLUSION

In recent years, there has been great interest in the possibility of chemoprevention of IBD-related CRC by mesalazine. Given the difficulty of performing double-blind, placebo-controlled, randomized clinical trials in patients, investigators have turned to experimental models of cancer, and indeed, the existing data suggest that mesalazine can reduce the risk of CRC by directly interfering with CRC cell biology, other than by simply controlling inflammation. However, definitive conclusions from experimental findings are not always completely correct, and therefore, future studies will be necessary to ascertain whether data generated from studies with cultured cells or animal models of CRC can be generalized to IBD-associated dysplasia or CRC.

Another important issue that needs further investigation regards the dosage/concentration of

mesalazine required to interfere with CRC cell growth and survival. *In vitro* studies have indicated that the antineoplastic effect of mesalazine is seen with relative high drug dosages (e.g. 10-50 mmol/L), which are not always reached within the colonic tissue under standard oral treatment. In this context, it is also relevant to take into consideration mesalazine metabolism, for instance, mesalazine oxidation and acetylation, which could differ considerably between *in vitro* and *in vivo* circumstances, and limit the amount of biologically active compound.

REFERENCES

1 **Eaden JA,** Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001; **48**: 526-535
2 **Jess T,** Gamborg M, Matzen P, Munkholm P, Sorensen TI. Increased risk of intestinal cancer in Crohn's disease: a meta-analysis of population-based cohort studies. *Am J Gastroenterol* 2005; **100**: 2724-2729
3 **Munkholm P.** Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease. *Aliment Pharmacol Ther* 2003; **18** Suppl 2: 1-5
4 **Rutter M,** Saunders B, Wilkinson K, Rumbles S, Schofield G, Kamm M, Williams C, Price A, Talbot I, Forbes A. Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. *Gastroenterology* 2004; **126**: 451-459
5 **Gupta RB,** Harpaz N, Itzkowitz S, Hossain S, Matula S, Kornbluth A, Bodian C, Ullman T. Histologic inflammation is a risk factor for progression to colorectal neoplasia in ulcerative colitis: a cohort study. *Gastroenterology* 2007; **133**: 1099-1105; quiz 1340-1341
6 **Eaden J.** Review article: colorectal carcinoma and inflammatory bowel disease. *Aliment Pharmacol Ther* 2004; **20** Suppl 4: 24-30
7 **Obrador A,** Ginard D, Barranco L. Review article: colorectal cancer surveillance in ulcerative colitis - what should we be doing? *Aliment Pharmacol Ther* 2006; **24** Suppl 3: 56-63
8 **Jess T,** Loftus EV Jr, Velayos FS, Harmsen WS, Zinsmeister AR, Smyrk TC, Schleck CD, Tremaine WJ, Melton LJ 3rd, Munkholm P, Sandborn WJ. Risk of intestinal cancer in inflammatory bowel disease: a population-based study from olmsted county, Minnesota. *Gastroenterology* 2006; **130**: 1039-1046
9 **Velayos FS,** Terdiman JP, Walsh JM. Effect of 5-aminosalicylate use on colorectal cancer and dysplasia risk: a systematic review and metaanalysis of observational studies. *Am J Gastroenterol* 2005; **100**: 1345-1353
10 **Van Staa TP,** Card T, Logan RF, Leufkens HG. 5-Aminosalicylate use and colorectal cancer risk in inflammatory bowel disease: a large epidemiological study. *Gut* 2005; **54**: 1573-1578
11 **Eaden J,** Abrams K, Ekbom A, Jackson E, Mayberry J. Colorectal cancer prevention in ulcerative colitis: a case-control study. *Aliment Pharmacol Ther* 2000; **14**: 145-153
12 **Bernstein CN,** Blanchard JF, Metge C, Yogendran M. Does the use of 5-aminosalicylates in inflammatory bowel disease prevent the development of colorectal cancer? *Am J Gastroenterol* 2003; **98**: 2784-2788
13 **Bantel H,** Berg C, Vieth M, Stolte M, Kruis W, Schulze-Osthoff K. Mesalazine inhibits activation of transcription factor NF-kappaB in inflamed mucosa of patients with ulcerative colitis. *Am J Gastroenterol* 2000; **95**: 3452-3457
14 **Kaiser GC,** Yan F, Polk DB. Mesalamine blocks tumor necrosis factor growth inhibition and nuclear factor kappaB activation in mouse colonocytes. *Gastroenterology* 1999; **116**: 602-609
15 **Mahida YR,** Lamming CE, Gallagher A, Hawthorne AB, Hawkey CJ. 5-Aminosalicylic acid is a potent inhibitor of interleukin 1 beta production in organ culture of colonic biopsy specimens from patients with inflammatory bowel disease. *Gut* 1991; **32**: 50-54
16 **Kennedy M,** Wilson L, Szabo C, Salzman AL. 5-aminosalicylic acid inhibits iNOS transcription in human intestinal epithelial cells. *Int J Mol Med* 1999; **4**: 437-443
17 **Miyachi Y,** Yoshioka A, Imamura S, Niwa Y. Effect of sulphasalazine and its metabolites on the generation of reactive oxygen species. *Gut* 1987; **28**: 190-195
18 **Reinacher-Schick A,** Schoeneck A, Graeven U, Schwarte-Waldhoff I, Schmiegel W. Mesalazine causes a mitotic arrest and induces caspase-dependent apoptosis in colon carcinoma cells. *Carcinogenesis* 2003; **24**: 443-451
19 **Reinacher-Schick A**, Seidensticker F, Petrasch S, Reiser M, Philippou S, Theegarten D, Freitag G, Schmiegel W. Mesalazine changes apoptosis and proliferation in normal mucosa of patients with sporadic polyps of the large bowel. *Endoscopy* 2000; **32**: 245-254
20 **Bus PJ,** Nagtegaal ID, Verspaget HW, Lamers CB, Geldof H, Van Krieken JH, Griffioen G. Mesalazine-induced apoptosis of colorectal cancer: on the verge of a new chemopreventive era? *Aliment Pharmacol Ther* 1999; **13**: 1397-1402
21 **Brown WA,** Farmer KC, Skinner SA, Malcontenti-Wilson C, Misajon A, O'Brien PE. 5-aminosalicyclic acid and olsalazine inhibit tumor growth in a rodent model of colorectal cancer. *Dig Dis Sci* 2000; **45**: 1578-1584
22 **MacGregor DJ,** Kim YS, Sleisenger MH, Johnson LK. Chemoprevention of colon cancer carcinogenesis by balsalazide: inhibition of azoxymethane-induced aberrant crypt formation in the rat colon and intestinal tumor formation in the B6-Min/+ mouse. *Int J Oncol* 2000; **17**: 173-179
23 **Ikeda I,** Tomimoto A, Wada K, Fujisawa T, Fujita K, Yonemitsu K, Nozaki Y, Endo H, Takahashi H, Yoneda M, Inamori M, Kubota K, Saito S, Nagashima Y, Nakagama H, Nakajima A. 5-aminosalicylic acid given in the remission stage of colitis suppresses colitis-associated cancer in a mouse colitis model. *Clin Cancer Res* 2007; **13**: 6527-6531
24 **Fina D,** Franchi L, Caruso R, Peluso I, Naccari GC, Bellinvia S, Testi R, Pallone F, Monteleone G. 5-aminosalicylic acid enhances anchorage-independent colorectal cancer cell death. *Eur J Cancer* 2006; **42**: 2609-2616
25 **Lengauer C,** Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature* 1998; **396**: 643-649
26 **Ionov Y,** Peinado MA, Malkhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 1993; **363**: 558-561
27 **Gasche C,** Goel A, Natarajan L, Boland CR. Mesalazine improves replication fidelity in cultured colorectal cells. *Cancer Res* 2005; **65**: 3993-3997
28 **Luciani MG,** Campregher C, Fortune JM, Kunkel TA, Gasche C. 5-ASA affects cell cycle progression in colorectal cells by reversibly activating a replication checkpoint. *Gastroenterology* 2007; **132**: 221-235
29 **Huls G,** Koornstra JJ, Kleibeuker JH. Non-steroidal anti-inflammatory drugs and molecular carcinogenesis of colorectal carcinomas. *Lancet* 2003; **362**: 230-232
30 **Koehne CH,** Dubois RN. COX-2 inhibition and colorectal cancer. *Semin Oncol* 2004; **31**: 12-21
31 **Dubois RN,** Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, Lipsky PE. Cyclooxygenase in biology and disease. *FASEB J* 1998; **12**: 1063-1073
32 **Tsujii M,** Kawano S, DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc Natl Acad Sci USA* 1997; **94**: 3336-3340
33 **Eaden J.** Review article: the data supporting a role for aminosalicylates in the chemoprevention of colorectal cancer in patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2003; **18** Suppl 2: 15-21
34 **Stolfi C,** Fina D, Caruso R, Caprioli F, Sarra M, Fantini MC, Rizzo A, Pallone F, Monteleone G. Cyclooxygenase-

2-dependent and -independent inhibition of proliferation of colon cancer cells by 5-aminosalicylic acid. *Biochem Pharmacol* 2008; **75**: 668-676
35 **Polakis P.** Wnt signaling and cancer. *Genes Dev* 2000; **14**: 1837-1851
36 **Behrens J,** Lustig B. The Wnt connection to tumorigenesis. *Int J Dev Biol* 2004; **48**: 477-487
37 **Oving IM,** Clevers HC. Molecular causes of colon cancer. *Eur J Clin Invest* 2002; **32**: 448-457
38 **Orford K,** Crockett C, Jensen JP, Weissman AM, Byers SW. Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. *J Biol Chem* 1997; **272**: 24735-24738
39 **Miller JR,** Hocking AM, Brown JD, Moon RT. Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca2+ pathways. *Oncogene* 1999; **18**: 7860-7872
40 **Ponzielli R,** Katz S, Barsyte-Lovejoy D, Penn LZ. Cancer therapeutics: targeting the dark side of Myc. *Eur J Cancer* 2005; **41**: 2485-2501
41 **Mann B,** Gelos M, Siedow A, Hanski ML, Gratchev A, Ilyas M, Bodmer WF, Moyer MP, Riecken EO, Buhr HJ, Hanski C. Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc Natl Acad Sci USA* 1999; **96**: 1603-1608
42 **Bos CL,** Diks SH, Hardwick JC, Walburg KV, Peppelenbosch MP, Richel DJ. Protein phosphatase 2A is required for mesalazine-dependent inhibition of Wnt/beta-catenin pathway activity. *Carcinogenesis* 2006; **27**: 2371-2382
43 **Jorissen RN,** Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp Cell Res* 2003; **284**: 31-53
44 **Bradley SJ,** Garfinkle G, Walker E, Salem R, Chen LB, Steele G Jr. Increased expression of the epidermal growth factor receptor on human colon carcinoma cells. *Arch Surg* 1986; **121**: 1242-1247
45 **Janmaat ML,** Giaccone G. The epidermal growth factor receptor pathway and its inhibition as anticancer therapy. *Drugs Today* (Barc) 2003; **39** Suppl C: 61-80
46 **Svrcek M,** Cosnes J, Tiret E, Bennis M, Parc Y, Flejou JF. Expression of epidermal growth factor receptor (EGFR) is frequent in inflammatory bowel disease (IBD)-associated intestinal cancer. *Virchows Arch* 2007; **450**: 243-244
47 **Martin ES,** Tonon G, Sinha R, Xiao Y, Feng B, Kimmelman AC, Protopopov A, Ivanova E, Brennan C, Montgomery K, Kucherlapati R, Bailey G, Redston M, Chin L, DePinho RA. Common and distinct genomic events in sporadic colorectal cancer and diverse cancer types. *Cancer Res* 2007; **67**: 10736-10743
48 **Mendelsohn J.** The epidermal growth factor receptor as a target for cancer therapy. *Endocr Relat Cancer* 2001; **8**: 3-9
49 **Mendelsohn J,** Baselga J. The EGF receptor family as targets for cancer therapy. *Oncogene* 2000; **19**: 6550-6565
50 **Monteleone G,** Franchi L, Fina D, Caruso R, Vavassori P, Monteleone I, Calabrese E, Naccari GC, Bellinvia S, Testi R, Pallone F. Silencing of SH-PTP2 defines a crucial role in the inactivation of epidermal growth factor receptor by 5-aminosalicylic acid in colon cancer cells. *Cell Death Differ* 2006; **13**: 202-211
51 **Moghal N,** Sternberg PW. Multiple positive and negative regulators of signaling by the EGF-receptor. *Curr Opin Cell Biol* 1999; **11**: 190-196
52 **Dubuquoy L,** Rousseaux C, Thuru X, Peyrin-Biroulet L, Romano O, Chavatte P, Chamaillard M, Desreumaux P. PPARgamma as a new therapeutic target in inflammatory bowel diseases. *Gut* 2006; **55**: 1341-1349
53 **Matthiessen MW,** Pedersen G, Albrektsen T, Adamsen S, Fleckner J, Brynskov J. Peroxisome proliferator-activated receptor expression and activation in normal human colonic epithelial cells and tubular adenomas. *Scand J Gastroenterol* 2005; **40**: 198-205
54 **Shimada T,** Kojima K, Yoshiura K, Hiraishi H, Terano A. Characteristics of the peroxisome proliferator activated receptor gamma (PPARgamma) ligand induced apoptosis in colon cancer cells. *Gut* 2002; **50**: 658-664
55 **Tanaka T,** Kohno H, Yoshitani S, Takashima S, Okumura A, Murakami A, Hosokawa M. Ligands for peroxisome proliferator-activated receptors alpha and gamma inhibit chemically induced colitis and formation of aberrant crypt foci in rats. *Cancer Res* 2001; **61**: 2424-2428
56 **Osawa E,** Nakajima A, Wada K, Ishimine S, Fujisawa N, Kawamori T, Matsuhashi N, Kadowaki T, Ochiai M, Sekihara H, Nakagama H. Peroxisome proliferator-activated receptor gamma ligands suppress colon carcinogenesis induced by azoxymethane in mice. *Gastroenterology* 2003; **124**: 361-367
57 **Jansson EA,** Are A, Greicius G, Kuo IC, Kelly D, Arulampalam V, Pettersson S. The Wnt/beta-catenin signaling pathway targets PPARgamma activity in colon cancer cells. *Proc Natl Acad Sci USA* 2005; **102**: 1460-1465
58 **Lu D,** Cottam HB, Corr M, Carson DA. Repression of beta-catenin function in malignant cells by nonsteroidal antiinflammatory drugs. *Proc Natl Acad Sci USA* 2005; **102**: 18567-18571
59 **Rousseaux C,** Lefebvre B, Dubuquoy L, Lefebvre P, Romano O, Auwerx J, Metzger D, Wahli W, Desvergne B, Naccari GC, Chavatte P, Farce A, Bulois P, Cortot A, Colombel JF, Desreumaux P. Intestinal antiinflammatory effect of 5-aminosalicylic acid is dependent on peroxisome proliferator-activated receptor-gamma. *J Exp Med* 2005; **201**: 1205-1215
60 **Desreumaux P,** Ghosh S. Review article: mode of action and delivery of 5-aminosalicylic acid - new evidence. *Aliment Pharmacol Ther* 2006; **24** Suppl 1: 2-9

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EDITORIAL

Thrombosis and inflammatory bowel disease-the role of genetic risk factors

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Abstract

Thromboembolism is a significant cause of morbidity and mortality in patients with inflammatory bowel disease (IBD). Recent data suggest thromboembolism as a disease-specific extraintestinal manifestation of IBD, which is developed as the result of multiple interactions between acquired and genetic risk factors. There is evidence indicating an imbalance of procoagulant, anticoagulant and fibrinolytic factors predisposing in thrombosis in patients with IBD. The genetic factors that have been suggested to interfere in the thrombotic manifestations of IBD include factor Ⅴ Leiden, factor Ⅱ (prothrombin, G20210A), methylenetetrahydrofolate reductase gene mutation (MTHFR, 6777T), plasminogen activator inhibitor type 1 (PAI-1) gene mutation and factor ⅩⅢ (val34leu). In this article we review the current data and future prospects on the role of genetic risk factors in the development of thromboembolism in IBD.

Key words: Crohn's disease; Factor V Leiden; Genetics; Thrombosis; Ulcerative colitis

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INTRODUCTION

Inflammatory bowel disease (IBD) is associated with an increased risk of vascular complications[1-4]. The most important of these complications are arterial and venous thromboembolisms, which represent a significant cause of morbidity and mortality in IBD patients. Thromboembolism is a disease-specific extraintestinal manifestation of IBD[5], which is developed as the result of multiple interactions between acquired and genetic risk factors. Several studies have shown an imbalance of procoagulant, anticoagulant and fibrinolytic factors predisposing in thrombosis in patients with IBD[6-9]. The incidence of thromboembolism in IBD ranges between 1% and 7.7% in clinical studies[1,10,11], rising to 39%-41% in postmortem studies[12].

The most common thrombotic manifestations in IBD are deep vein thrombosis, usually in the leg, and pulmonary embolism. The latter may be fatal. Thrombotic events occur less frequently in other sites, such as the cerebrovascular system, portal vein, mesenteric veins, and retinal vein. Arterial thrombotic complications occur less frequently than venous thromboembolism in patients with IBD, and most occur after surgery. An increased atherosclerotic risk in patients with IBD has also been suggested[13]. This could be associated with the elevated levels of C-reactive protein and plasma sCD40L, which are features of the IBD[14,15]. Common carotid artery intima-media thickness has been found significantly higher in IBD patients compared with healthy controls[16]. Premature atherosclerosis with lower extremity arterial occlusions has been reported in young patients with Crohn's disease[17]. An increased risk of cardiovascular arterial thromboembolic diseases in both Crohn's disease and ulcerative colitis and an increase in cerebrovascular arterial thromboembolic diseases in Crohn's disease recently have been reported[18]. IBD patients have been found to develop thrombembolisms earlier in life than non-IBD thrombotic patients[19]. Furthermore, many IBD patients with thromboembolic disorders either have active disease or have undergone recent major abdominal surgery[2]. Conversely, thromboembolisms may also occur in quiescent IBD[1]. It has been suggested that the extent of colonic disease is correlated with thromboembolic risk. Extensive ulcerative colitis and colonic involvement of Crohn's disease was significantly associated with the development of thromboembolism[2]. The risk of recurrence of thromboembolism in IBD patients has been

reported to be 10%-13% despite medical therapy for the thromboembolic event[2]. The mortality from this complication in IBD has been reported to be 8%-25% during the acute episode[1,2]. The etiology of thrombosis in IBD is multifactorial. Thrombosis is a complex event in which several mechanisms and causal factors, inherited and acquired, are implicated, complicating the identification of its causes[20,21]. Several acquired prothrombotic risk factors are frequently observed, such as the inflammatory process per se, prolonged immobilization, use of corticosteroids, surgical treatment, fluid depletion, central venous catheters, hyperhomocysteinemia, vitamin deficiencies, smoking and use of oral contraceptives[4]. Importantly, approximately half of the patients with inflammatory bowel disease that develop a thromboembolic event have no identifiable risk factor[10]. Genetic factors may also play a role in thrombosis of patients with IBD. Based on the unraveling of the biochemistry and cell biology of the coagulation system, major advances in the understanding of genetics of thrombosis have been applied also in IBD complicating by thrombosis. The most common genetic variants that have been found to affect the risk of thrombosis are: factor V Leiden, factor Ⅱ (prothrombin, G20210A), methylenetetrahydrofolate reductase gene mutation (MTHFR, 6777T), plasminogen activator inhibitor type 1 (PAI-1) gene mutation and factor XⅢ (val34leu). This review focuses on the role of genetic risk factors in the development of thromboembolism in IBD.

EVALUATING GENETIC RISK OF THROMBOSIS IN INFLAMMATORY BOWEL DISEASE

Genetic risk factors have been suggested to predispose to thromboembolism in IBD. Several genetic markers located in coagulation genes have been examined in an attempt to document whether these markers are associated with increased thrombotic risk in IBD. Genetic studies of vascular complications of IBD pose enormous challenges, including resolution of immense genotypic and phenotypic heterogeneity, and gene-environment and gene-gene interactions. Past efforts to identify thrombosis-related genes in IBD have utilized population-based association methods, but the substantial progress that has been made recently with the strategy of using positional cloning of genes based on linkage studies is expected to apply to this field. These methods focus on measuring quantitative traits that are correlated with the risk of disease.

Factor V Leiden

Factor V Leiden (FVL), an arginine to glutamine missense mutation in the factor V (FV) gene at position 506[22], is the most prominent risk factor for venous thromboembolism[23,24]. The amino acid substitution in the activated protein C (APC) cleavage site of FV leads to increased thrombin generation due to decreased APC-mediated inactivation of FV, and due to decreased FV cofactor activity for FVIIIa inactivation[25]. Factor V Leiden is found in approximately 5% of Caucasians, and increases the risk of thrombosis five- to eightfold for heterozygous carriers and 50- to 80-fold for homozygous carriers. The prevalence of FVL ranges from 20% to 30% in unselected patients with venous thrombosis. Genetic studies determining the prevalence of the FVL allele in IBD mostly have shown no difference in allele frequency between IBD patients and healthy controls[26-30]. The prevalence of FVL in thrombotic IBD patients was significantly higher than in IBD patients without thrombosis[31-33]. It is noteworthy that the FVL allele was associated mainly with venous thrombosis[33]. In addition, the prevalence of FVL in IBD patients with previous thromboembolism appears not to differ from that found in non-IBD patients with thromboembolism[33,34]. On the other hand, an Italian study showed a very low prevalence of FVL allele in thrombotic IBD patients[35]. The discrepancies between these results may be due to the different characteristics of the populations studied (genetic background, previous history of thrombosis, and small sample sizes). Furthermore, in a recent study of experimental colitis, the FVL allele had no effect in murine colitis and thus, the authors question the role of activated blood coagulation in IBD[36]. Although somewhat conflicting, these genetic studies suggest that the FVL as a risk factor for thrombosis in IBD patients matches that of the general population. Furthermore, FVL is not associated with IBD per se, but when present it increases the risk of thromboembolism[37]. Finally, it is important to realize that homozygous carriers of the FVL allele are rare and that all genetic studies are performed using heterozygotes, which have a milder thrombotic phenotype.

G20210A prothrombin gene mutation

The G20210A mutation is a genetic variation of the prothrombin (Factor Ⅱ) gene consisting of a single nucleotide change (guanine to adenine) at position 20210 of the 3'-untranslated region. The G20210A mutation is the second most frequent genetic prothrombotic mutation after FVL. It is present in approximately 2% of Caucasians, leads to greater prothrombin plasma levels (heterozygous carriers have about 30% higher PT levels than healthy controls), and increases the risk of venous thrombosis about threefold[38]. However, no definite association between this gene mutation and IBD has been detected in several studies[30,33-35,39]. Presence of this mutation is found at a similar prevalence in IBD patients as well as in IBD patients with thrombosis[27,32-33]. This mutation has only been studied in a limited number of thrombotic IBD patients so firm conclusions cannot be drawn.

Methylenetetrahydrofolate reductase C677T gene mutation

Methylenetetrahydrofolate reductase is a critical enzyme involved in the remethylation pathway of homocysteine metabolism. A common mutation (C677T) has been identified in the MTHFR gene. This variant leads to 10%-20% increases in homocysteine plasma levels in homozygous carriers, which are found in around 10% of

the population. The effect of MTHFR 6777T carriership on the risk of thrombosis varies among studies, and a recent meta-analysis found a weak effect (10%-20% risk increase)[40-43]. Studies of the prevalence of C677T homozygosity in IBD have found discordant results[34], probably because of regional and ethnic variations in the prevalence of polymorphism in the general population. In a recent population-based case-control study[30], although some differences were observed among patients with IBD and healthy controls in the prevalence of MTHFR 6777T (decrease in mutant allele carriership in UC), these did not explain an excess risk of thrombosis. The prevalence of C677T homozygosity between IBD thrombotic patients and non-IBD thrombotic patients showed no significant difference[32,33].

Factor XIII gene mutation

A common variant in subunit A of factor XIII, usually indicated by the amino acid position and change (val34leu), is associated with a greater FXIII activation rate and leads to a 20%-40% reduction of the risk of venous thrombosis for homozygous carriers. These are found in approximately 10% of the population[44,45]. This variant, which is protective against thrombosis, has been evaluated in IBD patients[46]. Available data suggest that the prevalence of this polymorphism is similar in patients with IBD compared to the general population[47,48]. A slightly greater prevalence of factor XIII mutation carriership in CD has been found in a recent population-based study[30], but this could not explain the greater risk of venous thrombosis in CD. Finally, the prevalence of XIII (val34leu) was similar in IBD patients with vascular complications and non-IBD thrombotic patients[33].

Plasminogen activator inhibitor type 1 gene mutation

Plasminogen activator inhibitor type 1 (PAI-1) is considered as inhibitor of fibrinolysis. The 4G/4G genotype is associated with an overexpression of PAI-1, which may cause a decreased fibrinolysis and, therefore, a hypercoagulability state contributing to the development of vascular complications. Several studies have demonstrated that the 4G/4G genotype is associated with an enhanced PAI-1 expression[49] and contributes as an additional risk factor to the development of myocardiac infarction[50], arterial thrombosis[51], and deep venous thrombosis[52]. However, the evidence regarding the relationship between an elevated PAI-1 plasma level or PAI-1 genetic polymorphism and the risk of venous thromboembolism is rather conflicting. The allelic frequency of PAI-1 4G has been reported higher in IBD patients than in the reference population[53]. Moreover, a recent study showed a significantly higher allelic frequency of PAI-1 4G in IBD patients with vascular complications compared with IBD and healthy controls. However, the prevalence of this genotype does not differ in thrombotic IBD patients compared to non-IBD thrombotic patients[33].

Janus kinase 2 gene mutation

Janus kinase 2 (JAK2) mutations have been described in several Philadelphia-negative myeloproliferative disorders (MPD)[54]. The point mutation in JAK2 encodes a valine to phenylalanine change at position 617 (JAK2 V617F) and confers constitutive tyrosine kinase activity[55]. It has been suggested that thrombosis in MPD may be due to JAK2 mutation. JAK2 is also important in vascular diseases, such as atherosclerosis, in which inflammation plays an important role. JAK2 V617F mutation has been found, in the absence of overt MPD, highly associated with splanchnic vein thrombosis and sporadically with cerebral thrombosis. A recent study investigated the role of JAK2 V617F mutation in 48 IBD patients with thrombotic complications, but no case with the JAK2 V617F mutation was found[56]. The small number of cases with splanchnic vein thrombosis in this series (but also in other IBD series) that are mainly associated JAK2 V617F mutation could be also an explanation of this finding.

Other genetic factors

The role of the well recognized inherited thrombophilic states such as deficiencies of plasma antithrombin III, protein C, and protein S has been examined in several studies. Although deficiency of the proteins C and S in IBD patients have been proposed by some studies[20], other studies failed to confirm these data[21]. These factors are rare (< 1% of the population) and are considered to play a less important role in the thrombosis, and therefore it is not surprising that their results in IBD are rather contradictory.

Future prospects

We are entering a new era in genetic studies of venous thrombosis. It is believed that, combined, all the known mutations account for about the half of the genetic thrombosis risk. There is every reason to believe that additional genetic causes of thrombosis remain to be discovered. As a complex disease, thrombosis is considered to be the result of flexible combinations of variations of multiple genes that interact with lifestyle or other environmental factors to produce the disease. Future studies should investigate these interactions. Moreover, in IBD we need collection of large case-control series of patients complicating with venous thrombosis. This will provide the high quality clinical information related both to IBD and thrombosis that forms the basis of any genetic project. On the other hand, large-scale DNA analysis systems are now becoming available, which will allow us to study in the setting of IBD the genetics of venous thrombosis down to the single nucleotide level. As an example, we recently have reported a multigenetic analysis of polymorphisms of thrombophilic and vasoactive genes in a group of IBD patients with vascular complications compared with IBD patients without vascular complications and both thrombotic and healthy controls[33]. This approach, in a multicenter basis with a large number of patients, will give more insight into the genetic architecture of thrombotic risk in IBD. The final aim is personalized thrombosis prediction and appropriate management in a patient with IBD.

CONCLUSION

Epidemiological data suggest that IBD is associated with an increased risk of thromboembolic complications. However, the cause for this strong association remains unclear. It can be speculated that gene mutations may underlie the greater risk for thromboembolic complications in IBD patients. Thus, several studies have investigated the role of genetic defects in the development of vascular complications in IBD. The most common genetic variants that affect the risk of thrombosis are factor V Leiden, factor Ⅱ (prothrombin, G20210A), MTHFR (6777T) and factor ⅩⅢ (val34leu). Furthermore, the role of other thrombophilic and vasoactive genes has been evaluated with the occurrence of thromboembolism in IBD. The available data in the literature have shown that genetic risk factors are generally not found more often in IBD patients than others. However, when they occur, those with IBD compared to healthy controls are more likely to suffer thromboembolic complications. The screening for genetic coagulation defects appears justified in all IBD patients with a history of thrombosis or a family history of venous thromboembolic events. Future multicentre studies with large number of cases and further investigation of the interaction between genetic and environmental factors might increase our understanding of the mechanisms of pathogenesis of thrombotic complications in IBD. Using the new large-scale DNA sequencing techniques that are becoming available and enable many genes to be studied in a single individual, we could expect an in-depth insight into how genetic risk factors are involved in thrombosis in IBD.

REFERENCES

1 **Talbot RW**, Heppell J, Dozois RR, Beart RW Jr. Vascular complications of inflammatory bowel disease. *Mayo Clin Proc* 1986; **61**: 140-145
2 **Solem CA**, Loftus EV, Tremaine WJ, Sandborn WJ. Venous thromboembolism in inflammatory bowel disease. *Am J Gastroenterol* 2004; **99**: 97-101
3 **Bernstein CN**, Blanchard JF, Houston DS, Wajda A. The incidence of deep venous thrombosis and pulmonary embolism among patients with inflammatory bowel disease: a population-based cohort study. *Thromb Haemost* 2001; **85**: 430-434
4 **Koutroubakis IE**. Therapy insight: Vascular complications in patients with inflammatory bowel disease. *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 266-272
5 **Miehsler W**, Reinisch W, Valic E, Osterode W, Tillinger W, Feichtenschlager T, Grisar J, Machold K, Scholz S, Vogelsang H, Novacek G. Is inflammatory bowel disease an independent and disease specific risk factor for thromboembolism? *Gut* 2004; **53**: 542-548
6 **Stadnicki A**, Gonciarz M, Niewiarowski TJ, Hartleb J, Rudnicki M, Merrell NB, Dela Cadena RA, Colman RW. Activation of plasma contact and coagulation systems and neutrophils in the active phase of ulcerative colitis. *Dig Dis Sci* 1997; **42**: 2356-2366
7 **van Bodegraven AA**, Schoorl M, Linskens RK, Bartels PC, Tuynman HA. Persistent activation of coagulation and fibrinolysis after treatment of active ulcerative colitis. *Eur J Gastroenterol Hepatol* 2002; **14**: 413-418
8 **van Bodegraven AA**. Haemostasis in inflammatory bowel diseases: clinical relevance. *Scand J Gastroenterol Suppl* 2003; 51-62
9 **Saibeni S**, Bottasso B, Spina L, Bajetta M, Danese S, Gasbarrini A, de Franchis R, Vecchi M. Assessment of thrombin-activatable fibrinolysis inhibitor (TAFI) plasma levels in inflammatory bowel diseases. *Am J Gastroenterol* 2004; **99**: 1966-1970
10 **Jackson LM**, O'Gorman PJ, O'Connell J, Cronin CC, Cotter KP, Shanahan F. Thrombosis in inflammatory bowel disease: clinical setting, procoagulant profile and factor V Leiden. *QJM* 1997; **90**: 183-188
11 **Webberley MJ**, Hart MT, Melikian V. Thromboembolism in inflammatory bowel disease: role of platelets. *Gut* 1993; **34**: 247-251
12 **Sloan WP Jr**, Bargen JA, Gage RP. Life histories of patients with chronic ulcerative colitis: a review of 2,000 cases. *Gastroenterology* 1950; **16**: 25-38
13 **Hatoum OA**, Binion DG. The vasculature and inflammatory bowel disease: contribution to pathogenesis and clinical pathology. *Inflamm Bowel Dis* 2005; **11**: 304-313
14 **Danese S**, Katz JA, Saibeni S, Papa A, Gasbarrini A, Vecchi M, Fiocchi C. Activated platelets are the source of elevated levels of soluble CD40 ligand in the circulation of inflammatory bowel disease patients. *Gut* 2003; **52**: 1435-1441
15 **Koutroubakis IE**, Theodoropoulou A, Xidakis C, Sfiridaki A, Notas G, Kolios G, Kouroumalis EA. Association between enhanced soluble CD40 ligand and prothrombotic state in inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 2004; **16**: 1147-1152
16 **Papa A**, Santoliquido A, Danese S, Covino M, Di Campli C, Urgesi R, Grillo A, Guglielmo S, Tondi P, Guidi L, De Vitis I, Fedeli G, Gasbarrini G, Gasbarrini A. Increased carotid intima-media thickness in patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2005; **22**: 839-846
17 **Levy PJ**, Tabares AH, Olin JW. Lower extremity arterial occlusions in young patients with Crohn's colitis and premature atherosclerosis: report of six cases. *Am J Gastroenterol* 1997; **92**: 494-497
18 **Bernstein CN**, Wajda A, Blanchard JF. The incidence of arterial thromboembolic diseases in inflammatory bowel disease: a population-based study. *Clin Gastroenterol Hepatol* 2008; **6**: 41-45
19 **Grip O**, Svensson PJ, Lindgren S. Inflammatory bowel disease promotes venous thrombosis earlier in life. *Scand J Gastroenterol* 2000; **35**: 619-623
20 **Koutroubakis IE**. Unraveling the mechanisms of thrombosis in inflammatory bowel disease. *Am J Gastroenterol* 2001; **96**: 1325-1327
21 **Danese S**, Papa A, Saibeni S, Repici A, Malesci A, Vecchi M. Inflammation and coagulation in inflammatory bowel disease: The clot thickens. *Am J Gastroenterol* 2007; **102**: 174-186
22 **Bertina RM**, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; **369**: 64-67
23 **Segers K**, Dahlback B, Nicolaes GA. Coagulation factor V and thrombophilia: background and mechanisms. *Thromb Haemost* 2007; **98**: 530-542
24 **Couturaud F**, Kearon C, Leroyer C, Mercier B, Abgrall JF, Le Gal G, Lacut K, Oger E, Bressollette L, Ferec C, Lamure M, Mottier D. Incidence of venous thromboembolism in first-degree relatives of patients with venous thromboembolism who have factor V Leiden. *Thromb Haemost* 2006; **96**: 744-749
25 **Nicolaes GA**, Dahlback B. Factor V and thrombotic disease: description of a janus-faced protein. *Arterioscler Thromb Vasc Biol* 2002; **22**: 530-538
26 **Turri D**, Rosselli M, Simioni P, Tormene D, Grimaudo S, Martorana G, Siragusa S, Mariani G, Cottone M. Factor V Leiden and prothrombin gene mutation in inflammatory bowel disease in a Mediterranean area. *Dig Liver Dis* 2001; **33**: 559-562

27 **Papa A**, De Stefano V, Gasbarrini A, Chiusolo P, Cianci R, Casorelli I, Paciaroni K, Cammarota G, Leone G, Gasbarrini G. Prevalence of factor V Leiden and the G20210A prothrombin-gene mutation in inflammatory bowel disease. *Blood Coagul Fibrinolysis* 2000; **11**: 499-503

28 **Haslam N**, Standen GR, Probert CS. An investigation of the association of the factor V Leiden mutation and inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 1999; **11**: 1289-1291

29 **Vecchi M**, Sacchi E, Saibeni S, Meucci G, Tagliabue L, Duca F, De Franchis R. Inflammatory bowel diseases are not associated with major hereditary conditions predisposing to thrombosis. *Dig Dis Sci* 2000; **45**: 1465-1469

30 **Bernstein CN**, Sargent M, Vos HL, Rosendaal FR. Mutations in clotting factors and inflammatory bowel disease. *Am J Gastroenterol* 2007; **102**: 338-343

31 **Koutroubakis IE**, Sfiridaki A, Mouzas IA, Maladaki A, Kapsoritakis A, Roussomoustakaki M, Kouroumalis EA, Manousos ON. Resistance to activated protein C and low levels of free protein S in Greek patients with inflammatory bowel disease. *Am J Gastroenterol* 2000; **95**: 190-194

32 **Guedon C**, Le Cam-Duchez V, Lalaude O, Menard JF, Lerebours E, Borg JY. Prothrombotic inherited abnormalities other than factor V Leiden mutation do not play a role in venous thrombosis in inflammatory bowel disease. *Am J Gastroenterol* 2001; **96**: 1448-1454

33 **Koutroubakis IE**, Sfiridaki A, Tsiolakidou G, Theodoropoulou A, Livadiotaki A, Paspatis G, Kouroumalis EA. Genetic risk factors in patients with inflammatory bowel disease and vascular complications: case-control study. *Inflamm Bowel Dis* 2007; **13**: 410-415

34 **Papa A**, Danese S, Grillo A, Gasbarrini G, Gasbarrini A. Review article: inherited thrombophilia in inflammatory bowel disease. *Am J Gastroenterol* 2003; **98**: 1247-1251

35 **Spina L**, Saibeni S, Battaglioli T, Peyvandi F, de Franchis R, Vecchi M. Thrombosis in inflammatory bowel diseases: role of inherited thrombophilia. *Am J Gastroenterol* 2005; **100**: 2036-2041

36 **Spek CA**, ten Kate FJ, te Velde AA. Factor V Leiden and the etiology of inflammatory bowel disease. *Thromb Haemost* 2007; **98**: 670-673

37 **Liebman HA**, Kashani N, Sutherland D, McGehee W, Kam AL. The factor V Leiden mutation increases the risk of venous thrombosis in patients with inflammatory bowel disease. *Gastroenterology* 1998; **115**: 830-834

38 **Poort SR**, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996; **88**: 3698-3703

39 **Mahmood A**, Needham J, Prosser J, Mainwaring J, Trebble T, Mahy G, Ramage J. Prevalence of hyperhomocysteinaemia, activated protein C resistance and prothrombin gene mutation in inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 2005; **17**: 739-744

40 **Frosst P**, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995; **10**: 111-113

41 **den Heijer M**, Koster T, Blom HJ, Bos GM, Briet E, Reitsma PH, Vandenbroucke JP, Rosendaal FR. Hyperhomocysteinemia as a risk factor for deep-vein thrombosis. *N Engl J Med* 1996; **334**: 759-762

42 **Kluijtmans LA**, den Heijer M, Reitsma PH, Heil SG, Blom HJ, Rosendaal FR. Thermolabile methylenetetrahydrofolate reductase and factor V Leiden in the risk of deep-vein thrombosis. *Thromb Haemost* 1998; **79**: 254-258

43 **Den Heijer M**, Lewington S, Clarke R. Homocysteine, MTHFR and risk of venous thrombosis: a meta-analysis of published epidemiological studies. *J Thromb Haemost* 2005; **3**: 292-299

44 **Catto AJ**, Kohler HP, Coore J, Mansfield MW, Stickland MH, Grant PJ. Association of a common polymorphism in the factor XIII gene with venous thrombosis. *Blood* 1999; **93**: 906-908

45 **Van Hylckama Vlieg A**, Komanasin N, Ariens RA, Poort SR, Grant PJ, Bertina RM, Rosendaal FR. Factor XIII Val34Leu polymorphism, factor XIII antigen levels and activity and the risk of deep venous thrombosis. *Br J Haematol* 2002; **119**: 169-175

46 **Franco RF**, Reitsma PH, Lourenco D, Maffei FH, Morelli V, Tavella MH, Araojo AG, Piccinato CE, Zago MA. Factor XIII Val34Leu is a genetic factor involved in the etiology of venous thrombosis. *Thromb Haemost* 1999; **81**: 676-679

47 **Helio T**, Wartiovaara U, Halme L, Turunen UM, Mikkola H, Palotie A, Farkkila M, Kontula K. Arg506Gln factor V mutation and Val34Leu factor XIII polymorphism in Finnish patients with inflammatory bowel disease. *Scand J Gastroenterol* 1999; **34**: 170-174

48 **Saibeni S**, Vecchi M, Faioni EM, Franchi F, Rondonotti E, Borsi G, de Franchis R. Val34Leu factor XIII polymorphism in Italian patients with inflammatory bowel disease. *Dig Liver Dis* 2003; **35**: 32-36

49 **Dawson S**, Hamsten A, Wiman B, Henney A, Humphries S. Genetic variation at the plasminogen activator inhibitor-1 locus is associated with altered levels of plasma plasminogen activator inhibitor-1 activity. *Arterioscler Thromb* 1991; **11**: 183-190

50 **Eriksson P**, Kallin B, van't Hooft FM, Bavenholm P, Hamsten A. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. *Proc Natl Acad Sci USA* 1995; **92**: 1851-1855

51 **Tassies D**, Espinosa G, Munoz-Rodriguez FJ, Freire C, Cervera R, Monteagudo J, Maragall S, Escolar G, Ingelmo M, Ordinas A, Font J, Reverter JC. The 4G/5G polymorphism of the type 1 plasminogen activator inhibitor gene and thrombosis in patients with antiphospholipid syndrome. *Arthritis Rheum* 2000; **43**: 2349-2358

52 **Stegnar M**, Uhrin P, Peternel P, Mavri A, Salobir-Pajnic B, Stare J, Binder BR. The 4G/5G sequence polymorphism in the promoter of plasminogen activator inhibitor-1 (PAI-1) gene: relationship to plasma PAI-1 level in venous thromboembolism. *Thromb Haemost* 1998; **79**: 975-979

53 **Magro F**, Dinis-Ribeiro M, Araujo FM, Pereira P, Fraga MC, Cunha-Ribeiro LM, Tome-Ribeiro A. High prevalence of combined thrombophilic abnormalities in patients with inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 2003; **15**: 1157-1163

54 **Levine RL**, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, Boggon TJ, Wlodarska I, Clark JJ, Moore S, Adelsperger J, Koo S, Lee JC, Gabriel S, Mercher T, D' Andrea A, Frohling S, Dohner K, Marynen P, Vandenberghe P, Mesa RA, Tefferi A, Griffin JD, Eck MJ, Sellers WR, Meyerson M, Golub TR, Lee SJ, Gilliland DG. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* 2005; **7**: 387-397

55 **Cools J**, DeAngelo DJ, Gotlib J, Stover EH, Legare RD, Cortes J, Kutok J, Clark J, Galinsky I, Griffin JD, Cross NC, Tefferi A, Malone J, Alam R, Schrier SL, Schmid J, Rose M, Vandenberghe P, Verhoef G, Boogaerts M, Wlodarska I, Kantarjian H, Marynen P, Coutre SE, Stone R, Gilliland DG. A tyrosine kinase created by fusion of the PDGFRA and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. *N Engl J Med* 2003; **348**: 1201-1214

56 **Karimi O**, Crusius JB, Coucoutsi C, Heijmans R, Sambuelli AM, Pena AS, Koutroubakis IE. JAK2 V617F mutation is not involved in thromboembolism in IBD. *Inflamm Bowel Dis* 2008; **14:** 1606-1607

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TOPIC HIGHLIGHT

Harry HX Xia, PhD, MD, *Series Editor*

Hepatocellular carcinoma: Defining the place of surgery in an era of organ shortage

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Abstract

Liver resection (LR) and transplantation offer the only potential chance of cure for patients with hepatocellular carcinoma (HCC). Historically, all patients were treated by hepatic resection. With the advent of liver transplantation (LT) patients with HCC were preferentially placed on the waiting list for LT. However, early experience with LT was associated with a high rate of tumour recurrence and poor long-term survival. The increasing scarcity of donor livers resulted in restrictions being placed on tumour size, and an improvement in patient survival. To date there have been no randomised clinical trials comparing LR to LT. We review the evidence supporting LR and/or LT for HCC and discuss the role of neoadjuvant therapy. The decision of whether to resect or transplant remains debatable and is often determined by centre experience, availability of LT and donor organs.

Key words: Hepatocellular carcinoma; Liver transplantation; Liver resection; Adjuvant therapy; Salvage liver transplantation; Radiofrequency ablation; Transarterial chemoembolization

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INTRODUCTION

Liver resection (LR) has been the only potentially curative treatment available for hepatocellular carcinoma (HCC). With the advent of liver transplantation (LT) as a clinical modality patients with HCC were preferentially placed on the waiting list for transplantation[1]. To date, there have been no randomised clinical trials comparing LR to LT in patients with potentially resectable HCC (Child-Pugh A, wedged hepatic venous pressure < 10 mmHg)[2]. The decision of whether to resect or transplant is often determined by centre experience and the availability of LT. Unfortunately, the majority of patients have extensive tumours at presentation and are not candidates for either LT or LR. The use of neoadjuvant therapies to downstage the disease has recently been shown to be effective in a small number of selected cases. The results of newer tumour specific therapies that are currently being developed are awaited and may expand the number of potentially resectable cases. If livers for LT were freely available, a case could be made for transplanting all patients with HCC confined to the liver. However, there are insufficient numbers of grafts to transplant even good risk candidates. Determining which patients should be managed by resection or transplantation remains a subject of much debate.

LIVER RESECTION

For the majority of patients with HCC, eligibility for LR is not only dependant upon anatomical location, but also on the extent of the underlying liver disease[3]. Consequently, only 15%-30% of patients with HCC are candidates for LR at the time of presentation[4]. Although the advantages of LR over LT are not clear-cut, patients with well-preserved liver function (Child-Pugh A) with small solitary (< 5 cm) HCCs should be considered for LR. The presence of hepatic decompensation (Child-Pugh C) is a contraindication to surgical LR, due to the high peri-operative mortality. The more difficult patients

Table 1 Recurrence and survival rates following surgical treatment for hepatocellular carcinoma

Treatment	Author	*n*	Recurrence (%)	5-yr survival (%)
Liver resection				
	Ercolani (2003)[61]	224	54.4	42.0
	Sim (2003)[62]	81	NS	59.0[1]
	Belghiti (2003)[63]	328	NS	37.0
	Bartlett (2007)[59]	53	47.0	42.6
	Nuzzo (2007)[64]	248	46.0	24.0
Deceased donor liver transplantation				
	Mazzaferro (1996) (Milan criteria)[33]	48	8.0	75.4[2]
	Jonas (2001) (Milan criteria)[65]	120	16.0	71.0
	Figueras (2001) (5 cm, localised)[66]	307	21.0	63.0
	Yao (2001) (UCSF criteria)[34]	70	11.4	75.2
	Decaens (2006)[36]			
	Milan criteria	279	NS	60.0
	Beyond Milan, but within UCSF	44	NS	45.6
	Beyond UCSF and Milan criteria	145	NS	34.7
Living donor liver transplantation				
	Todo (2007)[67]			
	Milan criteria	137	1.4	79.4[1]
	Extended criteria	172	22.2	60.0
	Hwang (2005)[68]			
	Milan criteria	173	NS	88.0
	Extended criteria	64	NS	60.0[1]
	Jonas (2007)[69]			
	Milan criteria	8	NS	75.0
	Extended criteria	13	NS	62.0
	Kwon (2007)[70]			
	Extended criteria	139	NS	79.9
	Sugawara (2007)[71]			
	Extended criteria (5 nodules <5cm)	78	10.0	75.0
	Soejima (2007)[40]			
	Milan criteria	16	0.0	100
	Unlimited criteria	44	18.2	74.0[1]
Salvage liver transplantation				
	Belghiti (2003)[55]	18	5.6	61.0
	Schwartz (2006)[19]	18	44.0	NS
	Hwang (2007)[54]	17	NS	NS

NS: Not stated; UCSF: University of California San Francisco. [1]3-year survival; [2]4-year survival.

are those with Child-Pugh B cirrhosis or those with large (> 5 cm) or multiple tumours.

The natural history of HCC has been studied in small series 20-30 years ago. Those patients with a solitary small (< 3 cm) tumour have good three-year survival irrespective of treatment modality. Of those that undergo LR approximately 70% will develop intrahepatic tumour recurrence within 5 years of resection[5]. This represents either tumour that was present, but not detected at the time of LR (synchronous), or a new tumour that has arisen in the diseased liver remnant (metachronous). The results of LR for HCC in some recent publications are summarised in Table 1.

A number of different clinico-pathological staging systems have been proposed to help predict survival outcomes for patients with HCC to assist clinicians in deciding whether patients are suitable for LR. The Barcelona Clinic Liver Cancer (BCLC) group stratifies patients with HCC into 4 categories (early, intermediate, advanced and terminal) and recommends different treatment options for each category[6]. Accordingly, LR is only indicated in patients with early stage HCC, that is; a single nodule ⩽ 5 cm or up to 3 nodules ⩽ 3 cm; Okuda stage 1 or 2[7]; Child-Pugh A or B[8]; Performance score of 0[9]; with no portal hypertension and a normal serum bilirubin level. The role of LR for BCLC group intermediate-stage HCC (single nodule > 5 cm or multinodular tumours) in the presence of preserved liver function (Okuda stage 1 or 2; Child-Pugh A; Performance score 0-2) remains controversial. Patients with large (> 5 cm) HCCs are not suitable for ablative therapies and are excluded from LT if the Milan criteria are used for patient selection. These patients are often deemed too high-risk to undergo LR due to the extent of the resection. The American Association for Study of Liver Diseases (AASLD)[10] and the European Association for Study of Liver (EASL)[11] guidelines state that LR is contraindicated for tumours > 5 cm due to the high incidence of vascular invasion and the associated poor prognosis. However, a number of centres have reported acceptable outcomes for patients with resected HCC greater than 5 cm or even 10 cm. A multicentre study of 300 patients with HCC > 10 cm reported a 5-year overall survival rate of 26.9%[12]. Poon *et al* reported a 5-year actual survival rate of 20.6% for 58 patients resected for tumours > 10 cm[13].

Much of the improvement in patient outcome following LR has been due to the adoption of a multidisciplinary approach to managing these patients with stringent preoperative evaluation of hepatic function and liver manipulation by selective portal vein or hepatic artery embolization. This has been borne out by nationally representative data which has shown inpatient mortality to be 40% less in high-volume hospitals compared to low-volume hospitals (odds ratio 0.60; $P = 0.02$)[14]. Supporting the concept that patients requiring hepatectomy, particularly in the presence of chronic liver disease, should be managed in high-volume centres.

Multi-focal HCC is associated with a poor outcome, due to the high rate of recurrence and is considered a relative contraindication to LR. A recent audit of LR in 380 patients with large and multifocal HCC and small (< 5 cm) single nodules ($n = 404$), revealed similar peri-operative morbidity and mortality rates between the two groups, but the three-year survival that was significantly better for small solitary HCC (76% *vs* 50%)[15]. Despite this the authors stated that survival of patients with multifocal HCC after LR was better than that achieved with trans-arterial chemoembolization (TACE), and suggested that if functional reserve was acceptable they should be considered for LR provided all identifiable tumour is able to be resected[15].

Patients with early Child-Pugh B cirrhosis without evidence of significant portal hypertension should be considered for minor LRs. The difficulty is objectively assessing liver function and the extent of LR likely to be tolerated. The indocyanine green (ICG) clearance test[16,17] has been well validated and the Model of End Stage Liver Disease (MELD) also appears to predict peri-operative mortality following LR. A MELD score of $\geqslant 9$ had a peri-operative mortality rate of 29%, whilst a score of < 9 had no mortality[18]. However, no technique has been shown to be superior to that of the judgement of an experienced clinician.

The aim of LR is to achieve local control of the index tumour, accepting that new or unrecognised tumours may subsequently appear. Thirty-nine percent of patients with a solitary HCC < 5 cm on preoperative cross sectional imaging are found after LT to have other lesions on histologic examination of the explanted liver[19]. Although preoperative imaging has improved, less than one third of HCCs < 1 cm can be identified using either contrast enhanced computer tomography (CT) or magnetic resonance imaging (MRI)[19]. This suggests that progression of established disease present at the time of LR accounts for a significant proportion of tumour recurrences post resection.

Parenchymal preservation is important in preserving hepatic function and reducing the risk of small for size syndrome[3]. However, HCC has a propensity for vascular invasion resulting in intrahepatic metastases. Anatomical (segmental) LR, results in resection of a greater volume of liver parenchyma, leads to the *en bloc* resection of the primary tumour and all the potentially tumour-bearing portal tributaries. In support of this, anatomical resection for solitary HCC has been shown to be associated with a lower rate of disease recurrence and improved overall survival[20-22]. In a retrospective study of 321 patients who underwent curative LR for solitary HCC < 5 cm, patients with preserved synthetic function (Liver damage group A) that underwent anatomical LR had improved overall and recurrence free 5-year survival compared to those treated by non-anatomical LR (87% *vs* 76%, $P = 0.02$ and 63% *vs* 35%, $P < 0.01$, respectively)[21]. Similarly, a recent retrospective analysis of 158 consecutive patients undergoing either anatomical ($n = 95$) or non-anatomical ($n = 63$) LR for HCC, demonstrated improved disease-free and long-term survival after anatomical LR, despite having larger tumours and higher prevalence of vascular invasion[22]. However, anatomical LR cannot always be performed due to limited hepatic reserve. The only option in these patients is a more limited (non-anatomical) LR or local ablative therapy. Patients with moderately impaired (Liver damage group B) synthetic function who underwent non-anatomical LR had significantly better 5-year overall and recurrence free survival compared to those treated by anatomical LR (72% *vs* 48%, $P < 0.01$ and 43% *vs* 28%, $P = 0.01$, respectively)[21]. Although the reason for this dichotomy remains speculative, it is possible that non-anatomical LR is associated with less physiological stress, which is better tolerated by patients with limited hepatic reserve.

More recently ablative therapies, such as radiofrequency ablation (RFA) or percutaneous ethanol injection (PEI), have been shown to offer similar outcomes to LR for small tumours (< 4 cm) without the associated operative morbidity. In a randomised controlled trial comparing PEI and RFA for HCCs < 3 cm, 4-year local recurrence and survival rates were 1.7% and 74%, respectively[23]. This was achieved despite a 63% incidence of disease recurrence elsewhere within the liver. Outcome appears to be operator dependent, both in terms of patient selection and technique, with rates of complete ablation varying from 20% to 96%. In a trial comparing PEI with RFA in HCCs $\leqslant$ 4 cm, RFA achieved initial complete ablation in 96% of lesions[24]. Looking at explant pathology, Lu *et al* reported complete necrosis in 83% of HCCs < 3 cm[25]. The higher failure rate of RFA compared to LR in larger lesions may be due to the presence of vascular invasion, which is present in 10%-15% and 46%-50% of 2 cm and 3-4 cm HCCs, respectively[26]. LR has the advantage of removing unrecognised regional metastases contained within the resected specimen and is supported by the finding of less intrahepatic recurrence after anatomical compared to non-anatomical LR[20].

INCREASING RESECTABILITY

Attempts to improve resectability include down staging the primary tumour and reducing the extent of surgery or increasing the size of the future liver remnant. Several techniques have been used to 'down-size' the tumour or to increase the size of the future liver remnant. Pre-operative portal vein embolization allows extensive resections to be performed by decreasing the likelihood

of post-operative liver insufficiency. This is achieved by embolizing the lobe of liver that is to be resected 6 wk prior to surgery, inducing hypertrophy in the future liver remnant. An increase of 40% to 60% in the size of the non-embolized liver is observed in non-cirrhotic livers.

TACE as a down-staging procedure in irresectable tumors has been shown to result in necrosis in 40%-100% of tumors with a three-year survival rate of 77%[26]. There is no clear evidence currently that chemoembolization is more effective than embolization alone[27]; however, combination of local ablative therapy with systemic chemotherapy or biological agents appears to increase resection rates in patients with compensated liver disease.

ADJUVANT TREATMENT FOLLOWING LIVER RESECTION

Several strategies have been employed in an attempt to reduce tumour recurrence following LR for HCC, including systemic chemotherapy, regional chemotherapy and internal radiotherapy. Intra-arterial 131-iodine labelled lipiodol has been shown to increase disease-free and overall survival in randomised controlled trials[27]. These findings have subsequently been confirmed in a retrospective analysis, where the 3-year disease-free survival rates were 68.4% and 41.5% in those that did and did not receive 131-iodine labelled lipiodol, respectively[28]. A large randomised study is awaited to confirm these results. More recently, menatetrenone, a vitamin K2 analogue with known anti-proliferative effects against hepatoma cell lines, reduced tumour recurrence and improved patient survival in patients with HCC following LR or local ablative therapy[29]. Sorafenib (Nexavar, Bayer Pharmaceuticals Corporation), an oral multi-kinase inhibitor, has been shown in a phase Ⅲ placebo-controlled randomised trial to improve overall survival by 44% in patients with stage IV HCC (Hazard Ratio = 0.69, $P = 0.0006$)[30]. Whether this will translate into an improvement in survival in patients following LR or ablation remains to be tested.

LIVER TRANSPLANTATION

LT is theoretically the best option for treating HCC as it allows for both radical resection of the primary tumour and treatment of the underlying liver disease, thus eliminating the risk of developing new HCCs and progression to end-stage liver failure. For many patients LR is not feasible because of tumour size, anatomical location or poor liver function, and LT is the only surgical option.

Early experience with LT for HCC was associated with a high rate of tumour recurrence and poor long-term survival[31]. Improving results for LT and the increasing scarcity donor grafts resulted in restrictions on tumour size as a 20%-40% survival at 5-years was deemed unacceptable. Bismuth[32] proposed and Mazzaferro[33] popularised the Milan criteria (single HCC $\leqslant$ 5 cm or up to 3 nodules $\leqslant$ 3 cm in diameter) for LT in patients with HCC to restrict access and to improve long-term outcome[33]. In many centres this has become the 'gold-standard' in determining eligibility for LT; however, some consider these criteria as too restrictive. Yao *et al* analyzed the outcome of 70 patients with HCC undergoing LT and found that patients with a single lesion $\leqslant$ 6.5 cm, 2 to 3 nodules with the largest $\leqslant$ 4.5 cm or a total tumor diameter $\leqslant$ 8 cm had a 75% 5-year survival[34]. Patients exceeding these University of California at San Francisco (UCSF) criteria, however, had a 1-year survival rate of 50% following LT[34]. Onaca *et al*, in an analysis of 1206 patients that underwent LT, found that patients with 2-4 tumours $\leqslant$ 5 cm or a solitary HCC $\leqslant$ 6 cm had tumour free survival similar to those that were within the Milan criteria[35]. Despite these encouraging reports adopting expanded criteria, a recent a retrospective study found that patients meeting the Milan criteria pre-transplant had a 5-year survival rate of 60% compared to 45% for those exceeding the Milan criteria, but meeting the UCSF criteria[36]. Although the difference was not statistically different, such a clinical difference warrants further examination. The results from a multicenter audit being undertaken by the 'Mazzaferro' group looking at the preoperative number and size of tumours and outcome is awaited[37].

Vascular invasion has been shown to be predictive of tumour recurrence and poor long-term survival in patients with HCC[38]. Vascular invasion is more common in large HCCs; however a significant proportion of tumours > 5 cm do not have histological evidence of vascular invasion[38]. Using size as a surrogate marker of biological behaviour may, therefore, result in patients with large well-differentiated HCC, who would potentially benefit from LT being excluded, and include small poorly differentiated tumours that are at high risk of recurrence[34]. An alternative method of assessing the risk of tumour recurrence is undertaking preoperative percutaneous biopsies, which places the patient at potential risk of local and hematogenous recurrence. Studies have demonstrated that percutaneous biopsy impairs the chance of curative resection. A retrospective review of 85 HCCs resected over 12 years found that preoperative biopsy resulted in the 5-year disease-free survival rate falling from 52% to 24%[38]. A recent review estimated the risk of needle tract seeding as being less than 2%, but acknowledged that biopsy carries a risk of hematogenous dissemination, but considered the degree of risk as speculative[39]. Further studies are needed to evaluate using histological criteria from biopsy for patients with tumours exceeding standard criteria. It is likely histological and other molecular analyses of tissue samples will increasingly be used preoperatively to characterise the biological behaviour of HCC.

As a consequence of donor shortage, live donor LT (LDLT) has become increasingly utilized for patients with end-stage liver disease. Despite the initial enthusiasm, the number of LRLT performed in the United States has fallen since 2002 as a consequence of

the realisation of donor risk and the implementation of the MELD system for organ allocation, which gave greater priority to patients with HCC. Out of necessity the number of LDLT undertaken in Asian countries, where cadaveric donation is not routinely available, has increased dramatically over the last decade.

For patients with early HCC for whom a suitable donor is available, LDLT offers a number of benefits. It can be performed in a timely manner eliminating the risk of waiting list dropout due to disease progression. LDLT does not rely upon cadaveric donation that is dependent upon equitable allocation for all patients with end-stage liver disease. Consequently, there have been a number of studies looking at LDLT using extended criteria, where the size and number is lesions is not limited[40,41]. The largest study reviewed 125 patients that underwent LDLT, 55 of which had tumours that exceeded Milan criteria[41]. Patients that exceeded Milan criteria, but had $\leqslant$ 10 tumours, all of which were $\leqslant$ 5 cm in diameter, had a 5-year recurrence rate similar to those that were within the Milan criteria (7.3% and 9.7%, respectively; $P = 0.89$). Multivariate analysis also demonstrated a preoperative des-gama-carboxy prothrombin (PIVKA-Ⅱ; protein induced by vitamin K antagonist-Ⅱ) value of > 400 mAU/mL as strongly associated with disease recurrence, and a level of < 400 mAU/mL be included in the selection criteria[41]. Similarly, Soejima *et al* reported on 60 patients that underwent LDLT for HCC, and found that there were no recurrences in those that were within the Milan Criteria. Multivariate analysis identified only tumour diameter of > 5 cm and PIVKA-Ⅱ of > 300 mAU/mL as strongly associated with disease recurrence[40]. Although these studies are small and have short follow-up, they suggest that expansion of the current tumour size and number with the use of preoperative PIVKA-Ⅱ, may be associated with acceptable outcomes in patients undergoing LDLT.

LOCAL ABLATIVE THERAPIES AS A BRIDGE TO LIVER TRANSPLANTATION

RFA and TACE have been used by many centres to downstage and/or prevent disease progression in patients with HCC. Currently there are no prospective randomised trials evaluating the effect of these therapies prior to LT.

RFA is operator dependent, both in terms of patient selection and technique, with rates of complete ablation varying from 20% to 96%[25]. Most studies have demonstrated a reduction in the dropout rate compared to historical controls. A study of 60 consecutive HCCs in 50 patients on the waiting list for LT treated by percutaneous and laparoscopic RFA demonstrated a 0% dropout rate and a 8% morbidity at a mean time to LT of 9.5 mo[42]. This compares favourably to an historical dropout rate of 10%-30% with waiting times of 6-12 mo[43]. More recently, a study of 52 patients treated by preoperative RFA reported a dropout rate of 5.7% at a mean of 12.7 mo with no evidence of tumour recurrence post-transplant[44]. Although there remains a potential risk for needle track dissemination and its efficacy has not been demonstrated in large HCC, RFA should be considered in patients on the waiting list with small (< 3 cm) solitary tumours and reasonable synthetic function (Child-Pugh A, and selected B).

A number of cohort studies have evaluated the efficacy of TACE, alone or in combination with systemic chemotherapy prior to LT[45,46]. The results are conflicting, and a recent meta-analysis of TACE as a bridge to LT found that there was insufficient evidence to support the use of neoadjuvant TACE prior to LT as it did not improve long-term survival, allow for the expansion of selection criteria or reduce the dropout rates on the waiting list[47]. TACE has been proposed as a method of selecting patients with favourable tumour biology. In a study of 96 consecutive patients with HCC, 62 of whom exceeded Milan criteria, tumour recurrence was influenced by the response to pre-transplant TACE. Patients who had a sustained response to TACE pre-transplant ($n = 39$) had a 5-year tumour free recurrence rate of 94.5%, whereas patients who had disease progression had a tumour free recurrence rate of 35.4% ($P = 0.0017$)[48]. Similarly, in a smaller study there were only 2 recurrences in 19 patients with tumours > 3 cm that had decreased the sum of two diameters by > 50% following pre-transplant TACE[49].

The current practice guidelines from the American Association for the Study of Liver Diseases (AASLD) state that local ablation, RFA and TACE, are safe and effective in patients who are not suitable for LR, or as a bridge to LT if the waiting list time exceeds 6 mo[10].

SALVAGE LIVER TRANSPLANTATION

Salvage LT has been promoted as a way of managing patients with HCC in an era of organ shortage. LR is performed as the primary procedure, keeping LT in reserve for those who develop further intrahepatic tumours or decompensation. The strategy offers a number of potential benefits. With increasing waiting times for LT patients with HCC face the prospect of disease progression beyond transplant criteria whilst waiting for a suitable donor. Overall, 5 year survival decreases by 10%-20% (from 81%-58% to 62%-47%) for waiting times of 6-12 mo, and dropout rates range from 10%-30%[43]. Undertaking LR in the first instance allows one to observe the natural history of the disease and allow those patients with aggressive disease to declare extrahepatic disease, thus avoiding inappropriate LT and eliminating the risk of disease progression beyond transplant criteria while on the waiting list. In addition, the potential exists to reduce the number of patients requiring LT. In the medium term, disease-free survival for patients undergoing LR with early stage HCC has been shown to be comparable to that of primary LT. LR also allows for histological analysis of the tumour and those with poor prognostic criteria, such as macroscopic vascular invasion or poor differentiation, should be excluded from LT due to the high likelihood

of tumour recurrence, while resected patients who had solitary well-differentiated tumours without vascular invasion can be managed by surveillance and offered LT only if there is tumour recurrence or hepatic decompensation.

Salvage LT for HCC relies upon the principal that patients that have tumour recurrence following LR are still amenable to LT. Tanaka *et al* found that 8% of patients who underwent LR within the Milan criteria had tumour recurrence that exceeded Milan criteria[50]. Conversely, only 22% of patients undergoing LR for tumours outside the Milan criteria develop post-resection recurrence that is within Milan criteria[50]. Multivariate analysis identified size of the primary tumour and degree of differentiation as risk factors for recurrence exceeding Milan criteria[50]. Others have identified the presence of portal vein invasion in the resected liver specimen as the most important predictor of tumour recurrence[51]. A number of molecular indices have been examined to try to predict tumour recurrence. A high level of telomerase activity is reported as an independent predictor for tumour recurrence[52]. However, no marker has been confirmed to predict the risk of tumour recurrence reliably.

Salvage LT appears to have higher morbidity and mortality and an increased incidence of tumour recurrence compared to primary LT[53]. Of 18 patients that under went salvage LT at Mount Sinai following LR, 2 died peri-operatively (11%), and 7 subsequently developed tumour recurrence (44%)[19]. Similarly, of 17 patients that underwent salvage LDLT, bleeding complications were more common, and the peri-operative mortality rate (5.9%) was significantly higher than after primary LT[54]. In contrast, Belghiti *et al* reported that LR prior to LT did not significantly increase the operative difficulty of the procedure[55]. Furthermore, they did not find any difference in disease-free or overall survival between primary and salvage LT. Patients who underwent salvage LT had a mean 20 mo disease-free interval before listing for LT[55]. The long-term outcome of these strategies is awaited.

EFFECT OF IMMUNOSUPPRESSION ON TUMOUR RECURRENCE

Calcineurin inhibitors, cyclosporine and tacrolimus, are currently the mainstay of immunosuppression in LT recipients. Sirolimus, a novel immunosuppressive drug that inhibits the mammalian target of rapamycin has been shown *in vitro* to allow for the maintenance of tumour immunosurveillance, and may theoretically offer survival benefit in patients transplanted for HCC. In a study of 70 patients transplanted for HCC receiving *de novo* sirolimus and low dose calcineurin inhibitor for 6-12 mo and either a short course (3 mo) or no steroids, tumour free survival at a median of 49 mo was comparable to that achieved with conventional immunosuppression[56]. However, 50% of patients had at least one episode of rejection and 34% developed an incisional hernia. A better understanding of tumour biology and particularly the role of immunosuppression and tumour growth will provide further improvement in the treatment of HCC.

DIRECT COMPARISONS: RESECTION VERSUS TRANSPLANTATION

The oncological advantage of LT compared to LR has not been universally demonstrated. For large tumours LR is not often possible and LT is the only potential treatment modality. Numerous retrospective studies from the 1990s have demonstrated that the results of LT for large HCCs are poor in relative terms, with 5-year survival rates of < 20%-30%[31,57,58]. In contrast the best therapeutic modality for small tumours (< 5 cm) is debatable. A retrospective analysis of 102 patients treated by LT (n = 50) and LR (n = 52) showed no difference in 3-year survival or recurrence rate for tumours < 5 cm[57]. In contrast, Bismuth found that LT was superior to LR for small (< 3 cm) tumours[32]. The 3-year survival rate for patients with tumours < 3 cm with 1 to 2 nodules was 83% and 41% for LT and LR, respectively[32]. The difference could be attributed to lower peri-operative mortality and tumour recurrence in the LT recipients. The operative mortality for LR for HCC varies from 0.5% and 21.5% and reflects the incidence of hepatic insufficiency-associated with underlying liver disease[59]. In addition, the rate of 'recurrent' disease is significantly higher after LR compared to LT with a 3-year recurrence-free survival rate of 83% and 18%, respectively[32]. Taken together, it is apparent that in the presence of chronic liver disease, LT offers the greatest chance of long-term survival for patients with small (< 5 cm) tumours. In the present climate of donor organ scarcity it is difficult to justify LT for large and/or advanced HCC.

CONCLUSION

Currently, in the absence of large randomised clinical trials, the treatment strategy for patients with HCC remains a matter of choice depending upon the interpretation of retrospective studies, anecdotal evidence, unit experience, and availability of therapeutic options.

To date, we have relied upon radiological criteria as a surrogate marker of tumour behaviour. What is needed is an accurate predictor of the biological behaviour of the tumour at the time of presentation. The molecular analysis of tumour biopsies has yet to deliver, and is associated with a risk of needle track recurrence. Less invasive markers that can accurately predict the risk of tumour recurrence are needed to help stratify patients for appropriate therapy.

One of the confounding factors in comparing the outcome of different treatment modalities for HCC is the lack of a uniform staging system. A large multicentre trial examining the commonly used staging systems, found that the American Joint Committee

on Cancer/Union Internationale Contre le Cancer AJCC/UICC (sixth edition) staging system provides the best stratification of prognosis following LR or LT[60]. Adoption of a uniform staging system by all centres would help to provide a better comparison of therapeutic modalities in the future.

Although there is no consensus as to the best treatment for patients with HCC, it is apparent that LR appears to be the most appropriate treatment for patients with small (< 5 cm) solitary HCC with well-preserved synthetic function (Child-Pugh A) and normal portal pressures (hepatic vein wedge pressure < 10 mmHg). On account of the high rate of complete ablation that can be achieved in small tumours (< 3 cm), with a similar rate of local control compared to LR, it is hard to justify LR for patients with HCCs < 3 cm, especially if they have significant co-morbidities. Given the scarcity of donor organs and the lack of prospective data demonstrating an acceptable outcome in extending the current criteria, LT should be reserved for early stage HCC (solitary < 5 cm; ⩽ 3 lesions 3 cm) that cannot be treated by LR. Medical treatments currently have limited efficacy, and their role, as a surgical adjuvant to LR and LT is yet to be determined.

REFERENCES

1 **Williams R**, O'Grady JG. Liver transplantation: results, advances and problems. *J Gastroenterol Hepatol* 1990; **5** Suppl 1: 110-126
2 **Burroughs A**, Hochhauser D, Meyer T. Systemic treatment and liver transplantation for hepatocellular carcinoma: two ends of the therapeutic spectrum. *Lancet Oncol* 2004; **5**: 409-418
3 **Tucker ON**, Heaton N. The 'small for size' liver syndrome. *Curr Opin Crit Care* 2005; **11**: 150-155
4 **Emond JC**, Samstein B, Renz JF. A critical evaluation of hepatic resection in cirrhosis: optimizing patient selection and outcomes. *World J Surg* 2005; **29**: 124-130
5 **Llovet JM**, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907-1917
6 **Bruix J**, Llovet JM. Prognostic prediction and treatment strategy in hepatocellular carcinoma. *Hepatology* 2002; **35**: 519-524
7 **Okuda K**, Ohtsuki T, Obata H, Tomimatsu M, Okazaki N, Hasegawa H, Nakajima Y, Ohnishi K. Natural history of hepatocellular carcinoma and prognosis in relation to treatment. Study of 850 patients. *Cancer* 1985; **56**: 918-928
8 **Pugh RN**, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646-649
9 **Sorensen JB**, Klee M, Palshof T, Hansen HH. Performance status assessment in cancer patients. An inter-observer variability study. *Br J Cancer* 1993; **67**: 773-775
10 **Bruix J**, Sherman M. Management of hepatocellular carcinoma. *Hepatology* 2005; **42**: 1208-1236
11 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodes J. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430
12 **Pawlik TM**, Poon RT, Abdalla EK, Zorzi D, Ikai I, Curley SA, Nagorney DM, Belghiti J, Ng IO, Yamaoka Y, Lauwers GY, Vauthey JN. Critical appraisal of the clinical and pathologic predictors of survival after resection of large hepatocellular carcinoma. *Arch Surg* 2005; **140**: 450-457; discussion 457-458
13 **Poon RT**, Fan ST, Wong J. Selection criteria for hepatic resection in patients with large hepatocellular carcinoma larger than 10 cm in diameter. *J Am Coll Surg* 2002; **194**: 592-602
14 **Dimick JB**, Cowan JA Jr, Knol JA, Upchurch GR Jr. Hepatic resection in the United States: indications, outcomes, and hospital procedural volumes from a nationally representative database. *Arch Surg* 2003; **138**: 185-191
15 **Ng KK**, Vauthey JN, Pawlik TM, Lauwers GY, Regimbeau JM, Belghiti J, Ikai I, Yamaoka Y, Curley SA, Nagorney DM, Ng IO, Fan ST, Poon RT. Is hepatic resection for large or multinodular hepatocellular carcinoma justified? Results from a multi-institutional database. *Ann Surg Oncol* 2005; **12**: 364-373
16 **Hemming AW**, Scudamore CH, Shackleton CR, Pudek M, Erb SR. Indocyanine green clearance as a predictor of successful hepatic resection in cirrhotic patients. *Am J Surg* 1992; **163**: 515-518
17 **Fan ST**, Lai EC, Lo CM, Ng IO, Wong J. Hospital mortality of major hepatectomy for hepatocellular carcinoma associated with cirrhosis. *Arch Surg* 1995; **130**: 198-203
18 **Teh SH**, Christein J, Donohue J, Que F, Kendrick M, Farnell M, Cha S, Kamath P, Kim R, Nagorney DM. Hepatic resection of hepatocellular carcinoma in patients with cirrhosis: Model of End-Stage Liver Disease (MELD) score predicts perioperative mortality. *J Gastrointest Surg* 2005; **9**: 1207-1215; discussion 1215
19 **Schwartz M**. Liver transplantation: the preferred treatment for early hepatocellular carcinoma in the setting of cirrhosis? *Ann Surg Oncol* 2007; **14**: 548-552
20 **Imamura H**, Matsuyama Y, Tanaka E, Ohkubo T, Hasegawa K, Miyagawa S, Sugawara Y, Minagawa M, Takayama T, Kawasaki S, Makuuchi M. Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. *J Hepatol* 2003; **38**: 200-207
21 **Yamashita Y**, Taketomi A, Itoh S, Kitagawa D, Kayashima H, Harimoto N, Tsujita E, Kuroda Y, Maehara Y. Longterm favorable results of limited hepatic resections for patients with hepatocellular carcinoma: 20 years of experience. *J Am Coll Surg* 2007; **205**: 19-26
22 **Wakai T**, Shirai Y, Sakata J, Kaneko K, Cruz PV, Akazawa K, Hatakeyama K. Anatomic resection independently improves long-term survival in patients with T1-T2 hepatocellular carcinoma. *Ann Surg Oncol* 2007; **14**: 1356-1365
23 **Shiina S**, Teratani T, Obi S, Sato S, Tateishi R, Fujishima T, Ishikawa T, Koike Y, Yoshida H, Kawabe T, Omata M. A randomized controlled trial of radiofrequency ablation with ethanol injection for small hepatocellular carcinoma. *Gastroenterology* 2005; **129**: 122-130
24 **Lin SM**, Lin CJ, Lin CC, Hsu CW, Chen YC. Radiofrequency ablation improves prognosis compared with ethanol injection for hepatocellular carcinoma < or = 4 cm. *Gastroenterology* 2004; **127**: 1714-1723
25 **Lu DS**, Yu NC, Raman SS, Limanond P, Lassman C, Murray K, Tong MJ, Amado RG, Busuttil RW. Radiofrequency ablation of hepatocellular carcinoma: treatment success as defined by histologic examination of the explanted liver. *Radiology* 2005; **234**: 954-960
26 **Urahashi T**, Lynch SV, Kim YH, Balderson GA, Fawcett JW, Crawford DH, Strong RW. Undetected hepatocellular carcinoma in patients undergoing liver transplantation: is associated with favorable outcome. *Hepatogastroenterology* 2007; **54**: 1192-1195
27 **Lau WY**, Leung TW, Ho SK, Chan M, Machin D, Lau J, Chan AT, Yeo W, Mok TS, Yu SC, Leung NW, Johnson PJ. Adjuvant intra-arterial iodine-131-labelled lipiodol for resectable hepatocellular carcinoma: a prospective randomised trial. *Lancet* 1999; **353**: 797-801
28 **Boucher E**, Corbinais S, Rolland Y, Bourguet P, Guyader D,

Boudjema K, Meunier B, Raoul JL. Adjuvant intra-arterial injection of iodine-131-labeled lipiodol after resection of hepatocellular carcinoma. *Hepatology* 2003; **38**: 1237-1241

29 **Mizuta T**, Ozaki I, Eguchi Y, Yasutake T, Kawazoe S, Fujimoto K, Yamamoto K. The effect of menatetrenone, a vitamin K2 analog, on disease recurrence and survival in patients with hepatocellular carcinoma after curative treatment: a pilot study. *Cancer* 2006; **106**: 867-872

30 **Llovet JM**, Di Bisceglie AM, Bruix J, Kramer BS, Lencioni R, Zhu AX, Sherman M, Schwartz M, Lotze M, Talwalkar J, Gores GJ. Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* 2008; **100**: 698-711

31 **Ringe B**, Pichlmayr R, Wittekind C, Tusch G. Surgical treatment of hepatocellular carcinoma: experience with liver resection and transplantation in 198 patients. *World J Surg* 1991; **15**: 270-285

32 **Bismuth H**, Chiche L, Adam R, Castaing D, Diamond T, Dennison A. Liver resection versus transplantation for hepatocellular carcinoma in cirrhotic patients. *Ann Surg* 1993; **218**: 145-151

33 **Mazzaferro V**, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693-699

34 **Yao FY**, Ferrell L, Bass NM, Watson JJ, Bacchetti P, Venook A, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: expansion of the tumor size limits does not adversely impact survival. *Hepatology* 2001; **33**: 1394-1403

35 **Onaca N**, Davis GL, Goldstein RM, Jennings LW, Klintmalm GB. Expanded criteria for liver transplantation in patients with hepatocellular carcinoma: a report from the International Registry of Hepatic Tumors in Liver Transplantation. *Liver Transpl* 2007; **13**: 391-399

36 **Decaens T**, Roudot-Thoraval F, Hadni-Bresson S, Meyer C, Gugenheim J, Durand F, Bernard PH, Boillot O, Sulpice L, Calmus Y, Hardwigsen J, Ducerf C, Pageaux GP, Dharancy S, Chazouilleres O, Cherqui D, Duvoux C. Impact of UCSF criteria according to pre- and post-OLT tumor features: analysis of 479 patients listed for HCC with a short waiting time. *Liver Transpl* 2006; **12**: 1761-1769

37 **Mazzaferro V**. Results of liver transplantation: with or without Milan criteria? *Liver Transpl* 2007; **13**: S44-S47

38 **Young AL**, Malik HZ, Abu-Hilal M, Guthrie JA, Wyatt J, Prasad KR, Toogood GJ, Lodge JP. Large hepatocellular carcinoma: time to stop preoperative biopsy. *J Am Coll Surg* 2007; **205**: 453-462

39 **Durand F**, Belghiti J, Paradis V. Liver transplantation for hepatocellular carcinoma: role of biopsy. *Liver Transpl* 2007; **13**: S17-S23

40 **Soejima Y**, Taketomi A, Yoshizumi T, Uchiyama H, Aishima S, Terashi T, Shimada M, Maehara Y. Extended indication for living donor liver transplantation in patients with hepatocellular carcinoma. *Transplantation* 2007; **83**: 893-899

41 **Ito T**, Takada Y, Ueda M, Haga H, Maetani Y, Oike F, Ogawa K, Sakamoto S, Ogura Y, Egawa H, Tanaka K, Uemoto S. Expansion of selection criteria for patients with hepatocellular carcinoma in living donor liver transplantation. *Liver Transpl* 2007; **13**: 1637-1644

42 **Mazzaferro V**, Battiston C, Perrone S, Pulvirenti A, Regalia E, Romito R, Sarli D, Schiavo M, Garbagnati F, Marchiano A, Spreafico C, Camerini T, Mariani L, Miceli R, Andreola S. Radiofrequency ablation of small hepatocellular carcinoma in cirrhotic patients awaiting liver transplantation: a prospective study. *Ann Surg* 2004; **240**: 900-909

43 **Llovet JM**, Schwartz M, Mazzaferro V. Resection and liver transplantation for hepatocellular carcinoma. *Semin Liver Dis* 2005; **25**: 181-200

44 **Lu DS**, Yu NC, Raman SS, Lassman C, Tong MJ, Britten C, Durazo F, Saab S, Han S, Finn R, Hiatt JR, Busuttil RW. Percutaneous radiofrequency ablation of hepatocellular carcinoma as a bridge to liver transplantation. *Hepatology* 2005; **41**: 1130-1137

45 **Lau WY**, Yu SC, Lai EC, Leung TW. Transarterial chemoembolization for hepatocellular carcinoma. *J Am Coll Surg* 2006; **202**: 155-168

46 **Taieb J**, Barbare JC, Rougier P. Medical treatments for hepatocellular carcinoma (HCC): what's next? *Ann Oncol* 2006; **17** Suppl 10: x308-x314

47 **Lesurtel M**, Mullhaupt B, Pestalozzi BC, Pfammatter T, Clavien PA. Transarterial chemoembolization as a bridge to liver transplantation for hepatocellular carcinoma: an evidence-based analysis. *Am J Transplant* 2006; **6**: 2644-2650

48 **Otto G**, Herber S, Heise M, Lohse AW, Monch C, Bittinger F, Hoppe-Lotichius M, Schuchmann M, Victor A, Pitton M. Response to transarterial chemoembolization as a biological selection criterion for liver transplantation in hepatocellular carcinoma. *Liver Transpl* 2006; **12**: 1260-1267

49 **Majno PE**, Adam R, Bismuth H, Castaing D, Ariche A, Krissat J, Perrin H, Azoulay D. Influence of preoperative transarterial lipiodol chemoembolization on resection and transplantation for hepatocellular carcinoma in patients with cirrhosis. *Ann Surg* 1997; **226**: 688-701; discussion 701-703

50 **Tanaka S**, Noguchi N, Ochiai T, Kudo A, Nakamura N, Ito K, Kawamura T, Teramoto K, Arii S. Outcomes and recurrence of initially resectable hepatocellular carcinoma meeting milan criteria: Rationale for partial hepatectomy as first strategy. *J Am Coll Surg* 2007; **204**: 1-6

51 **Shimada M**, Takenaka K, Taguchi K, Fujiwara Y, Gion T, Kajiyama K, Maeda T, Shirabe K, Yanaga K, Sugimachi K. Prognostic factors after repeat hepatectomy for recurrent hepatocellular carcinoma. *Ann Surg* 1998; **227**: 80-85

52 **Shan YS**, Hsieh YH, Lin PW. Telomerase activity in tumor and remnant liver as predictor of recurrence and survival in hepatocellular carcinoma after resection. *World J Surg* 2007; **31**: 1121-1128

53 **Adam R**, Azoulay D, Castaing D, Eshkenazy R, Pascal G, Hashizume K, Samuel D, Bismuth H. Liver resection as a bridge to transplantation for hepatocellular carcinoma on cirrhosis: a reasonable strategy? *Ann Surg* 2003; **238**: 508-518; discussion 518-519

54 **Hwang S**, Lee SG, Moon DB, Ahn CS, Kim KH, Lee YJ, Ha TY, Song GW. Salvage living donor liver transplantation after prior liver resection for hepatocellular carcinoma. *Liver Transpl* 2007; **13**: 741-746

55 **Belghiti J**, Cortes A, Abdalla EK, Regimbeau JM, Prakash K, Durand F, Sommacale D, Dondero F, Lesurtel M, Sauvanet A, Farges O, Kianmanesh R. Resection prior to liver transplantation for hepatocellular carcinoma. *Ann Surg* 2003; **238**: 885-892; discussion 892-893

56 **Toso C**, Meeberg GA, Bigam DL, Oberholzer J, Shapiro AM, Gutfreund K, Ma MM, Mason AL, Wong WW, Bain VG, Kneteman NM. De novo sirolimus-based immunosuppression after liver transplantation for hepatocellular carcinoma: long-term outcomes and side effects. *Transplantation* 2007; **83**: 1162-1168

57 **Otto G**, Heuschen U, Hofmann WJ, Krumm G, Hinz U, Herfarth C. Survival and recurrence after liver transplantation versus liver resection for hepatocellular carcinoma: a retrospective analysis. *Ann Surg* 1998; **227**: 424-432

58 **Komori K**, Nagino M, Nimura Y. Hepatocyte morphology and kinetics after portal vein embolization. *Br J Surg* 2006; **93**: 745-751

59 **Bartlett AS**, McCall JL, Koea JB, Holden A, Yeong ML, Gurusinghe N, Gane E. Liver resection for hepatocellular carcinoma in a hepatitis B endemic area. *World J Surg* 2007; **31**: 1775-1781

60 **Vauthey JN**, Ribero D, Abdalla EK, Jonas S, Bharat A, Schumacher G, Lerut J, Chapman WC, Hemming AW, Neuhaus P. Outcomes of liver transplantation in 490

patients with hepatocellular carcinoma: validation of a uniform staging after surgical treatment. *J Am Coll Surg* 2007; **204**: 1016-1027; discussion 1027-1028

61 **Ercolani G**, Grazi GL, Ravaioli M, Del Gaudio M, Gardini A, Cescon M, Varotti G, Cetta F, Cavallari A. Liver resection for hepatocellular carcinoma on cirrhosis: univariate and multivariate analysis of risk factors for intrahepatic recurrence. *Ann Surg* 2003; **237**: 536-543

62 **Sim HG**, Ooi LL. Results of resections for hepatocellular carcinoma in a new hepatobiliary unit. *ANZ J Surg* 2003; **73**: 8-13

63 **Belghiti J**, Regimbeau JM, Durand F, Kianmanesh AR, Dondero F, Terris B, Sauvanet A, Farges O, Degos F. Resection of hepatocellular carcinoma: a European experience on 328 cases. *Hepatogastroenterology* 2002; **49**: 41-46

64 **Nuzzo G**, Giuliante F, Gauzolino R, Vellone M, Ardito F, Giovannini I. Liver resections for hepatocellular carcinoma in chronic liver disease: experience in an Italian centre. *Eur J Surg Oncol* 2007; **33**: 1014-1018

65 **Jonas S**, Bechstein WO, Steinmuller T, Herrmann M, Radke C, Berg T, Settmacher U, Neuhaus P. Vascular invasion and histopathologic grading determine outcome after liver transplantation for hepatocellular carcinoma in cirrhosis. *Hepatology* 2001; **33**: 1080-1086

66 **Figueras J**, Ibanez L, Ramos E, Jaurrieta E, Ortiz-de-Urbina J, Pardo F, Mir J, Loinaz C, Herrera L, Lopez-Cillero P, Santoyo J. Selection criteria for liver transplantation in early-stage hepatocellular carcinoma with cirrhosis: results of a multicenter study. *Liver Transpl* 2001; **7**: 877-883

67 **Todo S**, Furukawa H. Living donor liver transplantation for adult patients with hepatocellular carcinoma: experience in Japan. *Ann Surg* 2004; **240**: 451-459; discussion 459-461

68 **Hwang S**, Lee SG, Joh JW, Suh KS, Kim DG. Liver transplantation for adult patients with hepatocellular carcinoma in Korea: comparison between cadaveric donor and living donor liver transplantations. *Liver Transpl* 2005; **11**: 1265-1272

69 **Jonas S**, Mittler J, Pascher A, Schumacher G, Theruvath T, Benckert C, Rudolph B, Neuhaus P. Living donor liver transplantation of the right lobe for hepatocellular carcinoma in cirrhosis in a European center. *Liver Transpl* 2007; **13**: 896-903

70 **Kwon CH**, Kim DJ, Han YS, Park JB, Choi GS, Kim SJ, Joh JW, Lee SK. HCC in living donor liver transplantation: can we expand the Milan criteria? *Dig Dis* 2007; **25**: 313-319

71 **Sugawara Y**, Tamura S, Makuuchi M. Living donor liver transplantation for hepatocellular carcinoma: Tokyo University series. *Dig Dis* 2007; **25**: 310-312

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CLINICAL RESEARCH

Clinical significance of NOD2/CARD15 and Toll-like receptor 4 gene single nucleotide polymorphisms in inflammatory bowel disease

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Abstract

AIM: To evaluate the role of genetic factors in the pathogenesis of Crohn's disease (CD) and ulcerative colitis (UC), we investigated the single nucleotide polymorphisms (SNPs) of NOD2/CARD15 (R702W, G908R and L1007finsC), and Toll-like receptor 4 (TLR4) genes (D299G and T399I) in a selected inflammatory bowel disease (IBD) population coming from Southern Italy.

METHODS: Allele and genotype frequencies of NOD2/CARD15 (R702W, G908R and L1007finsC) and TLR4 (D299G and T399I) SNPs were examined in 133 CD patients, in 45 UC patients, and in 103 healthy controls. A genotype-phenotype correlation was performed.

RESULTS: NOD2/CARD15 R702W mutation was significantly more frequent in CD (9.8%) than in controls (2.4%, $P = 0.001$) and in UC (2.3%, $P = 0.03$). No significant difference was found between UC patients and control group ($P > 0.05$). In CD and UC patients, no significant association with G908R variant was found. L1007finsC SNP showed an association with CD (9.8%) compared with controls (2.9%, $P = 0.002$) and UC patients (2.3%, $P = 0.01$). Moreover, in CD patients, G908R and L1007finsC mutations were significantly associated with different phenotypes compared to CD wild-type patients. No association of IBD with the TLR4 SNPs was found in either cohort (allele frequencies: D299G-controls 3.9%, CD 3.7%, UC 3.4%, $P > 0.05$; T399I-controls 2.9%, CD 3.0%, UC 3.4%, $P > 0.05$).

CONCLUSION: These findings confirm that, in our IBD patients selected from Southern Italy, the NOD2/CARD15, but not TLR4 SNPs, are associated with increased risk of CD.

Key words: Crohn's disease; Ulcerative colitis; NOD2/CARD15 gene; Toll-like receptor 4 gene; Single nucleotide polymorphisms

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are

idiopathic chronic inflammatory bowel disease (IBD). The molecular basis of their pathogenesis is not completely clear, but contributing factors may include persistent bacterial infection, a defective mucosal barrier, and an imbalance in the regulation of the intestinal immune response[1].

Animal models of IBD support the concept that genetic factors, environmental triggers, and immune dysregulation may have a potential role in developing uncontrolled intestinal inflammation that determines the typical endoscopic manifestations and mucosal lesions compatible with CD or UC[1-4].

Over the last decade, multiple genome-wide linkage searches have delineated numerous genomic regions containing putative IBD risk factors. In studies performed on unselected populations, an average of 8%-10% of CD patients and 6%-8% of UC patients have at least one relative affected by some type of IBD. However, these values vary from study to study and percentages of CD familial aggregation of less than 4% and more than 20% have been reported[2].

Moreover, studies on twins demonstrate a greater genetic influence for CD compared with UC; combined study concordance rates for monozygotic twins are 36% for CD and 16% for UC[5].

Recently, an association between CD and mutations in the NOD2/CARD15 gene located on chromosome 16q12 (IBD1) has been reported. NOD2/CARD15 acts as an intracellular receptor in monocytes for bacterial components, triggering activation of NFκB and thus leading to subsequent activation of the inflammatory response. Within the NOD2/CARD15 gene, three mutations have been identified as being associated with CD: two missense mutations (Arg702Trp in exon 4 and Gly908Arg in exon 8) and an insertion mutation of a C in exon 11 (1007finsC), the latter resulting in a truncated NOD2/CARD15 protein. These NOD2/CARD15 variants alter the structure of either the leucine-rich repeat (LRR) domain of the protein or the adjacent region. The activating function of nuclear factor NFκB is regulated by the carboxy-terminal LRR domain, which has an inhibitory role and also acts as an intracellular receptor for components of microbial pathogens. These observations suggest that the NOD2\CARD15 gene can confer susceptibility to CD by altering the recognition of these components and/or by over-activating NFκB in monocytes[6-9]. The question arises as to how NOD2/CARD15 mutations and impaired NFκB activation can confer susceptibility to CD. It has been suggested that the answer most likely lies within the leucine-rich repeats (LRR) of the NOD2/CARD15 gene and the family of Toll-like receptors (TLRs). These receptors, a family composed of at least 10 mammalian homologs of Drosophila Toll, serve as pattern recognition receptors for various microbial products and can mediate production of proinflammatory cytokines[10]. Toll-like receptor 4 (TLR4) functions as the main receptor for lipopolysaccharide (LPS) of Gram-negative bacteria[11]. After the recognition of pathogen-associated molecular patterns, the TLRs activate signal transduction pathways of the innate immune response genes including inflammatory cytokines and the NFκB signalling pathway[12]. TLR4 is expressed in macrophages, dendritic cells, endothelial cells, and, less abundantly, in intestinal epithelial cells, which are partly tolerant to LPS, thereby preventing an exaggerated immune response caused by the large number of bacteria in the intestinal lumen[13].

Mutations of the TLR4 gene are known to abolish responses to endotoxin in mice, as shown for the mice strains C3H/HeJ and C57BL/10SeCr[14]. Therefore, the ability to recognize bacterial wall products and to activate proinflammatory mechanisms by TLRs may be of great importance for immune reactions in the intestinal mucosa. The recently characterised D299G and T399I single nucleotide polymorphisms (SNPs) of TLR4 gene are probably associated with impaired LPS signalling and increased susceptibility to Gram negative infections[15].

In this study, we investigated the frequencies of the three NOD2/CARD15 gene mutations (Arg702Trp, Gly908Arg and 1007finsC) and of the TLR4 gene D299G and T399I SNPs in a group of 178 Italian adult patients affected by IBD: 133 patients with CD and 45 with UC. The allele frequencies of the NOD2/CARD15 and TLR4 genes were evaluated, and a detailed genotype-phenotype correlation was performed.

MATERIALS AND METHODS

Study population

The study population was comprised of 133 patients with CD (70 males, 63 females; mean age, 43.5 ± 10.7 years), 45 with UC (27 males, 18 females; mean age, 43.2 ± 11.0 years) and 103 healthy, unrelated controls (68 males, 35 females; mean age, 46.6 ± 9.8 years). Patients were consecutively recruited from Department of Paediatrics and Department of Medicine, University Hospital of Messina, Italy. All patients were from Eastern Sicily and Calabria (Southern Italy). Informed consent was obtained from each participant.

Diagnosis of CD and UC was established according to accepted clinical, endoscopic radiological, and histological criteria[16]. A detailed clinical questionnaire concerning different features of the disease was employed. The Vienna classification was used for CD phenotypes[17], while localization was defined based on the largest extent of the disease, according to X-ray, endoscopy, or surgical reports.

The following data of patients with CD and UC were collected: age, age at diagnosis, gender, familial or spontaneous disease (familial disease was considered if one first or second-degree relative had IBD), disease localization, disease behaviour, extraintestinal manifestations (arthritis, affections of eyes or skin, primary sclerosing cholangitis), type and site of surgery. Disease localization was defined as the maximum extent of digestive tract involvement at the latest follow-up.

Patients were eligible if IBD was confirmed, and they had undergone full colonoscopy with biopsy and/or surgical resection.

Table 1 Primers sequences and restriction enzymes used for genotyping TLR4 and NOD2/CARD15

Gene locus	SNPs		Sequence	Restriction enzyme
NOD2/CARD15	R702W	For	5' TTCAGATCACAGCAGCCTTC 3'	*Msp*I
		Rev	5' CCCACACTGCAAAATGTCAAC 3'	
	G908R	For	5' AGCCACTGAAAACTCTTGG 3'	*Hha*I
		Rev	5' TCTTCACCTGATCTCCCCAA 3'	
	L1007finsC	For	5' CCTGCAGTCTCTTTAACTGG 3'	*Nla*IV
		Rev	5' CTTACCAGACTTCCAGGATG 3'	
TLR4	D299G	For	5' TTAGAAATGAAGGAAACTTGGAAAAG 3'	*Bsa*BI
		Rev	5' TTTGTCAAACAATTAAATAAGTGATTAATA 3'	
	T399I	For	5' GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA 3'	*Hin*fI
		Rev	5' CCTGAAGACTGGAGAGTGAGTTAAATGCT 3'	

A group of 103 healthy, unrelated subjects coming from the Sicily and Calabria regions (mainly students, blood donors and hospital employees) were selected as controls.

DNA extraction

Genomic DNA was isolated from 1 mL of peripheral blood anticoagulated with EDTA as previously described[18]. DNA samples of the patients and control subjects were analyzed for the variants of NOD2/CARD15 and TLR4 genes by melting curve analysis.

Genotyping of the NOD2/CARD15 mutations

To detect the R702W, G908R, and L1007finsC mutations, we performed a polymerase chain reaction (PCR) using 0.5 U of Taq polymerase (Eurotaq, Euroclone Life Sciences Division, UK), 400 μmol/L dNTPs, and 0.1 μmol/L of each primer in a total volume of 25 μL. After an initial denaturation for 5 min at 95℃, PCR was performed by 35 cycles of denaturing at 95℃ for 30 s, annealing at 65℃ for 40 s, primer extension at 72℃ for 30 s. The final extension was performed at 72℃ for 7 min. PCR reactions were carried out using a GeneAmp PCR system 2700 (Applied Biosystem, CA, USA).

Genotyping of each SNP was performed by enzymatic digestion at 37℃, overnight. After enzymatic digestion, the fragments were separated and visualized by gel electrophoresis (3% NuSieve® GTG agarose gel BMA, Rockland, ME, USA).

The specific primers PCR and the restriction enzymes (New England Biolabs, Ipswich, MA) for each SNP are given in Table 1.

Wild-type/mutant genotype was confirmed by automatic sequencing using the ABI-PRISM Big Dye™ Terminator v. 3.0 Cycle sequencing Ready Reaction Kit (Applied Biosystems, CA, USA). The sequencing products were purified using DyeEx Spin Kits (Qiagen) and visualized on an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems, CA, USA).

Genotyping of the TLR4 polymorphisms

The two D299G and T399I SNPs of the TLR4 gene were determined by PCR-RFLP.

We performed PCR using 0.5 U of Taq polymerase (Eurotaq, Euroclone Life Sciences Division, UK), 400 μmol/L dNTPs, and 0.1 μmol/L of each primer in a total volume of 25 μL.

For D299G SNP, cycle conditions were an initial denaturation for 5 min at 95℃, followed by 32 cycles of denaturing at 95℃ for 30 s, annealing at 51℃ for 30 s, primer extension at 72℃ for 30 s, followed by a final extension at 72℃ for 7 min. For T399I SNP, cycle conditions were an initial denaturation for 5 min at 95℃, followed by 35 cycles of denaturing at 95℃ for 45 s, annealing at 55℃ for 30 s, primer extension at 72℃ for 45 s, followed by a final extension at 72℃ for 7 min.

The specific primers PCR and the restriction enzymes for each SNP are given in Table 1.

The amplified samples of TLR4 gene D299G and T399I SNPs were digested at 37℃, overnight, with the *Bsa*BI and *Hin*fI restriction enzymes (New England Biolabs, Ipswich, MA, USA), respectively.

After enzymatic digestion, the fragments were separated and visualized by gel electrophoresis (3% NuSieve® GTG agarose gel BMA, Rockland, ME, USA).

As previously described here, the results of enzymatic digestion were confirmed by DNA sequence analysis of representative samples of each SNP.

Statistical analysis

Data are given as mean ± SD. Allele and genotypes frequencies in patients and in controls were compared by χ^2 test or Fisher exact test, when an expected value was < 0.5; *P* values were considered significant at a level of < 0.05. Odds ratio (OR) and *P* values were calculated using a standard package (StataCorp. Stata Statistical Software: Release 8.0 College Station. TX: Stata Corporation 2001).

Allele frequencies were tested for the Hardy-Weinberg equilibrium. Cases and controls were compared using Pearson's χ^2 test.

RESULTS

Allele frequencies in IBD patients NOD2/CARD15 gene SNPs

In CD patients, the frequency of R702W mutation was significantly higher (9.8%) than in controls (2.4%, $P = 0.001$; OR, 4.09; 95% CI, 1.5-11.9) and in UC (2.3%, $P = 0.03$; OR, 4.49; 95% CI, 1.02-19.8; Table 2). No significant difference of the G908R mutation allele frequency was found

Table 2 NOD2/CARD15 and TLR4 SNPs allele frequencies of CD patients *vs* control group and UC patients

Polymorphisms of NOD2/CARD15 and TLR4 genes	CD (*n* = 133)	Allele frequency (%)	Controls (*n* = 103)	Allele frequency (%)	[1]*P*	OR (95% CI)	UC (*n* = 45)	Allele frequency (%)	[2]*P*	OR (95% CI)
R702W										
Wild-type	107 (80.4%)	9.8	98 (95.1%)	2.4	0.001	4.09 (1.5-11.9)	43 (95.5%)	2.3	0.03	4.49 (1.02-19.8)
Heterozygous	26 (19.6%)		5 (4.9%)				2 (4.5%)			
G908R										
Wild-type	120 (90.2%)	4.5	94 (91.2%)	4.3	NS	1.01 (0.3-3.5)	41 (91.1%)	4.4	NS	0.90 (0.28-2.92)
Heterozygous	13 (9.8%)		9 (8.8%)				4 (8.9%)			
L1007finsC										
Wild-type	107 (80.4%)	9.8	97 (94.1%)	2.9	0.002	3.92 (1.55-9.95)	43 (95.5%)	2.3	0.01	5.22 (1.19-22.98)
Heterozygous	26 (19.6%)		6 (5.9%)				2 (4.5%)			
D299G										
Wild-type	123 (92.5%)	3.7	95 (92.2%)	3.9%	NS	0.96 (0.37-2.54)	42 (93.3%)	3.4	NS	0.87 (0.23-3.35)
Heterozygous	10 (7.5%)		8 (7.8%)				3 (6.7 %)			
T399I										
Wild-type	125 (94%)	3.0	97 (94.1%)	2.9%	NS	1.15 (0.28-4.64)	42 (93.3%)	3.4	NS	1.11 (0.28-4.40)
Heterozygous	8 (6.0%)		6 (5.9%)				3 (6.7%)			

No patients homozygous for NOD2/CARD15 gene R702W, G908R and L1007finsC SNPs were found in this study population; No patients homozygous for TLR4 gene D299G and T399I SNPs were found in this study population. [1]CD patients *vs* control group; [2]CD patients *vs* UC patients. NS: No significance.

between CD (4.5%) and the control group (4.3%; $P > 0.05$; OR, 1.01; 95% CI, 0.3-3.5), and between CD and UC patients (4.4%, 0.05; OR, 0.90; 95% CI, 0.28-2.92; Table 2).

The frequency of the frameshift mutation L1007finsC was significantly higher in CD patients (9.8%) compared with controls (2.9%, $P = 0.002$; OR, 3.92; 95% CI, 1.55-9.95) or patients with UC (2.3%, $P = 0.01$; OR, 5.22; 95% CI, 1.19-22.98; Table 2).

In UC patients, the allele frequencies of the R702W, G908R, and 1007finsC mutations were not significantly different from the control group (R702W: $P > 0.05$; OR, 0.91; 95% CI, 0.17-4.88 and G908R: $P > 0.05$; OR, 1.01; 95% CI, 0.3-3.5 and L1007finsC: $P > 0.05$; OR, 0.75; 95% CI, 0.15-3.88).

No homozygous carriers of the three NOD2/CARD15 mutations were found in the study and control populations.

The NOD2/CARD15 allele frequencies were in Hardy-Weinberg equilibrium in all patients and in control subjects.

TLR4 gene SNPs

The results of the genotype analyses in 133 patients with CD, in 45 patients with UC and in 103 control individuals, with regard to the TLR4 D299G and T399I SNPs are shown in Table 2.

In CD patients, the frequency of the D299G SNP (3.7%) was not significantly different from the controls (3.9%, $P > 0.05$; OR, 0.96; 95% CI, 0.37-2.54) or from UC patients (3.4%, $P > 0.05$; OR, 0.87; 95% CI, 0.23-3.35; Table 2). The T399I SNP allele frequency was not significantly different between CD patients (3.0%) and control group (2.9%, $P > 0.05$; OR, 1.15; 95% CI, 0.28-4.64); or between CD (3.0%) and UC patients (3.4%, $P > 0.05$; OR 1.11, 95% CI 0.28-4.40; Table 2). No significant difference was found between UC patients and control group as regards the D299G SNP ($P > 0.05$; OR, 0.84; 95% CI, 0.21-3.36) or the T399I SNP ($P > 0.05$; OR, 1.15; 95% CI, 0.28-4.64).

No homozygous carriers of the two SNPs were found in the study and control populations.

The TLR4 allele frequencies were in Hardy-Weinberg equilibrium in all patients and in the control group.

Genotype-phenotype correlations

When the contribution of each SNP of the NOD2/CARD15 gene was investigated, the major support to the genotype-phenotype correlation could be ascribed to the G908R and the L1007finsC alleles (Table 3). In particular, in CD patients, the occurrence of one risk allele of G908R was associated with stenosing phenotype ($P = 0.03$) and resective surgery ($P = 0.003$).

An increased frequency of ileal localization (80.7%, $P = 0.001$) and resective surgery (53.9%, $P = 0.01$) was found in CD L1007finsC heterozygotes compared with CD patients with wild-type NOD2\CARD15 gene (ileum 36.8% and resective surgery 26.4%, respectively).

Moreover, the clinical features of all CD patients were analysed with respect to the presence of one or two risk alleles of each SNP (heterozygous or compound heterozygous) of any NOD2/CARD15 variants (Table 3).

By univariate analysis, the presence of one risk allele was significantly associated with ileal localization ($P = 0.04$) and resective surgery ($P = 0.03$). These significant associations increased in the compound heterozygotes ($P = 0.03$ and $P < 0.0001$, respectively). Moreover, the presence of two risk alleles was significantly associated with stenosing disease ($P = 0.02$, Table 3).

In CD patients, TLR4 D299G and T399I SNPs were not found to be associated with age at diagnosis, sex, localization, disease type, resective surgery and extraintestinal manifestations.

Similarly, in UC patients, these TLR4 gene SNPs were not associated with any studied clinicopathological parameter.

Table 3 Genotype-phenotype correlations in CD patients

Total CD patients (n = 133)	CARD15 no risk alleles (n = 68, 51.1%)	R702W 1 risk allele (n = 26, 19.6%)	G908R 1 risk allele (n = 13, 9.7%)	L1007finsC 1 risk allele (n = 26, 19.6%)	CARD15 at least 1 risk allele (n = 65, 48.8%)	CARD15 compound heterozygous (n = 48, 36.1%)
Age (mean ± SD)	41.5 ± 11.2	41.2 ± 11.9	43.02 ± 10.9	42.0 ± 12.8	42.3 ± 12.1	42.7 ± 11.9
Sex (m/f, 70/63)	32/36	13/13	8/5	10/16	30/35	25/23
Localization (%)						
Ileum (n = 61)	25 (36.8)	11 (42.3)	4 (30.8)	21 (80.7)	38 (58.5)	30 (62.5)
Ileo-colon (n = 39)	22 (32.3)	10 (38.5)	4 (30.8)	3 (11.5)	15 (23.0)	10 (20.8)
Colon (n = 30)	18 (26.5)	5 (19.5)	5 (38.4)	2 (7.8)	12 (18.5)	8 (16.7)
Upper GI (n = 3)	3 (4.4%)				0	0
P		> 0.05[1]	> 0.05[1]	0.001[1]	0.04[2]	0.03[3]
Disease type (%)						
Inflammatory (n = 37)	14 (20.6)	7 (27.0)	6 (46.0)	10 (38.5)	23 (35.4)	8 (16.6)
Stenosing (n = 56)	32 (47.0)	11 (42.3)	7 (54.0)	6 (23.0)	24 (37.0)	34 (70.8)
Fistulizing (n = 40)	22 (32.4)	8 (30.7)	0	10 (38.5)	18 (27.6)	6 (12.6)
P		> 0.05[1]	0.03[1]	> 0.05[1]	> 0.05[2]	0.02[3]
Resective Surgery (%)	18 (26.4)	9 (34.6)	9 (69.2)	14 (53.9)	32 (49.2)	31 (64.6)
P		> 0.05[1]	0.003[1]	0.01[1]	0.01[2]	0.000[3]
Extraintestinal manifestations (n = 18, %)	10 (55.5)	3 (11.5)	1 (7.7)	4 (15.3)	8 (13.3)	3 (6.2)

No patients homozygous for R702W, G908R and L1007finsC SNPs were found in this study population. [1]CD patients no risk allele *vs* CD patients with risk allele; [2]CARD15 at least 1 risk allele *vs* CD patients no risk allele; [3]CARD15 compound heterozygous *vs* CD patients no risk allele.

DISCUSSION

In our study, we investigated the prevalence of NOD2/CARD15 and TLR4 genetic variants in CD and UC patients. Moreover, we compared the results with clinical phenotype characteristics of IBD of our patients to identify a possible genotype-phenotype association.

There are several controversial data about the role of the SNPs of the NOD2/CARD15 (R702W, G908R and L1007finsC), and TLR4 genes (D299G and T399I) in the pathogenesis of CD. Indeed, there are significant phenotypic differences that exist among populations.

The NOD2/CARD15 mutations are absent in Asian CD populations and controls[19-21]. In this case, the findings indicate that the NOD2/CARD15 is not a major contributor to CD susceptibility in the Japanese population. Similar data have been found in Turkish patients with IBD[22].

The highest recorded frequencies are reported in a small study of 55 paediatric patients in Europe with two thirds of the patients having at least one NOD2/CARD15 mutation[23]. Within Europe, there is evidence of a north-south gradient with lower allele frequencies in the Celtic and Scandinavian countries compared to Southern Europe[23].

To our knowledge, this is the first study in a large series of sporadic IBD patients coming from Eastern Sicily and Calabria. Indeed, previous reports regarded a Sicilian, small town population in which a familial study was performed[24]. Other studies have examined a group of sporadic Sicilian IBD patients, but the number of cases was smaller than in our study[25].

In our study, the reported rates of 48.8 % of patients carrying at least one NOD2/CARD15 mutation in CD and 19.4% in controls are consistent with previously reported rates of 30%-50% in CD and 7%-20% in controls from other European regions[26-31]. Moreover, 36.1% had two mutations (compound heterozygotes). Recently, Renda *et al* examined a group of 182 CD patients coming from Western Sicily and they found that 56 patients (30%) had at least one mutation of the NOD2/CARD15 gene[32].This percentage was lower in respect to our data (48.8%). This difference may be ascribed to a different ethnic background. Indeed, the patients of our study coming both Eastern Sicily and Calabria. Today, populations genetically similar to that of the Northern Italy (as well as of the Northern Africa) are present in the Eastern Sicily. This heterogeneous population, during the middle age, might explain the genetic differences in the patient CD samples of the Eastern in respect to Western Sicily[33].

The allele frequencies of the R702W (9.8%) and L1007finsC (9.8%) mutations were significantly higher in CD patients compared to UC patients and controls, whereas the frequency of the G908R (4.5%) mutation was similar in CD and UC patients, and in the control group. Collectively, our study confirmed previous studies, which reported increased mutation carrier frequencies of one of the three variant alleles in CD patients compared to UC patients or healthy controls[2,28,32,34-36].

We also found that different risk alleles might be associated with different clinical features: in particular, the G908R allele seems to be associated with stenosing phenotype and need for surgery. The L1007finsC seems to correlate with ileal localization and resective surgery. These data suggest a more aggressive course of the disease in carriers of risk alleles. The strongest observed effect for ileal location is consistent with the proposed involvement of ileal Paneth cells in the pathophysiology of NOD2/CARD15-mediated disease susceptibility[30,36,37]. NOD2/CARD15 mutations may, thus, abrogate normal Paneth cell behaviour, explaining preferential involvement of the terminal ileum[26].

Moreover, in our study, the risk of developing CD with a more aggressive course was increased in compound heterozygotes. In other populations, stronger associations

have been reported for homozygotes and compound heterozygotes than for simple heterozygotes. One copy of the risk alleles confers a 2-4-fold risk for developing CD, whereas double-dose carriage increases the risk by 20-40-fold[23]. Our study is in agreement with such a gene-dosage effect, although at lower levels.

In our IBD patients, we also examined the allele frequencies of the TLR4 D299G and T399I SNPs and the possible genotype-phenotype correlation.

With regard to the role of the TLR4 gene in the pathogenesis of IBD, several studies have been conducted leading to divergent results[23]. The allele frequency of the D299G mutation ranges between 8%-13% in CD, 0%-10% in UC and 3%-15% in healthy controls[38]. This TLR4 SNP was associated with CD and UC in a Belgian study[39]. This association was replicated in Dutch, German, Australian and Greek populations with CD, and an association with colonic disease has been described[8,34,35,40]. In one German cohort, an association was demonstrated between UC and the TLR4 T399I SNP[41]. However, there is substantial heterogeneity between populations, and no association was noted in Scottish CD patients[30].

In our study, we found no difference in the prevalence of these mutations in our CD and UC patients, and controls. Recently, other studies have failed to find the association of the D299G and T399I SNPs of TLR4 gene[24,42-45]. In a retrospective German and Hungarian cohort study, patients with CD and UC were genotyped for the presence of the CD14 c.1-260C>T promoter variant and the TLR4 D299G variant. In this study, in German and Hungarian populations, IBD appears to be associated with the CD14 c.1-260C>T promoter variant, but not with the TLR4 D299G variant[45]. Recent data suggest that neither of these 2 variants is causal, but they may be in linkage disequilibrium with, as yet unidentified, causal variants[46-48].

We examined also whether the TLR4 D299G and T399I SNPs could be related to particular CD or UC phenotypes. Detailed analysis did not show any association of the examined TLR4 gene variants with either CD or UC patient subgroups. In other studies, in CD patients, an association has been reported between D299G SNP and ileal localization and structuring phenotype[49]. Our data are similar to those previously reported[28]. These contrasting results can be ascribed to the different ethnic background of the various IBD populations studied.

Although several studies have been performed, further research is warranted to clarify the role of the genetic variants of NOD2/CARD15 and TLR4, and to investigate whether these genetic risk factors might be confirmed and considered clinically relevant. Indeed, an eventual goal in the genomic study of IBD is to identify these biologically relevant genotype-phenotype associations and to apply them to clinical practice.

COMMENTS

Background

Crohn's disease (CD) and ulcerative colitis (UC) are idiopathic inflammatory bowel disease (IBD) with genetic risk factors. There is evidence that NOD2/CARD15 and Toll-like receptor 4 (TLR4) genes may be involved in their pathogenesis.

Research frontiers

In our study, we found that some single nucleotide polymorphisms (SNPs) of the NOD2/CARD15 gene, but not of the TLR4 gene, were significantly more frequent in CD patients. Therefore, it is possible that the NOD2/CARD15 gene plays an important role in the pathogenesis of IBD.

Innovations and breakthroughs

We evaluated the allele and genotype frequencies of the more frequent SNPs of NOD2/CARD15 and TLR4 genes in a selected IBD population coming from Eastern Sicily and Calabria (Southern Italy), a geographical area for which very few data exist.

Applications

Genotyping of patients with CD could be an important diagnostic tool in clinical practice for identifying high-risk patients with specific diagnostics and therapeutic needs.

Peer review

This study underlines the association of the NOD2/CARD15 genotype with the behaviour and location of CD also in patients coming from Eastern Sicily and Calabria (Italy). Moreover, the CD patients carrying at least one major variant of NOD/CARD15 gene had an aggressive clinical course. Test strategies with NOD2/CARD15 variations to predict the clinical course of CD could lead to the development of new therapeutic paradigms.

REFERENCES

1 **Hugot JP**. Inflammatory bowel disease: a complex group of genetic disorders. *Best Pract Res Clin Gastroenterol* 2004; **18**: 451-462

2 **Van Limbergen J**, Russell RK, Nimmo ER, Ho GT, Arnott ID, Wilson DC, Satsangi J. Genetics of the innate immune response in inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**: 338-355

3 **Trinh TT**, Rioux JD. Understanding association and causality in the genetic studies of inflammatory bowel disease. *Gastroenterology* 2005; **129**: 2106-2110

4 **Henckaerts L**, Pierik M, Joossens M, Ferrante M, Rutgeerts P, Vermeire S. Mutations in pattern recognition receptor genes modulate seroreactivity to microbial antigens in patients with inflammatory bowel disease. *Gut* 2007; **56**: 1536-1542

5 **Bonen DK**, Cho JH. The genetics of inflammatory bowel disease. *Gastroenterology* 2003; **124**: 521-536

6 **Kobayashi KS**, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, Flavell RA. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 2005; **307**: 731-734

7 **Vermeire S**. NOD2/CARD15: relevance in clinical practice. *Best Pract Res Clin Gastroenterol* 2004; **18**: 569-575

8 **Brand S**, Staudinger T, Schnitzler F, Pfennig S, Hofbauer K, Dambacher J, Seiderer J, Tillack C, Konrad A, Crispin A, Goke B, Lohse P, Ochsenkuhn T. The role of Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms and CARD15/NOD2 mutations in the susceptibility and phenotype of Crohn's disease. *Inflamm Bowel Dis* 2005; **11**: 645-652

9 **Braat H**, Stokkers P, Hommes T, Cohn D, Vogels E, Pronk I, Spek A, van Kampen A, van Deventer S, Peppelenbosch M, Hommes D. Consequence of functional Nod2 and Tlr4 mutations on gene transcription in Crohn's disease patients. *J Mol Med* 2005; **83**: 601-609

10 **Meylan E**, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. *Nature* 2006; **442**: 39-44

11 **Akira S**, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124**: 783-801

12 **Medzhitov R**, Janeway CA Jr. Innate immunity: impact on the adaptive immune response. *Curr Opin Immunol* 1997; **9**: 4-9

13 **Otte JM**, Cario E, Podolsky DK. Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. *Gastroenterology* 2004; **126**: 1054-1070

14 **Poltorak A**, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998; **282**: 2085-2088

15 **Gaya DR**, Russell RK, Nimmo ER, Satsangi J. New genes in inflammatory bowel disease: lessons for complex diseases? *Lancet* 2006; **367**: 1271-1284

16 **Podolsky DK**. Inflammatory bowel disease 2. *N Engl J Med* 1991; **325**: 1008-1016

17 **Gasche C**, Scholmerich J, Brynskov J, D'Haens G, Hanauer SB, Irvine EJ, Jewell DP, Rachmilewitz D, Sachar DB, Sandborn WJ, Sutherland LR. A simple classification of Crohn's disease: report of the Working Party for the World Congresses of Gastroenterology, Vienna 1998. *Inflamm Bowel Dis* 2000; **6**: 8-15

18 **Sambrook J**, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1989: E3-E4

19 **Inoue N**, Tamura K, Kinouchi Y, Fukuda Y, Takahashi S, Ogura Y, Inohara N, Nunez G, Kishi Y, Koike Y, Shimosegawa T, Shimoyama T, Hibi T. Lack of common NOD2 variants in Japanese patients with Crohn's disease. *Gastroenterology* 2002; **123**: 86-91

20 **Sugimura M**, Kinouchi Y, Takahashi S, Aihara H, Takagi S, Negoro K, Obana N, Kojima Y, Matsumoto K, Kikuchi T, Hiroki M, Oomori S, Shimosegawa T. CARD15/NOD2 mutational analysis in Japanese patients with Crohn's disease. *Clin Genet* 2003; **63**: 160-162

21 **Yamazaki K**, Takazoe M, Tanaka T, Kazumori T, Nakamura Y. Absence of mutation in the NOD2/CARD15 gene among 483 Japanese patients with Crohn's disease. *J Hum Genet* 2002; **47**: 469-472

22 **Ozen SC**, Dagli U, Kilic MY, Toruner M, Celik Y, Ozkan M, Soykan I, Cetinkaya H, Ulker A, Ozden A, Bozdayi AM. NOD2/CARD15, NOD1/CARD4, and ICAM-1 gene polymorphisms in Turkish patients with inflammatory bowel disease. *J Gastroenterol* 2006; **41**: 304-310

23 **Riis L**, Vind I, Vermeire S, Wolters F, Katsanos K, Politi P, Freitas J, Mouzas IA, O'Morain C, Ruiz-Ochoa V, Odes S, Binder V, Munkholm P, Moum B, Stockbrugger R, Langholz E. The prevalence of genetic and serologic markers in an unselected European population-based cohort of IBD patients. *Inflamm Bowel Dis* 2007; **13**: 24-32

24 **Cottone M**, Renda MC, Mattaliano A, Oliva L, Fries W, Criscuoli V, Modesto I, Scimeca D, Maggio A, Casa A, Maisano S, Mocciaro F, Sferrazza A, Orlando A. Incidence of Crohn's disease and CARD15 mutation in a small township in Sicily. *Eur J Epidemiol* 2006; **21**: 887-892

25 **Fries W**, Renda MC, Lo Presti MA, Raso A, Orlando A, Oliva L, Giofre MR, Maggio A, Mattaliano A, Macaluso A, Cottone M. Intestinal permeability and genetic determinants in patients, first-degree relatives, and controls in a high-incidence area of Crohn's disease in Southern Italy. *Am J Gastroenterol* 2005; **100**: 2730-2736

26 **Ahmad T**, Armuzzi A, Bunce M, Mulcahy-Hawes K, Marshall SE, Orchard TR, Crawshaw J, Large O, de Silva A, Cook JT, Barnardo M, Cullen S, Welsh KI, Jewell DP. The molecular classification of the clinical manifestations of Crohn's disease. *Gastroenterology* 2002; **122**: 854-866

27 **Brant SR**, Picco MF, Achkar JP, Bayless TM, Kane SV, Brzezinski A, Nouvet FJ, Bonen D, Karban A, Dassopoulos T, Karaliukas R, Beaty TH, Hanauer SB, Duerr RH, Cho JH. Defining complex contributions of NOD2/CARD15 gene mutations, age at onset, and tobacco use on Crohn's disease phenotypes. *Inflamm Bowel Dis* 2003; **9**: 281-289

28 **Lesage S**, Zouali H, Cezard JP, Colombel JF, Belaiche J, Almer S, Tysk C, O'Morain C, Gassull M, Binder V, Finkel Y, Modigliani R, Gower-Rousseau C, Macry J, Merlin F, Chamaillard M, Jannot AS, Thomas G, Hugot JP. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. *Am J Hum Genet* 2002; **70**: 845-857

29 **Annese V**, Palmieri O, Latiano A, Ardizzone S, Castiglione F, Cottone M, D'Inca R, Gionchetti P, Papi C, Riegler G, Vecchi M, Andriulli A. Frequency of NOD2/CARD15 variants in both sporadic and familial cases of Crohn's disease across Italy. An Italian Group for Inflammatory Bowel Disease Study. *Dig Liver Dis* 2004; **36**: 121-124

30 **Cuthbert AP**, Fisher SA, Mirza MM, King K, Hampe J, Croucher PJ, Mascheretti S, Sanderson J, Forbes A, Mansfield J, Schreiber S, Lewis CM, Mathew CG. The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease. *Gastroenterology* 2002; **122**: 867-874

31 **Abreu MT**, Taylor KD, Lin YC, Hang T, Gaiennie J, Landers CJ, Vasiliauskas EA, Kam LY, Rojany M, Papadakis KA, Rotter JI, Targan SR, Yang H. Mutations in NOD2 are associated with fibrostenosing disease in patients with Crohn's disease. *Gastroenterology* 2002; **123**: 679-688

32 **Renda MC**, Orlando A, Civitavecchia G, Criscuoli V, Maggio A, Mocciaro F, Rossi F, Scimeca D, Modesto I, Oliva L, Cottone M. The role of CARD15 mutations and smoking in the course of Crohn's disease in a Mediterranean area. *Am J Gastroenterol* 2008; **103**: 649-655

33 **Ramachandran S**, Deshpande O, Roseman CC, Rosenberg NA, Feldman MW, Cavalli-Sforza LL. Support from the relationship of genetic and geographic distance in human populations for a serial founder effect originating in Africa. *Proc Natl Acad Sci USA* 2005; **102**: 15942-15947

34 **Gazouli M**, Mantzaris G, Kotsinas A, Zacharatos P, Papalambros E, Archimandritis A, Ikonomopoulos J, Gorgoulis VG. Association between polymorphisms in the Toll-like receptor 4, CD14, and CARD15/NOD2 and inflammatory bowel disease in the Greek population. *World J Gastroenterol* 2005; **11**: 681-685

35 **Hume GE**, Fowler EV, Doecke J, Simms LA, Huang N, Palmieri O, Griffiths LR, Florin TH, Annese V, Radford-Smith GL. Novel NOD2 haplotype strengthens the association between TLR4 Asp299Gly and Crohn's disease in an Australian population. *Inflamm Bowel Dis* 2008; **14**: 585-590

36 **Annese V**, Latiano A, Palmieri O, Lombardi G, Andriulli A. NOD2/CARD15 in healthy relatives of IBD patients. *Eur Rev Med Pharmacol Sci* 2006; **10**: 33-36

37 **Odes S**, Friger M, Vardi H, Claessens G, Bossuyt X, Riis L, Munkholm P, Wolters F, Yona H, Hoie O, Beltrami M, Tsianos E, Katsanos K, Mouzas I, Clofent J, Monteiro E, Messori A, Politi P, O'Morain C, Limonard C, Russel M, Vatn M, Moum B, Stockbrugger R, Vermeire S. Role of ASCA and the NOD2/CARD15 mutation Gly908Arg in predicting increased surgical costs in Crohn's disease patients: a project of the European Collaborative Study Group on Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2007; **13**: 874-881

38 **Russell RK**, Nimmo ER, Satsangi J. Molecular genetics of Crohn's disease. *Curr Opin Genet Dev* 2004; **14**: 264-270

39 **Franchimont D**, Vermeire S, El Housni H, Pierik M, Van Steen K, Gustot T, Quertinmont E, Abramowicz M, Van Gossum A, Deviere J, Rutgeerts P. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. *Gut* 2004; **53**: 987-992

40 **Oostenbrug LE**, Drenth JP, de Jong DJ, Nolte IM, Oosterom E, van Dullemen HM, van der Linde K, te Meerman GJ, van der Steege G, Kleibeuker JH, Jansen PL. Association between Toll-like receptor 4 and inflammatory bowel disease. *Inflamm Bowel Dis* 2005; **11**: 567-575

41 **Torok HP**, Glas J, Tonenchi L, Mussack T, Folwaczny C. Polymorphisms of the lipopolysaccharide-signaling

complex in inflammatory bowel disease: association of a mutation in the Toll-like receptor 4 gene with ulcerative colitis. *Clin Immunol* 2004; **112**: 85-91

42 **Arnott ID**, Ho GT, Nimmo ER, Satsangi J. Toll-like receptor 4 gene in IBD: further evidence for genetic heterogeneity in Europe. *Gut* 2005; **54**: 308; author reply 309

43 **Browning BL**, Huebner C, Petermann I, Gearry RB, Barclay ML, Shelling AN, Ferguson LR. Has toll-like receptor 4 been prematurely dismissed as an inflammatory bowel disease gene? Association study combined with meta-analysis shows strong evidence for association. *Am J Gastroenterol* 2007; **102**: 2504-2512

44 **Hong J**, Leung E, Fraser AG, Merriman TR, Vishnu P, Krissansen GW. TLR2, TLR4 and TLR9 polymorphisms and Crohn's disease in a New Zealand Caucasian cohort. *J Gastroenterol Hepatol* 2007; **22**: 1760-1766

45 **Baumgart DC**, Buning C, Geerdts L, Schmidt HH, Genschel J, Fiedler T, Gentz E, Molnar T, Nagy F, Lonovics J, Lochs H, Wiedenmann B, Nickel R, Witt H, Dignass A. The c.1-260C>T promoter variant of CD14 but not the c.896A>G (p.D299G) variant of toll-like receptor 4 (TLR4) genes is associated with inflammatory bowel disease. *Digestion* 2007; **76**: 196-202

46 **Arnott ID**, Nimmo ER, Drummond HE, Fennell J, Smith BR, MacKinlay E, Morecroft J, Anderson N, Kelleher D, O' Sullivan M, McManus R, Satsangi J. NOD2/CARD15, TLR4 and CD14 mutations in Scottish and Irish Crohn's disease patients: evidence for genetic heterogeneity within Europe? *Genes Immun* 2004; **5**: 417-425

47 **De Jager PL**, Franchimont D, Waliszewska A, Bitton A, Cohen A, Langelier D, Belaiche J, Vermeire S, Farwell L, Goris A, Libioulle C, Jani N, Dassopoulos T, Bromfield GP, Dubois B, Cho JH, Brant SR, Duerr RH, Yang H, Rotter JI, Silverberg MS, Steinhart AH, Daly MJ, Podolsky DK, Louis E, Hafler DA, Rioux JD. The role of the Toll receptor pathway in susceptibility to inflammatory bowel diseases. *Genes Immun* 2007; **8**: 387-397

48 **Henckaerts L**, Pierik M, Joossens M, Ferrante M, Rutgeerts P, Vermeire S. Mutations in pattern recognition receptor genes modulate seroreactivity to microbial antigens in patients with inflammatory bowel disease. *Gut* 2007; **56**: 1536-1542

49 **Annese V**, Lombardi G, Perri F, D'Inca R, Ardizzone S, Riegler G, Giaccari S, Vecchi M, Castiglione F, Gionchetti P, Cocchiara E, Vigneri S, Latiano A, Palmieri O, Andriulli A. Variants of CARD15 are associated with an aggressive clinical course of Crohn's disease--an IG-IBD study. *Am J Gastroenterol* 2005; **100**: 84-92

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BASIC RESEARCH

Metabolomic changes in fatty liver can be modified by dietary protein and calcium during energy restriction

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Abstract

AIM: To characterise the effect of energy restriction (ER) on liver lipid and primary metabolite profile by using metabolomic approach. We also investigated whether the effect of energy restriction can be further enhanced by modification of dietary protein source and calcium.

METHODS: Liver metabolomic profile of lean and obese C57Bl/6J mice (n = 10/group) were compared with two groups of weight-reduced mice. ER was performed on control diet and whey protein-based high-calcium diet (whey + Ca). The metabolomic analyses were performed using the UPLC/MS based lipidomic platform and the HPLC/MS/MS based primary metabolite platform.

RESULTS: ER on both diets significantly reduced hepatic lipid accumulation and lipid droplet size, while only whey + Ca diet significantly decreased blood glucose (P < 0.001) and serum insulin (P < 0.01). In hepatic lipid species the biggest reduction was in the level of triacylglycerols and ceramides while the level of cholesterol esters was significantly increased during ER. Interestingly, diacylglycerol to phospholipid ratio, an indicator of relative amount of diabetogenic diglyceride species, was increased in the control ER group, but decreased in the whey + Ca ER group (P < 0.001, *vs* obese). ER on whey + Ca diet also totally reversed the obesity induced increase in the relative level of lipotoxic ceramides (P < 0.001, *vs* obese; P > 0.05, *vs* lean). These changes were accompanied with up-regulated TCA cycle and pentose phosphate pathway metabolites.

CONCLUSION: ER-induced changes on hepatic metabolomic profile can be significantly affected by dietary protein source. The therapeutic potential of whey protein and calcium should be further studied.

Key words: Fatty liver; Metabolomics; Energy restriction; Whey protein; Dietary calcium

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INTRODUCTION

Obesity is closely associated with different components of metabolic syndrome[1]. However, not all obese

individuals develop metabolic syndrome and not all individuals with metabolic syndrome are obese. It has recently been suggested that fat accumulation in the liver is the key feature distinguishing those individuals who develop metabolic syndrome from those who do not[2]. The mechanisms leading to hepatic fat accumulation are not fully understood and, hence, the means of preventing and treating this condition are limited. Once fat has accumulated, the liver is insulin resistant and overproduces major cardiovascular risk factors, such as C-reactive protein, very low density lipoprotein and plasminogen activator inhibitor-1[3]. At the moment, improving insulin resistance through energy restriction and subsequent weight loss remains the cornerstone of therapy for non-alcoholic fatty liver disease[4].

Lipids are a highly diverse class of molecules, which have important roles as signalling and structural molecules in addition to serving as energy storage. Therefore, it is crucial to identify the variety of lipid species accumulating in the liver in order to understand the complex process of hepatic insulin resistance. The level of triacylglycerides (TAG) and diacylglycerides (DAG) has been shown to be increased in non-alcoholic fatty liver disease in humans, while total amount of phosphatidylcholines is decreased[5]. Similar changes in liver lipids have been detected in ob/ob mice with up-regulation of TAG and DAG, diacylphosphoglycerols and specific ceramide species and down-regulation of sphingomyelins[6]. Interestingly, a recent human study revealed that a high level of liver fat is also associated with changes in the lipidomic profile of subcutaneous adipose tissue[7]. Increased adipose tissue ceramides, SM, ether phospholipids and long-chain TAG were associated with higher liver fat level. Hence, the accumulation of ceramides and TAG also in the subcutaneous adipose tissue seems to reflect the development of fatty liver. However, more studies are needed to support the findings of fatty liver lipidomics.

Even though weight loss is the main therapeutic way to reduce liver fat, the information on the effect of energy restriction on liver lipidomic profile is scarce. Also the beneficial effect of different dietary components on liver fat species is nearly an unexplored area[8]. High intake of dairy products is related to lower risk of insulin resistance[9], type two diabetes[10,11] and metabolic syndrome[12-15], but the mechanism of action has not been established. The increased intake of dairy products or calcium has also been shown to augment weight loss both in humans and mice[16-19]. Although part of the effect of dairy products on weight loss can be attributed to calcium, it has been repeatedly demonstrated that the anti-obesity effect of dairy is superior to that of calcium alone[18,20]. It has been suggested that the whey protein fraction of milk is a source of bioactive peptides or other compounds capable of regulating adipose tissue metabolism, energy expenditure or satiety signals[21]. In our previous study, we showed that whey protein in combination with calcium attenuates weight gain[22], but the effects of whey protein during energy restriction have not been reported. Also, the effect of whey protein containing high-calcium diet on hepatic lipid profile has not been previously described.

The aim of this study was to characterise the effect of high-fat diet-induced obesity and the subsequent ER on liver lipidomic and primary metabolite profile in C57Bl/6 mice, a widely studied model of diet induced obesity. In addition we investigated whether the effect of ER may be significantly improved by modulating the protein source and calcium content of the weight loss diet.

MATERIALS AND METHODS

Animals and diets

Eight-week old male C57Bl/6J mice (n = 40) were purchased from Harlan (Horst, The Netherlands). The mice were housed five in a cage in a standard experimental animal laboratory, illuminated from 6:30-18:30, temperature 22 ± 1℃. The protocols were approved by the Animal Experimentation Committee of the University of Helsinki, Finland and the principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed. The mice had free access to feed and tap water during the experiment. After a one-week acclimatisation period on a normal chow diet (Harlan Tekland 2018, Harlan Holding, Inc, Wilmington, DE, USA) thirty mice (25.5 g ± 0.3 g) were put on a high-fat diet (60% of energy from fat, D05031101M, Research Diets Inc., New Brunswick, NJ, USA) for 14 wk. Ten remaining mice continued on normal chow diet (*ad libitum*) throughout the study and served as a lean control group. After the weight gain period on high-fat diet one group of mice (obese group, n = 10) was sacrificed, and the remaining mice were put on a calorie restriction diet for 7 wk. During the calorie restriction period, the mice were given 70% of the energy they ate during the ad libitum feeding. In the beginning of the calorie restriction period, the body weight matched mice were divided into two groups: whey + Ca group and control group. Whey + Ca group received high-fat diet (D05031104M, Research Diets Inc., New Brunswick, NJ, USA) with 1.8% $CaCO_3$ and all protein (18% of energy) from whey protein isolate (Alacen™ 895, NZMP, Auckland, New Zealand). The control group continued with the same high-fat diet (D05031101M) as during the weight gain period. The powdered diets were moistened with tap water (200 mL/kg in whey + Ca, 110 mL/kg in control and 700 mL/kg in normal chow diet) using industrial dough mixer, packed in one-day portions and stored at -20℃.

The body weight was monitored weekly during the weight gain period, and twice per week during the calorie restriction period using a standard table scale (Ohaus Scout™ Pro, SP4001, Nänikon, Switzerland). The consumption of feed was monitored daily using the same table scale. The body fat content was analysed by dual-energy X-ray absorptiometry (DEXA, Lunar PIXImus, GE Healthcare, Chalfont St. Giles, UK) at the end of the weight gain and calorie restriction periods.

Calorimetry and metabolic performance

The dietary protein-induced differences in metabolic performance, energy expenditure, physical activity and drinking and feeding behaviour were analysed by housing an additional group of animals (n = 4/whey group and n = 3/casein group) in a home cage-based monitoring system for laboratory animals (LabMaster®, Bad Homburg, Germany). The instrument consists of a combination of highly sensitive feeding and drinking sensors for automated online measurement. The calorimetry system is an open-circuit system that determines O_2 consumption, CO_2 production, and respiratory quotient (RQ = VCO_2/VO_2, where V is volume), respiratory exchange rate and heat. A photobeam-based activity monitoring system detects and records every ambulatory movement, including rearing and climbing movements in every cage. The sensors for detection of movement operate efficiently in both light and dark phases, allowing continuous recording. All of the parameters were measured continuously and simultaneously in all animals over the subsequent 7 d after 5 d of adaptation in identical training cages.

Fecal fat excretion

For the collection of feces, the mice were housed in metabolic cages for 72 h at the end of the weight gain and weight reduction periods. The intake of feed and drink was monitored daily and feces collected at the end of the 72 h period. The feces were weighed and stored in -70℃ until assayed. The fat content of the fecal samples was determined by SBR (Schmid-Bondzynski-Ratzlaff) method modified for fecal sample analysis[23]. The apparent fat absorption was calculated from the amount of feed consumed and the amount of fat excreted during the housing in metabolic cages. Apparent fat absorption (%) was determined as 100 × (fat intake-fecal fat)/(fat intake). To estimate the effect of fat excretion on energy absorption during the whole study period, we calculated the apparent cumulative energy absorption from fat using the cumulative energy intake data (apparent fat absorption % × cumulative energy intake from fat) as described previously[24].

Blood glucose and serum insulin

Blood glucose and was analysed from the blood samples taken at the termination of the animals. Blood glucose was determined by glucometer (Super Glucocard™ II, GT-1630, Arkray Factory Inc., Shiga, Japan). Serum insulin was analysed from frozen serum samples by ELISA kit for mouse insulin (Ultra sensitive Mouse Insulin ELISA kit 90080, Crystal Chem Inc., IL, USA).

The sample preparation

At the end of the treatment period, the mice were rendered unconscious with CO_2/O_2 (95%/5%; AGA, Riihimäki, Finland) and decapitated. The blood samples were taken into chilled plastic tubes, and the serum was separated by centrifugation at 4℃ for 15 min. The livers and subcutaneous, epididymal, abdominal and perirenal fat pads were removed, washed with saline, blotted dry and weighted. The tissue samples for lipidomic and primary metabolite analysis were snap-frozen in liquid nitrogen and stored at -80℃ until assayed. The samples for oil red O-staining were frozen in isopentane (-38℃) and stored at -80℃ until further processed. The samples for histology were fixed in 40 g/L formaldehyde and embedded in paraffin with routine techniques.

Liver histology and Oil Red O staining

For histological evaluation of the liver samples 4 μm sections of the paraffin embedded samples were cut with a microtome, stained with H&E and examined with a light microscopy. The severity of the observed lesions was graded as previously described [25].

In order to determine the relative amount of lipids in the liver samples, frozen sections (4 μm) were stained with Oil Red O, mounted and photographed. From the obtained microscopic images, the amount of Oil Red O-positive staining was determined with AnalySIS Pro-software (Soft Imaging System, Münster, Germany).

Lipidomics

The lipidomic analysis of liver tissue samples (n = 10/group) was performed as described previously described[26]. Liver tissue lipid extracts were examined by a Q-Tof Premier mass spectrometer by introducing the sample through an Acquity UPLCTM system equipped with an Acquity UPLCTM BEH C18 1 mm × 50 mm column with 1.7 μm particles. The compounds were detected by using electrospray ionization in positive ion mode (ESI+). Data was collected at m/z 300-1200 with a scan duration of 0.2 s. Data was processed using MZmine software version 0.60[27,28], and metabolites were identified using internal spectral library or with tandem mass spectrometry as previously described[6,29].

Primary metabolites

Twenty mg of frozen liver tissue (n = 10/group) was weighed into Eppendorf tubes and 200 μL of methanol (-80℃) and 10 μL of ^{13}C labeled internal standard was added. Sample was homogenized with Micro Dismembrator S (Sartorius, Germany) by using glass beads (0.5-0.75 mm) and 3000 r/min for 3 min. Homogenized samples were boiled immediately in 80℃ for 3 min and at 10000 r/min for 5 min. Supernatant was collected and evaporated to dryness under a stream of nitrogen. Samples were reconstituted in 100 μL of ultra pure water.

The liver extracts were analyzed with HPLC-MS/MS method for quantitative analysis of phosphorous and TCA-cycle compounds. The system consisted of HT-Alliance HPLC (Waters, Milford, MA, USA) working at high pH. The analytes were resolved by anion exchange chromatography combined with post column ASRS Ultra II 2 mm ion suppressor (Dionex, Sunnyvale, CA) and detected with Quattro Micro triple quadruple mass spectrometry (Waters, Milford, MA, USA) operating in electrospray negative ion mode. The analytical column

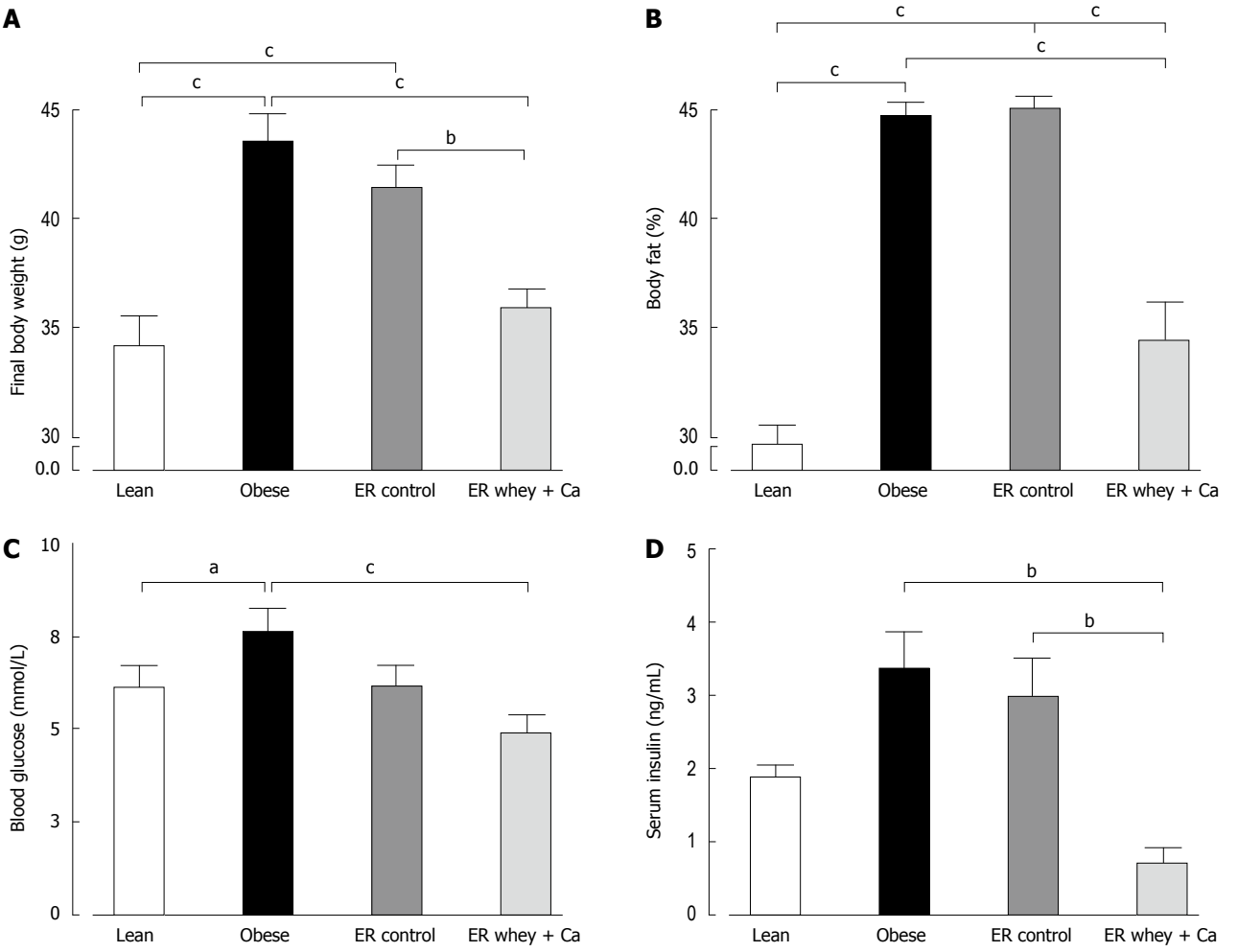

Figure 1 **A**: The body weight of C57Bl/6J mice at the end of the study; **B**: The body fat content of C57Bl/6J mice measured by DEXA at the end of the study; **C**: The blood glucose of C57Bl/6J mice at the end of the study; **D**: The serum insulin of C57Bl/6J mice at the end of the study. Data is presented as mean ± SE. The letters denote a significant difference between the groups ($^{a}P < 0.05$; $^{b}P < 0.01$, $^{c}P < 0.001$; $n = 10$/group).

was IonPac AS11 (2 mm × 250 mm, Dionex, Sunnyvale, CA) and guard column IonPac AG11 (2 mm × 50 mm, Dionex, Dionex Sunnyvale, CA). Flow rate was 250 μL/min and injection volume 5 μL. The temperature of the column was 35℃ and autosampler 10℃.

The compounds were detected in Multiple Reaction Monitoring mode for optimal sensitivity and selectivity. Mass spectrometric parameters, cone voltage and collision energy were optimized for each component. A small aliquot of ^{13}C-labelled metabolites from yeast-fed batch cultivation was used as an internal standard for both calibration standards and samples. Hexose phosphates (glucose-6-phosphate, fructose-6-phosphate, mannose-6-phosphate and 6-glucose-1-phosphate), pentose phosphates (ribose-5-phosphate and ribulose-5-phosphate), fructose bisphosphate, glycerate-2-phosphate and glycerate-3-phosphate, phosphoenolpyruvate, 6-phosphogluconate, succinate, malate, α-ketoglutarate, oxaloacetate, citrate, iso-citrate, glyoxylate and pyruvate were quantitatively measured with this method. Data was processed with MassLynx 4.1 software and internal calibration curves were calculated based on response of ^{12}C analyte and ^{13}C labelled analogue.

Statistical analysis

Data are presented as mean ± SEM. Statistically significant difference in mean values were tested by ANOVA followed by Tukey's test. The data were analysed using GraphPad Prism, version 4.02 (GraphPad Software, Inc., San Diego, CA, USA). Statistical analyses of metabolomics data were performed using R statistical software (www.r-project.org).

RESULTS

Weight and fat loss and fat absorption during ER

The body weight of the high-fat fed mice increased significantly during the 14 wk ad libitum feeding. At the end of the weight gain period the high-fat fed mice weighed significantly more than the chow fed control mice (Figure 1A). The obese mice also had significantly more fat tissue than the lean controls (Figure 1B). The 7-week ER reduced the body weight in the whey + Ca group, to the level of lean controls, but the decrease in body weight was not statistically significant in the control group. Whey + Ca also reduced the fat pad weights more than the weight loss on control diet

Table 1 Fat pad weights (g)

	Lean	Obese	ER		ANOVA *P* value
			Control	Whey + Ca	
Subcutaneous fat	0.4 ± 0.1[c]	1.6 ± 0.1	1.4 ± 0.1[d,e]	1.0 ± 0.1[c,d]	< 0.0001
Epididymal fat	1.4 ± 0.1[a]	1.9 ± 0.1	1.8 ± 0.1[f]	1.3 ± 0.05[b]	0.0006
Perirenal fat	0.7 ± 0.1[c]	1.3 ± 0.1	1.4 ± 0.1[d,f]	0.9 ± 0.1[b]	< 0.0001

Data is presented as mean ± SE (n = 10/group). [a]P < 0.05, [b]P < 0.01, [c]P < 0.001, *vs* obese, respectively; [d]P < 0.001 *vs* lean; [e]P < 0.05, [f]P < 0.01 *vs* whey + Ca, respectively.

Table 2 Incidences of the observed histopathological lesions in liver samples

	Lean	Obese	ER	
			Control	Whey + Ca
Number of samples	8	10	10	10
No abnormalities detected	8	0	0	0
Macrovesicular fatty change (total)	0	10	10	10
Severe	0	1	0	0
Marked	0	1	0	0
Moderate	0	5	5	4
Slight	0	3	5	5
Minimal	0	0	0	1
Infiltration of inflammatory cells, minimal	0	1	1	3
Focal hepatocyte necrosis, total	0	2	0	2
Slight	0	1	0	0
Minimal	0	1	0	2

(Table 1). Apparent fat absorption was reduced in the whey + Ca group in comparison with the control diet (96.9% ± 0.3% *vs* 98.4% ± 0.1% in whey + Ca and control diet, respectively; P = 0.0004).

Blood glucose, serum insulin and liver histology

ER on both diets reduced the blood glucose to the level of lean controls, but the decrease was statistically significant only in the whey + Ca group (Figure 1C). Also the serum insulin was significantly decreased only in the whey + Ca group (Figure 1D). In the obese group, the liver histology showed an evident macrovesicular fatty change of diffuse pattern, with severity ranging from slight to severe (Table 2). In the ER groups, the observed fatty change was less severe. The fat droplets were smaller and mainly present in the perivenular regions. Minimal foci of inflammatory cells and necrotic hepatocytes were occasionally noted, but fibrosis was absent. Oil Red O-staining demonstrated that ER on control and whey + Ca diet significantly reduced hepatic lipid accumulation and lipid droplet size (Figure 2), but the amount of fat did not reach the level observed in the lean mice.

The effect of protein source on metabolic performance

In order to investigate whether the more pronounced weight loss effect in the whey protein fed mice was a result of differences in drinking and feeding behaviour, increased activity or changes in metabolic performance, an additional group of mice were housed in a home cage based monitoring system. A 7-day monitoring did not reveal differences in cumulative feed or water intake, respiratory exchange rate, heat production, O_2 consumption, CO_2 production, total or rearing activity or ambulatory movements during the observation period (Figure 3).

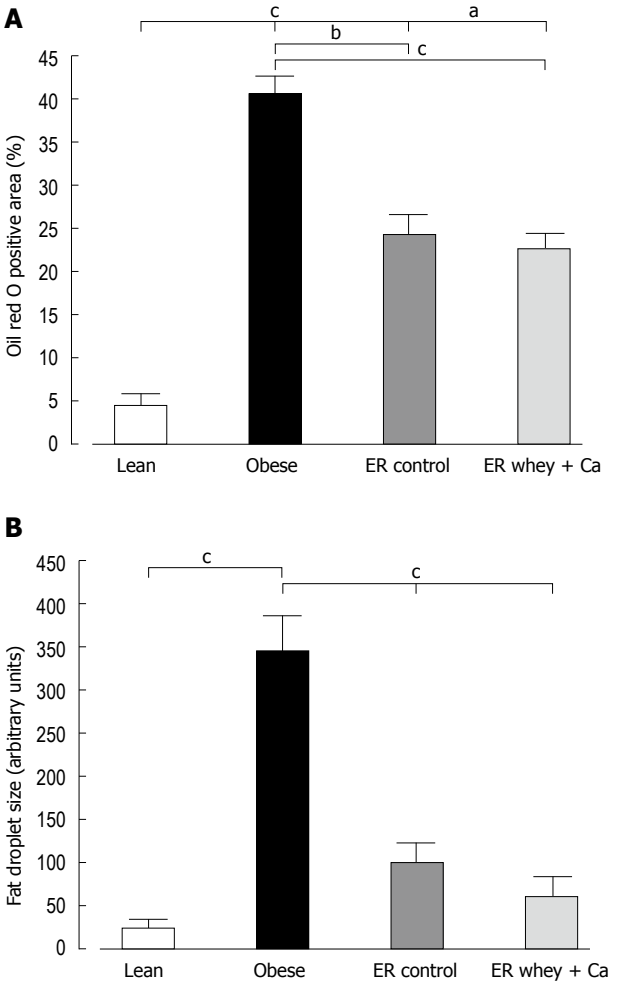

Figure 2 **A**: The Oil Red O positive area of paraffin embedded liver samples of C57Bl/6J mice at the end of the study; **B**: The mean fat droplet area (arbitrary units) of paraffin embedded liver samples of C57Bl/6J mice at the end of the study. Data is presented as mean ± SE. The letters denote the significant difference between the groups ([a]P < 0.05; [b]P < 0.01, [c]P < 0.001; n = 10/group).

The effect of ER on liver lipid profile

Of the total 2498 hepatic lipid peaks detected, 391 major peaks were identified and included in further analysis. The reduction of lipids was mainly seen in the level of triacylglycerols (TAG) and ceramides and ER on whey + Ca diet even restored the level of ceramides to the level of lean mice (Figure 4). The amount of cholesterol esters was significantly increased in both ER groups. The TAG to phospholipid ratio, which reflects the relation of membrane lipids to storage lipids, was significantly reduced only in whey + Ca group, but it was still higher than in the lean controls (Figure 5A). Interestingly, diacylglycerol (DAG) to phospholipid ratio was increased in the control ER group, but decreased in

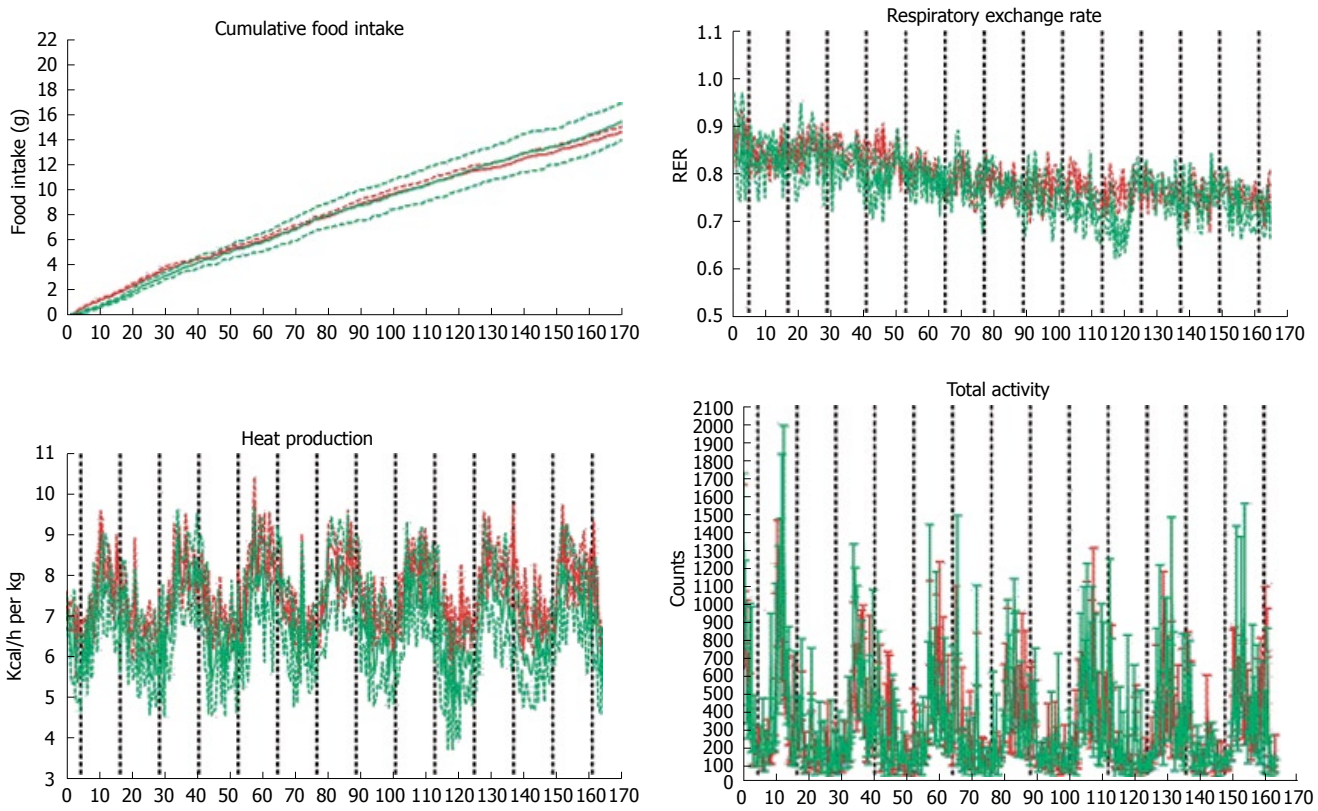

Figure 3 **A**: Cumulative food intake over 7 d analyzed by LabMaster system. The cumulative total food intake was similar between the groups; **B**: LabMaster analysis of respiratory exchange rate (RER); **C**: Heat production measured by LabMaster; **D**: Total ambulatory movement did not differ between the groups. In all figures data is presented as mean ± SE, *n* = 4 in casein group (red) and *n* = 3 in whey group (green).

the whey + Ca ER group (Figure 5B). ER on whey + Ca diet also totally reversed the obesity-induced increase in the ceramide to sphingomyelin ratio (Figure 5C). Phosphatidic acid (GPA) and phosphatidylglycerol (GPGro) peaks could not be uniquely distinguished by our method. The level of GPA/GPGro, phosphatidylcholines and lysophosphatidylethanolamines was not affected by obesity or ER.

The most significantly changed lipids are presented in Figure 6. Interestingly TAG (58:3) and TAG (58:2) were at higher level in the control weight loss group even though they were already increased as a result of obesity, and so was the level of TAG (50:8) in the whey + Ca group. Weight loss further increased the level of GPCho (32:2) even though its level was already over 10 times higher in the obese group than in the lean mice. On the other hand, the level of TAG (52:0) decreased during ER even though its level was already lower in obese than in the lean animals.

The most distinct features of whey + Ca ER group were the significant increases in the level of total phosphatidylserines, phosphatidylethanolamines and sphingomyelins (Figure 6). It is also of note that the level of certain phosphatidylcholine species was significantly decreased during ER on control diet whereas there was no change in whey + Ca group. Whey + Ca specifically affected certain ceramide species [Cer (d18:0/22:5), Cer (d18:0/22:6), Cer (d18:1/23:3), Cer (d18:1/23:5), Cer (d18:1/26:4)], whose level was reduced to the level of lean mice, whereas their level was unaffected by ER on control diet. Cer (d18:1/25:4) was even increased by ER on control diet while its level did not differ between lean, obese and whey + Ca group.

The effect of ER on primary metabolites

The primary metabolite analysis led to identification of 13 metabolites (glucose-6-phosphate, fructose-6-phosphate, mannose-6-phosphate, fructose bisphosphate, glycerate-3-phosphate, ribose-5-phosphate, succinate, malate, citrate, pyruvate, phosphoenolpyruvate, 6-phosphogluconate and fumarate). The high-fat diet feeding and subsequent obesity led to reduction of glycolytic metabolites, such as glucose-6-phosphate, fructose-6-phosphate and pyruvate (Table 3, Figure 7). ER with whey + Ca diet was associated with significant increases of succinate, which belongs to the TCA cycle and of ribose-5-phosphate, which is a product of pentose phosphate pathway. Whey + Ca diet also decreased the level of glycolytic metabolites glucose-6-phosphate, fructose-6-phosphate and fructose bisphosphate in contrast with ER on control diet, which did not affect the level of these metabolites.

DISCUSSION

In this study, we showed that decreasing liver fat by ER significantly modulates the overall profile of liver

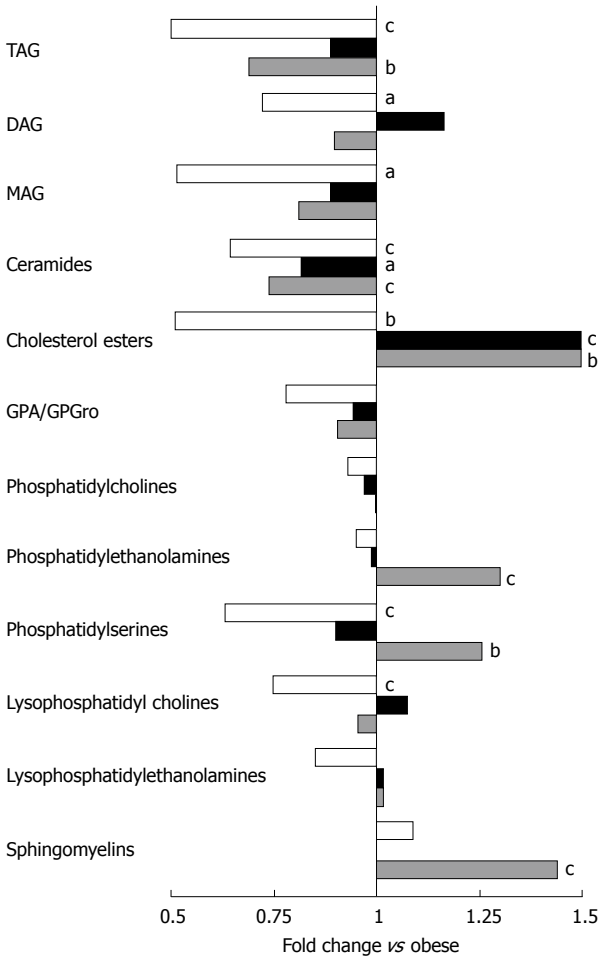

Figure 4 Mean fold changes in lipid classes in lean (white), ER control (black) and ER whey + Ca (grey) groups in relation to the obese group (n = 10/group). The letters denote a significant difference in comparison with the obese group ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$).

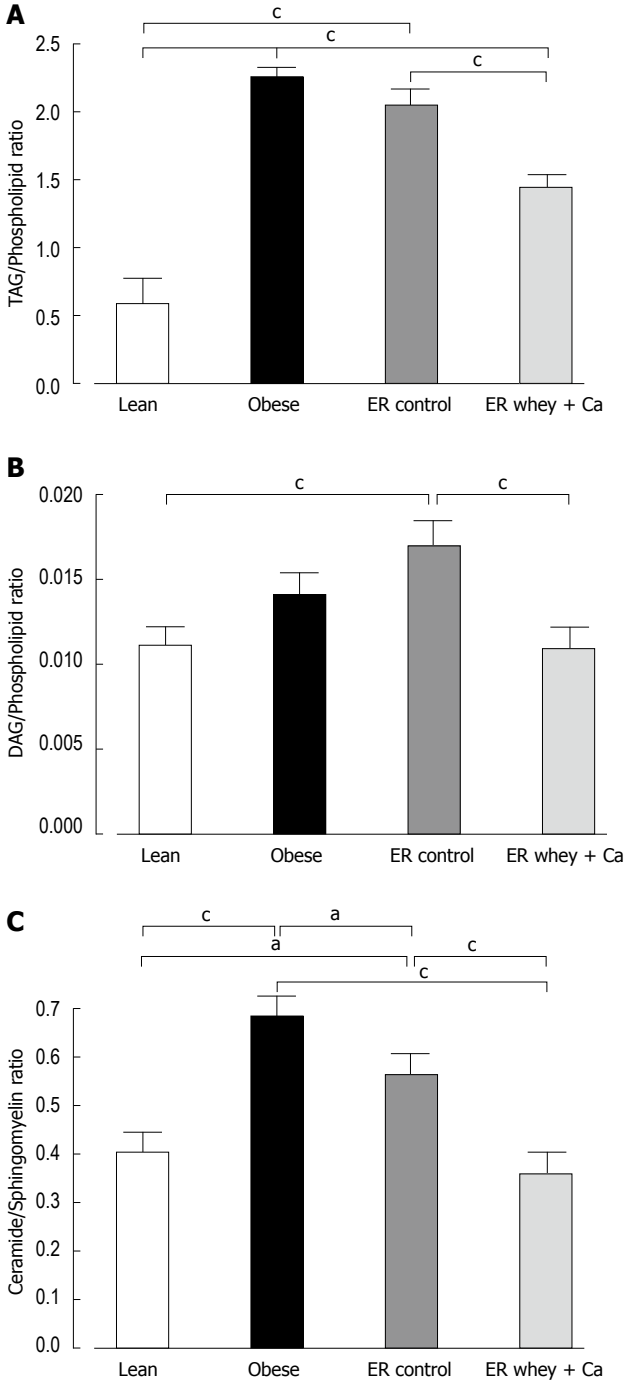

Figure 5 **A**: The liver TAG/Phospholipid ratio; **B**: The liver DAG/Phospholipid ratio; **C**: The liver Ceramide/Sphingomyelin ratio. Lipids measured by UPLC/MS. Data is presented as mean ± SE. The letters denote a significant difference between the groups ($^aP < 0.05$; $^bP < 0.01$, $^cP < 0.001$; n = 10/group).

lipid species. The main finding of the study was that the protein source and calcium content of the diet had a significant effect on the ER-induced hepatic lipid changes. Even though the histological analysis did not reveal significant differences in the amount of liver fat between the ER groups, the metabolomic data demonstrated that ER on whey + Ca diet was able to reduce the relative level of potentially diabetogenic ceramides and diacylglycerols to the level observed in lean animals. This finding is in accordance with the decreased level of serum insulin in this group. These changes were accompanied by a decrease in glycolytic metabolites while the metabolites from the pentose phosphate pathway and TCA cycle were increased together with a shift towards gluconeogenesis.

The UPLC/MS based lipidomics platform and the HPLC/MS/MS based primary metabolite platform techniques were used to characterise the hepatic lipid and primary metabolite changes in this study. These techniques provide an overview of key metabolites involved in energy metabolism, including a broad profile covering all major lipid classes present in liver as well as key metabolites of the central carbon metabolism. Traditional analyses of lipids have been generally limited to investigations of lipid class-or fatty acid-specific changes[30]. These new analytical methods combined with information technology provide extremely sensitive tools to measure the extended metabolome, and may help to explore the mechanisms of many complex diseases[31,32]. However, one evident shortcoming of the method is that a major part of the spectral peaks are still unidentified.

To our knowledge this is the first study to demonstrate the effect of ER on fatty liver lipidomic and primary metabolite profile in diet induced obese mice. In

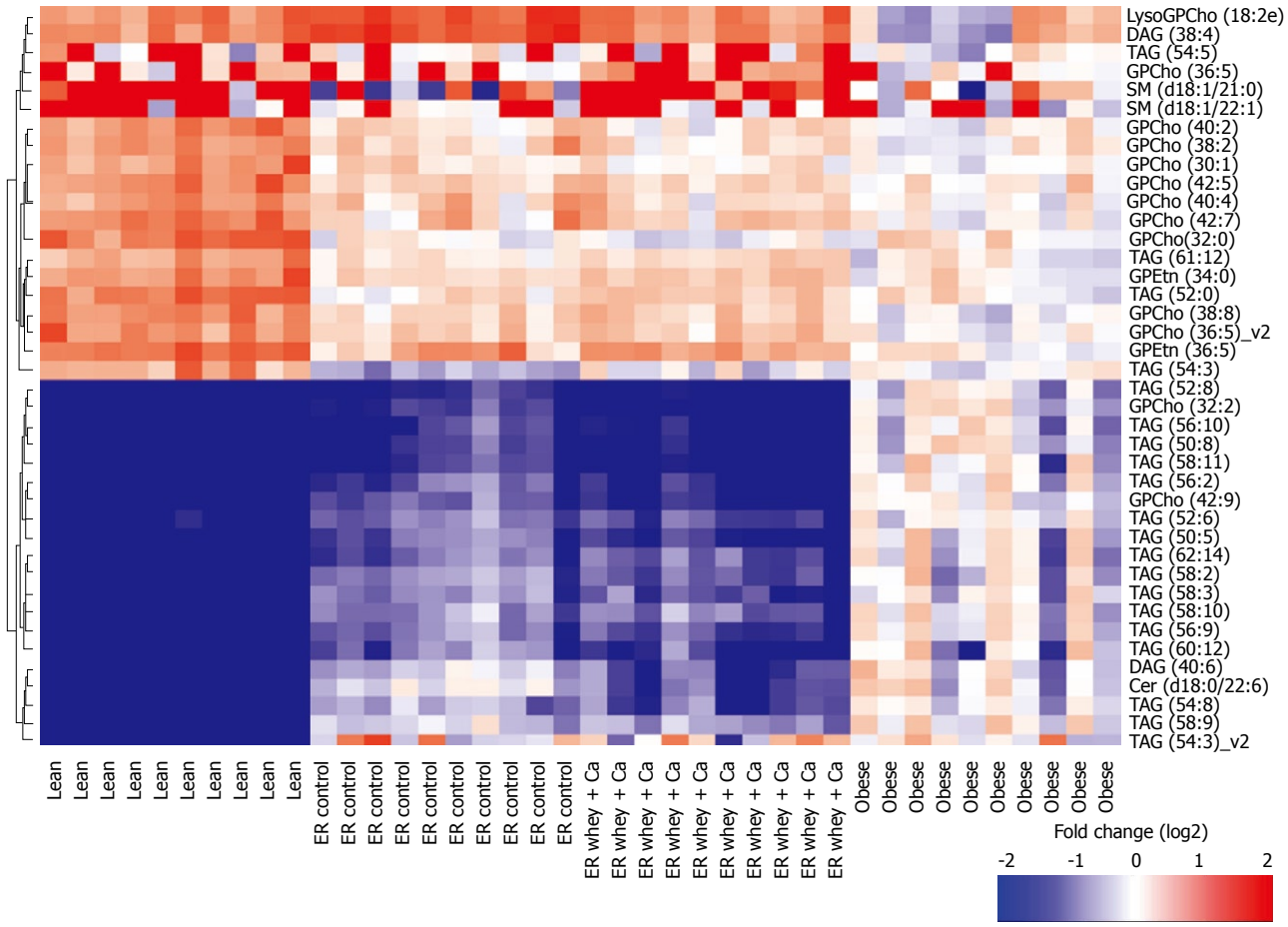

Figure 6 Twenty most significantly up- and down-regulated lipids between obese and lean group. Fold change for each individual mouse within each group as a log2 ratio between the lipid concentration in individual sample and the median lipid concentration in the obese group. Hierarchical clustering using Ward linkage was applied.

Table 3 Concentrations of primary metabolites in liver samples (μmol/g tissue)

	Lean	Obese	ER		ANOVA *P* value
			Control	Whey + Ca	
Glucose-6-phosphate	28.6 ± 2.7[a]	19.2 ± 3.5	22.7 ± 1.7[h]	11.2 ± 3.2[f]	< 0.0001
Fructose-6-phosphate	7.0 ± 0.7	4.9 ± 0.6	6.9 ± 0.7[h]	3.2 ± 0.9[e]	0.001
Mannose-6-phosphate	1.7 ± 0.2	1.7 ± 0.2	1.4 ± 0.1[g]	0.8 ± 0.2[c,f]	0.0003
Fructose bisphosphate	3.2 ± 0.6	8.7 ± 2.0	9.5 ± 2.4[d,h]	0.5 ± 0.1[b]	0.0005
Glycerate-3-phosphate	26.2 ± 3.7	21.0 ± 0.8	17.4 ± 1.2[d]	13.1 ± 1.4[f]	0.0008
Ribose-5-phosphate	0.3 ± 0.02[a]	0.7 ± 0.1	0.7 ± 0.1[d,i]	1.3 ± 0.1[c,f]	< 0.0001
Succinate	24.5 ± 3.3[c]	5.3 ± 1.1	6.7 ± 2.4[f,i]	24.0 ± 4.0[c]	< 0.0001
Malate	42.0 ± 5.2[a]	54.5 ± 3.7	61.3 ± 4.7[d,g]	40.5 ± 5.1[a]	0.008
Citrate	4.9 ± 0.7[c]	2.0 ± 0.3	1.6 ± 0.2[f]	1.8 ± 0.3[f]	< 0.0001
Pyruvate	5.6 ± 0.8[c]	0.9 ± 0.2	2.6 ± 0.4[e]	1.5 ± 0.3[f]	< 0.0001
Phosphoenolpyruvate	2.8 ± 0.8	2.3 ± 0.3	1.4 ± 0.2	1.2 ± 0.2	0.0426
6-phosphogluconate	3.5 ± 0.2[a]	2.4 ± 0.2	3.7 ± 0.2[e,g]	2.6 ± 0.4[a]	0.0023
Fumarate	9.6 ± 1.0[a]	10.4 ± 1.1	17.8 ± 1.7[b,f,g]	11.9 ± 1.3[a]	0.0003

Data is presented as mean ± SE (n = 10/group). [a]P < 0.05, [b]P < 0.01, [c]P < 0.001 *vs* obese, respectively; [d]P < 0.05, [e]P < 0.01, [f]P < 0.001 *vs* lean, respectively; [g]P < 0.05, [h]P < 0.01, [i]P < 0.001 *vs* whey + Ca, respectively.

accordance with previous studies on lipidomic profile of non-alcoholic fatty liver disease, we also found increased levels of TAG, DAG and specific ceramide species and down-regulation of sphingomyelins in the obese group[6]. Interestingly, only ceramides were significantly decreased by ER on control diet, while the level of DAG increased non-significantly and sphingomyelins stayed un-changed. However, ER on whey + Ca diet significantly increased the level of sphingomyelins and decreased the level of DAG changing the ceramide/sphingomyelin and DAG/phospholipid ratios to the level of lean animals. The accumulation of both ceramides and DAG in peripheral tissues contribute to insulin resistance[33-35] and, therefore, the decrease of these lipids can be considered

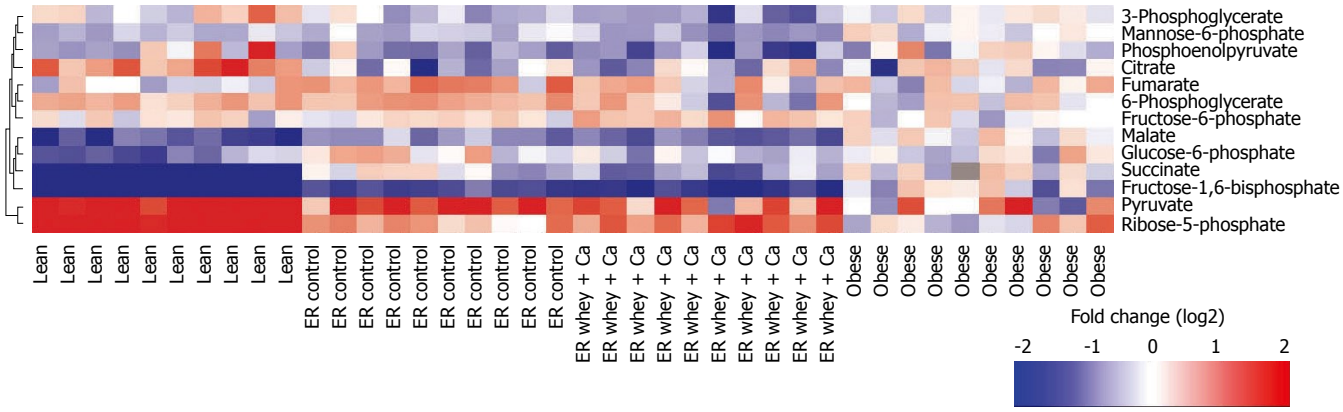

Figure 7 Primary metabolite profiles for each individual mouse as a log2 ratio between the metabolite concentration in individual samples and the median metabolite concentration in the obese group. Hierarchical clustering using Ward linkage was applied.

particularly beneficial.

Additionally an increase in liver cholesterol ester level, which was seen in both of the ER groups, has been demonstrated to occur also as a result of acute 24 h food deprivation[36]. One of the main functions of cholesterol is maintaining of membrane fluidity by interacting with other membrane lipid components such as phosphatidylcholines and sphingomyelin[37]. However, only cholesterol esters were found to be increased as a result of ER in the control group.

The level of primary metabolites was particularly affected in the whey + Ca group. Energy restriction in normal weight, healthy mice, is known to enhance hepatic gluconeogenesis[38,39] and suppress glycolysis[40]. This effect was particularly pronounced in the whey + Ca group as indicated by the striking decrease of fructose bisphosphate, the key regulator of gluconeogenesis, and significant decrease of glycolytic intermediates glucose-6-phosphate and fructose-6-phosphate. An increased level of ribose-5-phosphate in the whey + Ca group indicates enhanced flux through pentose phosphate pathway, which is known to be triggered by low concentrations of fructose-2,6-bisphosphate[41]. ER on whey + Ca diet also decreased the level of succinate to the level of lean animals, whereas the level of succinate did not change in the ER control group. The significance of the decrease of mannose-6-phosphate in whey + Ca groups remains to be elucidated.

One of the few dietary components which are known to influence the liver fat profile during ER is the type and amount of dietary fat[8]. In this study there were no differences in either the type or amount of dietary fat between the ER groups. However, the apparent fat absorption was decreased in the whey + Ca group. Calcium preferentially binds saturated fatty acids in the intestine[42], and therefore, also the quality of the absorbed fat might have been influenced in the whey + Ca group.

Even though these findings may help to understand why increased dairy calcium intake may lower the risk of metabolic syndrome, the molecular mechanism by which whey protein and calcium modulate the liver lipid profile remain unanswered. Whey protein consists of several small protein types, including alfa-lactalbumin, beta-lactoglobulin, bovine serum albumin, lactoferrin and other minor peptides[43]. In order to investigate the possible effects of whey protein on energy expenditure and food intake, we measured the metabolic performance of mice fed either casein or whey based diet in a calorimetry system, but did not see any differences between the proteins. The principal question regarding the mechanism is whether the beneficial effect is derived only from the amino acids or if bioactive peptides are formed during the digestion and absorption of the protein.

The present study demonstrates that ER-induced changes in fatty liver are significantly affected by dietary protein source and calcium. Reducing liver fat by ER is currently the main treatment for non-alcoholic fatty liver disease and therefore, it is crucial to understand which dietary factors have significant effects on the outcome of ER in liver. These results indicate that whey protein and calcium could be beneficial in the dietary treatment of fatty liver, and are likely contribute to the inverse relationship between dairy intake and the risk of insulin resistance. The therapeutic potential of whey protein and calcium in clinical setting, and the mechanism of action remain to be elucidated.

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COMMENTS

Background

Fatty liver is considered to be an important link between obesity and the development of metabolic syndrome and insulin resistance. Liver fat can be reduced by weight loss, but the effect of weight loss on the quality of hepatic lipid profile is currently not well established.

Research frontiers

Some dietary factors, like the quantity and quality of fat during weight loss have been shown to have an effect on liver fat. However, the importance of dietary protein source and calcium content has not been investigated previously.

Innovations and breakthroughs

This study characterises the changes in hepatic lipid profile during energy

restriction in a mouse model of diet induced obesity. The effect of protein source and calcium content of the weight loss diet is also studied. This study demonstrates for the first time that dietary protein source may beneficially modulate the lipid profile of fatty liver during energy restriction.

Applications

Weight loss by life style changes is the main therapeutic approach to reduce liver fat. Therefore, it is crucial to identify dietary components which may improve the outcome of weight loss in the level of hepatic lipid profile. These results indicate that whey protein and calcium beneficially modulate the hepatic lipid profile targeting specifically the lipotoxic diacylglycerol and ceramide species.

Peer review

The study is well conducted and has important information about the pathophysiology of fatty liver.

REFERENCES

1 **Maison P**, Byrne CD, Hales CN, Day NE, Wareham NJ. Do different dimensions of the metabolic syndrome change together over time? Evidence supporting obesity as the central feature. *Diabetes Care* 2001; **24**: 1758-1763

2 **Kotronen A**, Yki-Jarvinen H. Fatty liver: a novel component of the metabolic syndrome. *Arterioscler Thromb Vasc Biol* 2008; **28**: 27-38

3 **Yki-Jarvinen H**. Fat in the liver and insulin resistance. *Ann Med* 2005; **37**: 347-356

4 **Parekh S**, Anania FA. Abnormal lipid and glucose metabolism in obesity: implications for nonalcoholic fatty liver disease. *Gastroenterology* 2007; **132**: 2191-2207

5 **Puri P**, Baillie RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O, Sargeant C, Contos MJ, Sanyal AJ. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* 2007; **46**: 1081-1090

6 **Yetukuri L**, Katajamaa M, Medina-Gomez G, Seppanen-Laakso T, Vidal-Puig A, Oresic M. Bioinformatics strategies for lipidomics analysis: characterization of obesity related hepatic steatosis. *BMC Syst Biol* 2007; **1**: 12

7 **Kolak M**, Westerbacka J, Velagapudi VR, Wegsater D, Yetukuri L, Makkonen J, Rissanen A, Hakkinen AM, Lindell M, Bergholm R, Hamsten A, Eriksson P, Fisher RM, Oresic M, Yki-Jarvinen H. Adipose tissue inflammation and increased ceramide content characterize subjects with high liver fat content independent of obesity. *Diabetes* 2007; **56**: 1960-1968

8 **Zivkovic AM**, German JB, Sanyal AJ. Comparative review of diets for the metabolic syndrome: implications for nonalcoholic fatty liver disease. *Am J Clin Nutr* 2007; **86**: 285-300

9 **Pereira MA**, Jacobs DR Jr, Van Horn L, Slattery ML, Kartashov AI, Ludwig DS. Dairy consumption, obesity, and the insulin resistance syndrome in young adults: the CARDIA Study. *JAMA* 2002; **287**: 2081-2089

10 **Liu S**, Choi HK, Ford E, Song Y, Klevak A, Buring JE, Manson JE. A prospective study of dairy intake and the risk of type 2 diabetes in women. *Diabetes Care* 2006; **29**: 1579-1584

11 **Choi HK**, Willett WC, Stampfer MJ, Rimm E, Hu FB. Dairy consumption and risk of type 2 diabetes mellitus in men: a prospective study. *Arch Intern Med* 2005; **165**: 997-1003

12 **Ruidavets JB**, Bongard V, Dallongeville J, Arveiler D, Ducimetiere P, Perret B, Simon C, Amouyel P, Ferrieres J. High consumptions of grain, fish, dairy products and combinations of these are associated with a low prevalence of metabolic syndrome. *J Epidemiol Community Health* 2007; **61**: 810-817

13 **Elwood PC**, Pickering JE, Fehily AM. Milk and dairy consumption, diabetes and the metabolic syndrome: the Caerphilly prospective study. *J Epidemiol Community Health* 2007; **61**: 695-698

14 **Liu S**, Song Y, Ford ES, Manson JE, Buring JE, Ridker PM. Dietary calcium, vitamin D, and the prevalence of metabolic syndrome in middle-aged and older U.S. women. *Diabetes Care* 2005; **28**: 2926-2932

15 **Azadbakht L**, Mirmiran P, Esmaillzadeh A, Azizi F. Dairy consumption is inversely associated with the prevalence of the metabolic syndrome in Tehranian adults. *Am J Clin Nutr* 2005; **82**: 523-530

16 **Zemel MB**, Richards J, Mathis S, Milstead A, Gebhardt L, Silva E. Dairy augmentation of total and central fat loss in obese subjects. *Int J Obes* (Lond) 2005; **29**: 391-397

17 **Zemel MB**, Richards J, Milstead A, Campbell P. Effects of calcium and dairy on body composition and weight loss in African-American adults. *Obes Res* 2005; **13**: 1218-1225

18 **Shi H**, Dirienzo D, Zemel MB. Effects of dietary calcium on adipocyte lipid metabolism and body weight regulation in energy-restricted aP2-agouti transgenic mice. *FASEB J* 2001; **15**: 291-293

19 **Parra P**, Bruni G, Palou A, Serra F. Dietary calcium attenuation of body fat gain during high-fat feeding in mice. *J Nutr Biochem* 2008; **19**: 109-117

20 **Sun X**, Zemel MB. Calcium and dairy products inhibit weight and fat regain during ad libitum consumption following energy restriction in Ap2-agouti transgenic mice. *J Nutr* 2004; **134**: 3054-3060

21 **Zemel MB**. The role of dairy foods in weight management. *J Am Coll Nutr* 2005; **24**: 537S-546S

22 **Pilvi TK**, Korpela R, Huttunen M, Vapaatalo H, Mervaala EM. High-calcium diet with whey protein attenuates body-weight gain in high-fat-fed C57Bl/6J mice. *Br J Nutr* 2007; **98**: 900-907

23 **Cheese and processed cheese products-determination of fat content-gravimetric method (reference method).** International Standard IDF 5B, ISO 1735. 2004

24 **Papakonstantinou E**, Flatt WP, Huth PJ, Harris RB. High dietary calcium reduces body fat content, digestibility of fat, and serum vitamin D in rats. *Obes Res* 2003; **11**: 387-394

25 **Herbert RA**, Hailey JR, Seely JC, Shackelford CC, Jokinen MP, Wolf JC, et al. Nomenclature. In: Haschek WM RC, Wallig MA. Handbook of Toxicologic Pathology. San Diego: Academic Press, 2002: 157-167

26 **Medina-Gomez G**, Gray SL, Yetukuri L, Shimomura K, Virtue S, Campbell M, Curtis RK, Jimenez-Linan M, Blount M, Yeo GS, Lopez M, Seppanen-Laakso T, Ashcroft FM, Oresic M, Vidal-Puig A. PPAR gamma 2 prevents lipotoxicity by controlling adipose tissue expandability and peripheral lipid metabolism. *PLoS Genet* 2007; **3**: e64

27 **Katajamaa M**, Miettinen J, Oresic M. MZmine: toolbox for processing and visualization of mass spectrometry based molecular profile data. *Bioinformatics* 2006; **22**: 634-636

28 **Katajamaa M**, Oresic M. Processing methods for differential analysis of LC/MS profile data. *BMC Bioinformatics* 2005; **6**: 179

29 **Laaksonen R**, Katajamaa M, Paiva H, Sysi-Aho M, Saarinen L, Junni P, Lutjohann D, Smet J, Van Coster R, Seppanen-Laakso T, Lehtimaki T, Soini J, Oresic M. A systems biology strategy reveals biological pathways and plasma biomarker candidates for potentially toxic statin-induced changes in muscle. *PLoS ONE* 2006; **1**: e97

30 **Neuberger A**, Van Deenen LLM. New comprehensive biochemistry. In Vance DE, Vance JE. Biochemistry of lipids, lipoproteins and membranes. New York: Elsevier, 1991: 403-426

31 **Watson AD**. Thematic review series: systems biology approaches to metabolic and cardiovascular disorders. Lipidomics: a global approach to lipid analysis in biological systems. *J Lipid Res* 2006; **47**: 2101-2111

32 **Schnackenberg LK**, Beger RD. Monitoring the health to disease continuum with global metabolic profiling and systems biology. *Pharmacogenomics* 2006; **7**: 1077-1086

33 **Summers SA**. Ceramides in insulin resistance and lipotoxicity. *Prog Lipid Res* 2006; **45**: 42-72

34 **Stratford S**, Hoehn KL, Liu F, Summers SA. Regulation of insulin action by ceramide: dual mechanisms linking ceramide accumulation to the inhibition of Akt/protein

kinase B. *J Biol Chem* 2004; **279**: 36608-36615
35 **Zhang D**, Liu ZX, Choi CS, Tian L, Kibbey R, Dong J, Cline GW, Wood PA, Shulman GI. Mitochondrial dysfunction due to long-chain Acyl-CoA dehydrogenase deficiency causes hepatic steatosis and hepatic insulin resistance. *Proc Natl Acad Sci USA* 2007; **104**: 17075-17080
36 **van Ginneken V**, Verhey E, Poelmann R, Ramakers R, van Dijk KW, Ham L, Voshol P, Havekes L, Van Eck M, van der Greef J. Metabolomics (liver and blood profiling) in a mouse model in response to fasting: a study of hepatic steatosis. *Biochim Biophys Acta* 2007; **1771**: 1263-1270
37 **Ohvo-Rekila H**, Ramstedt B, Leppimaki P, Slotte JP. Cholesterol interactions with phospholipids in membranes. *Prog Lipid Res* 2002; **41**: 66-97
38 **Selman C**, Kerrison ND, Cooray A, Piper MD, Lingard SJ, Barton RH, Schuster EF, Blanc E, Gems D, Nicholson JK, Thornton JM, Partridge L, Withers DJ. Coordinated multitissue transcriptional and plasma metabonomic profiles following acute caloric restriction in mice. *Physiol Genomics* 2006; **27**: 187-200
39 **Hagopian K**, Ramsey JJ, Weindruch R. Caloric restriction increases gluconeogenic and transaminase enzyme activities in mouse liver. *Exp Gerontol* 2003; **38**: 267-278
40 **Dhahbi JM**, Mote PL, Wingo J, Tillman JB, Walford RL, Spindler SR. Calories and aging alter gene expression for gluconeogenic, glycolytic, and nitrogen-metabolizing enzymes. *Am J Physiol* 1999; **277**: E352-E360
41 **Boada J**, Roig T, Perez X, Gamez A, Bartrons R, Cascante M, Bermudez J. Cells overexpressing fructose-2,6-bisphosphatase showed enhanced pentose phosphate pathway flux and resistance to oxidative stress. *FEBS Lett* 2000; **480**: 261-264
42 **Bhattacharyya AK**, Thera C, Anderson JT, Grande F, Keys A. Dietary calcium and fat. Effect on serum lipids and fecal excretion of cholesterol and its degradation products in man. *Am J Clin Nutr* 1969; **22**: 1161-1174
43 **Marshall K**. Therapeutic applications of whey protein. *Altern Med Rev* 2004; **9**: 136-156

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BASIC RESEARCH

Lysophosphatidic acid induced nuclear translocation of nuclear factor-κB in Panc-1 cells by mobilizing cytosolic free calcium

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Abstract

AIM: To clarify whether Lysophosphatidic acid (LPA) activates the nuclear translocation of nuclear factor-κB (NF-κB) in pancreatic cancer.

METHODS: Panc-1, a human pancreatic cancer cell line, was used throughout the study. The expression of LPA receptors was confirmed by reverse-transcript polymerase chain reaction (RT-PCR). Cytosolic free calcium was measured by fluorescent calcium indicator fura-2, and the localization of NF-κB was visualized by immunofluorescent method with or without various agents, which effect cell signaling.

RESULTS: Panc-1 expressed LPA receptors, LP_{A1}, LP_{A2} and LP_{A3}. LPA caused the elevation of cytosolic free calcium dose-dependently. LPA also caused the nuclear translocation of NF-κB. Cytosolic free calcium was attenuated by pertussis toxin (PTX) and U73122, an inhibitor of phospholipase C. The translocation of NF-κB was similarly attenuated by PTX and U73122, but phorbol ester, an activator of protein kinase C, alone did not translocate NF-κB. Furthermore, the translocation of NF-κB was completely blocked by Ca^{2+} chelator BAPTA-AM. Thapsigargin, an endoplasmic-reticulum Ca^{2+}-ATPase pump inhibitor, also promoted the translocation of NF-κB. Staurosporine, a protein kinase C inhibitor, attenuated translocation of NF-κB induced by LPA.

CONCLUSION: These findings suggest that protein kinase C is activated endogenously in Panc-1, and protein kinase C is essential for activating NF-κB with cytosolic calcium and that LPA induces the nuclear translocation of NF-κB in Panc-1 by mobilizing cytosolic free calcium.

Key words: Lysophosphatidic acid; Nuclear translocation; Nuclear factor-κB; Cytosolic free calcium; Panc-1

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INTRODUCTION

Lysophosphatidic acid (LPA) is the smallest and structurally simplest of all glycerophospholipids, and exists in serum at concentrations between 1-5 μmol/L[1] LPA is mainly released by activated platelets[2], and immediately complexes with high affinity to serum albumin[3]. It is well known that LPA exhibits hormone- and growth factor-like activities[4,5]. LPA represents a major bioactive constituent of serum. LPA increases [^{3}H]-thymidine incorporation, inositol phosphates, intracellular calcium, and protein kinase C activities[4,5]. Indeed, LPA acts on a large number of cells to achieve a broad range of immediate and long lasting effects. Specific responses to LPA include changes in cell shape and tension, chemotaxis, proliferation and differentiation[6,7]. LPA binds to putative G protein-coupled receptors found on nearly all cell types, including cancer cell lines. Numerous other cellular

and biochemical responses to LPA have also been documented[8].

The molecular actions of LPA have been characterized best in rodent fibroblasts, where LPA stimulates phosphoinositide hydrolysis[4] and promotes the Rho-dependent formation of stress fibers and focal adhesions[9]. The stimulation of phosphoinositide hydrolysis is thought to occur through the GTP-binding regulatory protein (G protein) Go or Gq. The formation of stress fibers and focal adhesions is consistent with activation of Rho through G_{12} or G_{13}[8]. Whether G proteins are sufficient for this action is unclear, but the sensitivity of the phenomenon to pertussis toxin (PTX) implies that $G_{i/o}$ represents at least one necessary input. Receptors that recognize LPA have been identified that conform to the seven-transmembrane domain motif characteristic of G protein-coupled receptors, and identified as LP_{A1}/Edg-2, LP_{A2}/Edg-4, LP_{A3}/Edg-7[10].

On the other hand, nuclear factor-κB (NF-κB) is the prototype of a family of dimers whose constituents are members of the Rel family of transcription factors[11]. In most types of cells, NF-κB is present as a heterodimer comprising p50 (NF-κB1) and p65 (RelA). NF-κB is normally retained in the cytosol in an inactive form through interaction with IκB inhibitory proteins. Release of NF-κB for translocation to the nucleus and interaction with cognate DNA sequences is accomplished through a signal-induced phosphorylation and subsequent degradation of IκB. Originally described as a necessary element for expression of the immunoglobulin gene in mature B cells, NF-κB is now recognized to be an important transcriptional regulatory protein in a variety of cell types.

It is reported that LPA translocates NF-κB to the nucleus in fibroblasts[12], lymphocytes[13], breast cancer cells[14], colon cancer cells[15], prostate cancer cells[16], and ovarian cancer cells[17]. However, until now it is not clear whether LPA translocates NF-κB in pancreatic cancer cells or not. In this study, we demonstrate for the first time that LPA induces translocation of NF-κB to nucleus in the pancreatic cancer cell line, Panc-1, by mobilizing cytosolic free calcium.

MATERIALS AND METHODS

Materials

The human pancreatic cancer cell line, Panc-1 (ATCC CRL 1469), was obtained from American Type Culture Collection (MD, USA). Media and supplements were from GIBCO BRL (New York, USA). Fetal bovine serum and fetal calf serum were from Hyclone (Utah, USA). Glass-bottomed chambers were from Costar (Massachusetts, USA). LPA and fatty-acid free albumin were from Sigma (Montana, USA). PTX, staurosporine, genistein and PD98059 were from Calbiochem (California, USA). U73122, U73343 and Fura-2/AM were from Seikagakukogyo (Tokyo, Japan). Rabbit anti-human NF-κB p65 polyclonal antibody was obtained from Upstate Biotechnology (New York, USA); and rhodamine-conjugated donkey anti-rabbit polyclonal antibody and normal donkey serum from Chemicon International (California, USA), respectively.

Methods

Cell culture: Panc-1 was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin (50 units/mL), streptomycin (50 units/mL), and 10% fetal bovine serum (FBS). Panc-1 was maintained in DMEM without FBS for 48 h prior to use for the following experiments.

Total RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR) analysis for detection of LPA receptors: Total RNA was extracted from Panc-1 using ISOGEN (Nippon Gene, Tokyo, Japan). The first strand cDNA synthesis was carried out using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen™ life technologies, California, USA). One hundred nanogram of the synthesized first strand cDNA was subjected to PCR using Platinum® Taq DNA Polymerase (Invitrogen™ Life Technologies, California, USA). The first strand cDNA was then amplified with specific primers; GAPDH (as an internal control), Edg-2, Edg-4, and Edg-7. The PCR primers and conditions are listed in Table 1. In all cases after the RT step the templates were heated at 94℃ for 30 s, annealed for another 30 s, and finally subjected to a 72℃ extension for 60 s. The PCR products were separated in 2% agarose gel and visualized under UV illumination.

Measurement of cytosolic free calcium: Cytosolic free calcium concentration was measured as previously described[18]. Confluent monolayers of Panc-1 cells grown in 175 cm^2 flasks were harvested by incubation in 0.9% (w/v) NaCl, 0.02% (w/v) EGTA and 10 mmol/L Hepes, pH7.2. Cells were washed twice with PBS, resuspended to a density of 1×10^6 cells/mL, and then incubated with 6 μmol/L fura-2/AM for 1 h at room temperature. Aliquots of this suspension were washed twice with Krebs-Ringer's HEPES buffer in a stirred fluorimetry cuvette at 37℃. Fluorescence of fura-2 was measured with Shimazu RF-5000 luminescence spectrometer (alternate excitation wavelengths of 340 nm and 380 nm: emission wavelength, 505 nm). R_{max} was determined by the addition of 0.1% Triton X-100, then R_{min} was estimated using 4 mmol/L EGTA.

Immunofluorescence: Panc-1 cells were cultured on glass-bottomed culture dishes, and experiments were conducted as described above. Panc-1 cells were washed twice with ice-cold PBS and fixed with methanol for 5 min, permeabilized with 0.2% Triton X-100. Once the dishes had air-dried, the cells were incubated in 10% FCS for 2 h to block nonspecific antibody binding. The cells were then incubated with rabbit anti-human NF-κB p65 polyclonal antibody (1:50) in PBS containing 0.2% BSA for 6 h at room temperature. The dishes were then washed three times in PBS with 0.2% BSA for 5 min at room temperature. Cells were then incubated with rhodamine-conjugated donkey anti-rabbit polyclonal

Table 1 The PCR primers and conditions for detection of LPA receptors

	Sense primer	Antisense primer	Temperature (℃)	Size (bp)
GAPDH	5'-AATGCATCCTGCACCACCAACTGC-3'	5'-GGAGGCCATGTAGGCCATGAGGTC-3′	59	554
Edg-2	5'-TCCACACACGGATGAGCAAC-3′	5'-GTGATCATTGCTGTGAACTCC-3′	62	620
Edg-4	5'-CCACCAGCCCATCTACTACCT-3′	5'-CTCACAGCCTAAACCATCCAG -3′	62	619
Edg-7	5'-GCTGGAATTGCCTCTGCAAC -3′	5'-ACCACAAACGCCCCTAAGAC -3′	62	253

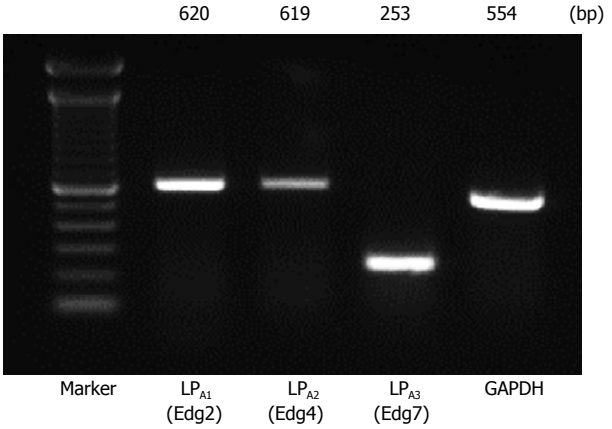

Figure 1 Expression of LPA receptors in Panc-1 cells. LPA-specific receptors LP_{A1}/Edg-2, LP_{A2}/Edg-4, and LP_{A3}/Edg-7 are expressed in PANC-1 cells. Total mRNA of PANC-1 cells was extracted and used for RT and PCR using primers designed from the sequence of LP_{A1}, LP_{A2}, and LP_{A3}, and GAPDH. PCR products were separated by electrophoresis in 2.0% agarose gel. The molecular size of the amplification products is inferred from the electrophoretic migration of the DNA markers.

antibody (1:100) in PBS containing 0.2% BSA for 1 h at room temperature. The dishes were then washed three times in PBS containing 0.2% BSA for 5 min at room temperature and mounted with glass coverslips using Vectashield mounting medium from Vector Laboratories (California, USA). Immunofluorescence was observed by using an LSM410 Confocal Laser Scanning Microscope from Carl Zeiss, Inc. (Oberkochen, Germany).

Treatment of Panc-1 cells: Panc-1 cells were treated with various agents before measuring cytosolic free calcium or detecting translocation of NF-κB. For PTX-pretreatment, monolayers of Panc-1 cells were cultured in DMEM with 100 ng/mL PTX overnight, and then washed twice with PBS. Panc-1 cells were treated with other agents; 10 μmol/L U73122 for 10 min, 10 μmol/L U73343 for 10 min, 10 μmol/L BAPTA for 15 min, 10 nmol/L staurosporine for 10 min, 50 μg/mL genistein for 1 h, and 10 nmol/L PD98059 for 1 h before stimulation of LPA.

Statistical analysis

The Student's *t*-test was used to determine significant differences between the groups.

RESULTS

Panc-1 cells express LP_{A1}, LP_{A2}, and LP_{A3} mRNA

The expression of LP_{A1}/Edg-2, LP_{A2}/Edg-4, and LP_{A3}/Edg-7 receptors was determined by RT-PCR in Panc-1 cells. As shown in Figure 1, a significant expression of mRNA encoding LP_{A1}/Edg-2, LP_{A2}/Edg-4, and LP_{A3}/Edg-7 was observed, as judged from the appearance of unique cDNA bands of the expected size.

LPA elevates $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from intracellular stores

LPA, at concentration of 10 μmol/L, elevated $[Ca^{2+}]_i$ by 600 ± 45 nmol/L (n = 5) above a resting $[Ca^{2+}]_i$ of 108 ± 30 nmol/L within 20 s of addition to intact fura2-loaded Panc-1 cell suspension (Figure 2A). This increase in $[Ca^{2+}]_i$ was followed by a return to near resting levels within 3 min. The elevation of $[Ca^{2+}]_i$ by LPA in Panc-1 cells was concentration-dependent, with an EC_{50} of 870 nmol/L (Figure 3). Responses to LPA were dependent upon the presence of fatty acid-free BSA (0.1% w/v), without which $[Ca^{2+}]_i$ increases were a quarter-maximal. Fatty acid-free BSA itself did not cause a significant increase in $[Ca^{2+}]_i$ for 30 min (data not shown), as other researchers previously reported[4]. LPA-induced elevation of $[Ca^{2+}]_i$ resulted predominantly from mobilization of intracellular $[Ca^{2+}]_i$ store. In the condition of Ca^{2+}-free with 0.2 mmol/L EDTA, LPA increased $[Ca^{2+}]_i$ with a similar potency and maximal effect to the condition with extracellular Ca^{2+} (Figure 2B). After overnight treatment with PTX (100 ng/mL), LPA did not evoke $[Ca^{2+}]_i$ in Panc-1 cells (Figures 2C and 3). Treatment of Panc-1 cells with a phospholipase C inhibitor, U73122, at a concentration of 10 μmol/L for 10 min, abolished LPA-induced increase in $[Ca^{2+}]_i$ (Figure 2D and 3). Pretreated with U73343, an inactive analogue of U73122, at a concentration of 10 μmol/L for 10 min had no effect on LPA-induced elevation of $[Ca^{2+}]_i$ in Panc-1 cells (Figure 2E). These findings suggest that LPA induces elevation of $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from Ca^{2+} store through PTX-sensitive G-protein (Gi or Go) and phospholipase C. Treatment of Panc-1 cells with thapsigargin, the endoplasmic-reticulum Ca^{2+}-ATPase pump inhibitor, at a concentration of 500 nmol/L caused a slow increase in $[Ca^{2+}]_i$ followed by a sustained plateau (data not shown).

Effect of LPA on translocation of NF-κB to nuclei in Panc-1 cells

It has been reported that LPA activates NF-κB in fibroblasts[19] and endothelial cells[20]. We, therefore, investigated the possibility that LPA activates the transcription factor NF-κB in Panc-1 cells.

NF-κB is normally retained in the cytosol in an inactive form in Panc-1 cells (Figure 4A). LPA, at a

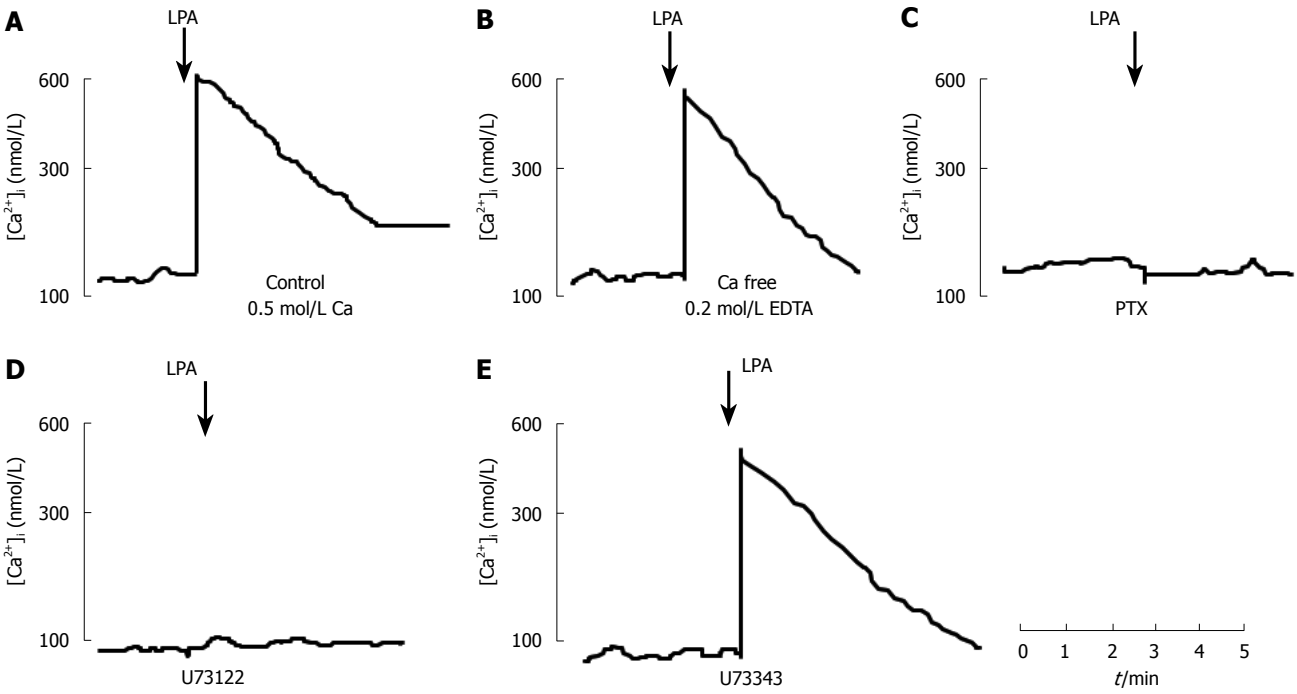

Figure 2 Effect of LPA on cytosolic free calcium concentration $[Ca^{2+}]_i$ in Panc-1 cells. **A**: Representative Ca^{2+} signal was evoked by 10 μmol/L LPA in Panc-1 cells; **B**: Effect of chelation of extracellular Ca^{2+} by addition of 0.2 mmol/L EDTA on LPA-induced increases in Ca^{2+}; **C**: PTX-sensitive effect of LPA on cytosolic free calcium. Panc-1 cells were pretreated with 100 ng/mL of PTX for overnight and loaded with fura-2/AM; **D**: Effect of U73122, a phospholipase C inhibitor, on LPA-induced increases in cytosolic free calcium in Panc-1 cells. Panc-1 cells were treated with U73122 at a concentration of 10 μmol/L for 10 min, and then stimulated by 10 μmol/L LPA; **E**: Effect of U73343, an inactive analogue of U73122, on LPA-evoked increases in cytosolic free calcium in Panc-1 cells.

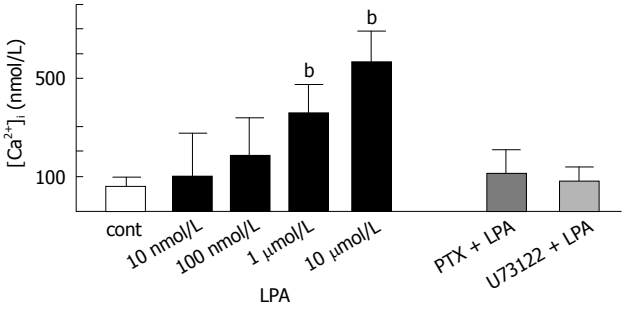

Figure 3 Effect of LPA on cytosolic free calcium in Panc-1. LPA evoked cytosolic free calcium in concentration-dependent manner. PTX-treatment at a concentration of 100 ng/ml for overnight abolished 10 μmol/L LPA-induced mobilization of $[Ca^{2+}]_i$. Pretreatment of U73122 (10 μmol/L) for 10 min also abolished LPA-induced mobilization of $[Ca^{2+}]_i$. $^bP < 0.01$.

concentration of 10 μmol/L, translocated NF-κB to the nucleus within 30 min (Figure 4B). NF-κB was re-established in the cytosol after 3 h (data not shown).

After Panc-1 cells were preincubate with 100 ng/mL PTX, LPA did not translocated NF-κB to nuclei (Figure 5A). A phospholipase C inhibitor, U73122, also abolished the translocation induced by LPA (Figure 5B). Pretreatment of Panc-1 with BAPTA-AM, an intracellular calcium chelator, at a concentration of 10 μmol/L for 15 min, completely abolished NF-κB translocation (Figure 5C). Thapsigargin is known to block a Ca^{2+}-ATP pump of calcium stores, and elevates cytosolic free calcium slowly. In the present study, thapsigargin alone caused NF-κB translocation in Panc-1 cells at a concentration of 500 nmol/L (Figure 5D). These data suggest that elevation of cytosolic free calcium is necessary for activation of NF-κB in Panc-1 cells.

Phorbol myristate, which is well known to be a potent activator of protein kinase C, alone failed to induce the translocation at a concentration of 100 nmol/L (Figure 5E). On the other hand, staurosporine, a protein kinase C inhibitor, attenuated translocation of NF-κB induced by LPA (Figure 5F). These findings suggested that protein kinase C is activated endogenously in cancer cells, such as Panc-1, and that protein kinase C is essential for activating NF-κB with cytosolic calcium. It is reported that PMA could activate NF-κB in some types of cells including rat pancreatic acinar cells. However, the present data revealed that cytosolic calcium might have a more crucial role in activating NF-κB than protein kinase C in Panc-1 cells.

A tyrosine kinase inhibitor genistein or a MEK inhibitor PD98059 did not have significant effects on the translocation of NF-κB induced by LPA (data not shown).

DISCUSSION

In the present study, we first confirmed that Panc-1 cells express LPA receptors, and then showed that LPA induced Ca^{2+} mobilization and translocation of NF-κB into the nucleus.

LPA acts as an intercellular messenger molecule bound to serum albumin[3]. LPA exerts multiple biological functions through G protein-coupled receptors. It has been identified a new family of

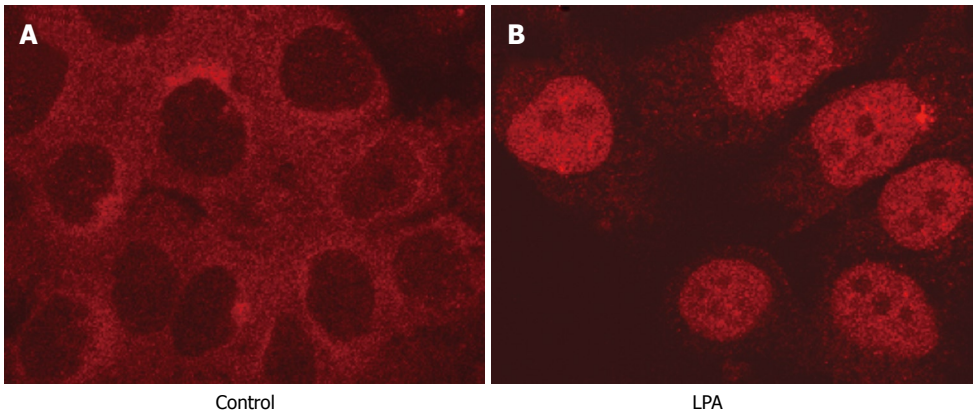

Figure 4 Effect of LPA on nuclear translocation of NF-κB. LPA-induced nuclear translocation of p65 in Panc-1 cells. Localization of p65 was visualized by indirect immunofluorescence staining using rabbit anti-p65 polyclonal antibodies (1:50) which only recognized NF-κB p65. Donkey anti-rabbit antibodies (1:100) conjugated to rhodamine was performed, and visualized under a confocal microscope. **A**: Cytosolic localization of p65 in an inactive form in unstimulated Panc-1 cells; **B**: Nuclear localization of p65 in Panc-1 cells after treatment of 10 μmol/L LPA was observed at 60 min.

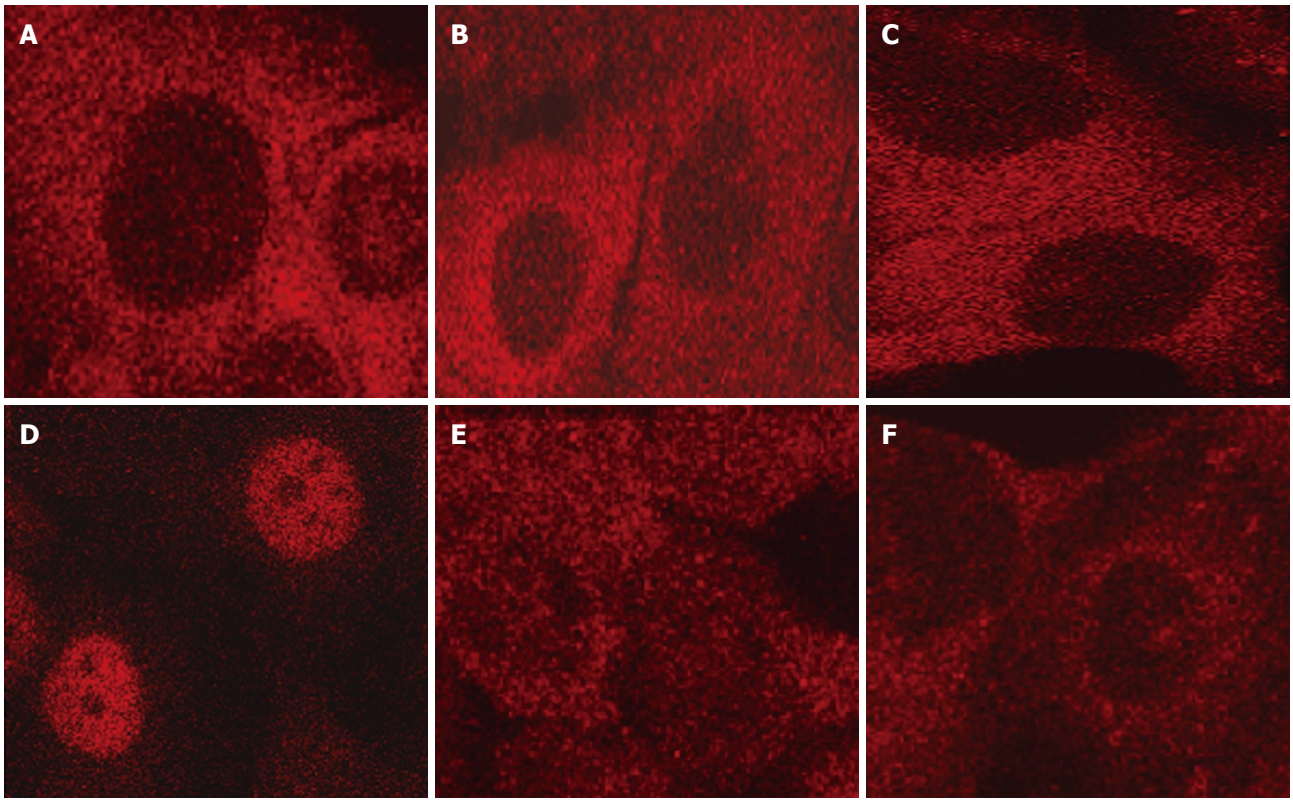

Figure 5 Localization of NF-κB p65 in Panc-1 cells. **A**: PTX-treatment prevented nuclear translocation of NF-κB p65 induced by LPA in Panc-1 cells at 1 h. Panc-1 cells were preincubated with 100 ng/mL PTX, and then stimulated with 10 μmol/L LPA; **B**: U73122-treatment prevented nuclear translocation of NF-κB p65 induced by LPA in Panc-1 cells at 1 h. Panc-1 cells were pretreated with 10 μmol/L U73122 for 10 min before adding 10 μmol/L LPA; **C**: Nuclear translocation of p65 induced by LPA was reduced by an intracellular calcium chelator, BPTA-AM at 1 h. Panc-1 cells were treated with 10 μmol/L BAPTA-AM for 15 min and then stimulated with 10 μmol/L LPA; **D**: Thapsigargin alone caused nuclear translocation of p65 at 1 h. Panc-1 cells were treated with 500 nmol/L thapsigarigin; **E**: Phorbol myristate (PMA) alone failed to mobilize p65 into nuclei at 1 h. Panc-1 cells were treated with 100 nmol/L PMA; **F**: Staurosporine reduced nuclear localization of p65 induced by LPA at 1 h. Panc-1 cells were treated with 10 nmol/L staurosporine for 10 min and then stimulated with 10 μmol/L LPA.

receptor genes for LPA. Members of this family include at least three G-protein-coupled receptors belonging to the endothelial differentiation gene (Edg) family, Edg-2/LP_{A1}, Edg-4/LP_{A2}, and Edg-7/LP_{A3}[10]. Recent investigation has revealed that Panc-1 cells also express functionally active LPA receptors (LP_{A1}/Edg-2, LP_{A2}/Edg-4, LP_{A3}/Edg-7)[21]. LPA has a major role in migration of Panc-1 cells via phosphorylation of ERK[21], but was reported to not act as mitogen in pancreatic cancer cell lines, Panc-1 cells and Bx-PC cells[21]. LPA also mobilized cytosolic calcium in Panc-1 cells as neuropeptides including neurotensin, bombesin, cholecystokinin, and vasopressin[22]. However, it is still unclear of roles of calcium mobilization in Panc-1 cells.

Some researchers have tried to identify which subtype of LPA receptors is crucial for calcium mobilization. An investigation using human-recombinant G protein has suggested that LP_{A1}/EDG-2 transduces Ca^{2+} mobilization largely through PTX sensitive $G_{i/o}$ proteins[18]. LP_{A2}/Edg-4 was supposed to be linked with G_q and phospholipase C[18] and cooperated with other G proteins[19]. Another study, using a knock-out technique, revealed that nearly all PLC activation in response to LPA is dependent on endogenous expression of LP_{A1} and LP_{A2}[20].

The results presented in this paper show that PTX and U73122 attenuated LPA-induced calcium mobilization in Panc-1 cells, which suggests that LPA evokes cytosolic calcium via PTX-sensitive G protein and inositol phosphate production. We have not yet determined which subtypes of LPA receptors have a major role in mobilizing cytosolic calcium in Panc-1 cells. It is likely that LP_{A1} or LP_{A2} might have a major role in mobilization of cytosolic calcium in Panc-1 cells.

The present data suggests that LPA-induced cytosolic calcium mobilization is necessary for activation of NF-κB. PTX and U73122 attenuated both cytosolic calcium mobilization and nuclear translocation of NF-κB induced by LPA. Thapsigargin exerted more potent and long lasting actions than those achieved by LPA. PMA alone failed to stimulate the nuclear translocation of NF-κB. This result suggests that evoked cytosolic calcium concentration is crucial for this translocation, and that a resting level of cytosolic calcium might be insufficient for activation of NF-κB by phorbol ester-stimulated protein kinase C.

Activation of NF-κB by LPA may function as a counterpart to proliferative signaling. It is well known that activation of NF-κB is related to resistance to apoptosis. Recent studies have revealed that chemosensitivity of human pancreatic cancer cells, including Panc-1, is enhanced by I-κBα super-repressor[23] and that NF-κB has a major role in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance of pancreatic cancer cell lines[24].

LPA is supposed to utilize G proteins to achieve its activation of NF-κB. The fact that PTX attenuates activation of NF-κB would suggest a role for $G_{i/o}$. NF-κB can be activated by oncogenic Ras[25], and both Ras and ERKs are activated by LPA through pathways partly sensitive to PTX[20]. Cummings *et al* has shown that protein kinase C is also related with activation of NF-κB, which was coupled to $G_{i/o}$ and $G_{12/13}$ proteins[25].

$G_{i/o}$ might have a major role in the activation of NF-κB[25]. It is conceivable that low concentrations of LPA activates $G_{i/o}$ and that higher concentrations activates G_q. G_q protein may be responsible for the activation of protein kinase C and mobilization of Ca^{2+} of sufficient magnitude or duration to bring, together with signals from $G_{i/o}$, activation of NF-κB[26].

In conclusion, our results show for the first time that LPA induces nuclear translocation of NF-κB in Panc-1 cells by mobilizing cytosolic free calcium. This effect might be caused by activation of phospholipase C *via* PTX-sensitive G protein.

ACKNOWLEDGMENTS

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COMMENTS

Background

Lysophosphatidic acid (LPA) is a growth factor that exerts a number of biological actions on some cancer cell lines. However, the effect of LPA on pancreatic cancer cells has not been well estimated. In the present study, the authors investigate the effects of LPA on cytosolic calcium and translocation of NF-κB in Panc-1 cells.

Research frontiers

The article focuses on the relationship of cytosolic calcium mobilization and NF-κB in Panc-1 cells induced by LPA.

Innovations and breakthroughs

The present study shows that LPA-induced cytosolic calcium mobilization is necessary for activation of NF-κB in pancreatic cancer cells, and that a resting level of cytosolic calcium might be insufficient for activation of NF-κB by phorbol ester-stimulated protein kinase C. It is suggested that protein kinase C is activated endogenously in Panc-1, and protein kinase C is essential for activating NF-κB with cytosolic calcium and that LPA induces the nuclear translocation of NF-κB in Panc-1 by mobilizing cytosolic free calcium.

Applications

It is known that LPA represents a major bioactive constituent of serum, and that activation of NF-κB is related to resistance to apoptosis. By knowing the mechanism of action of LPA, it can be used that target apoptosis in pancreatic cancer cells.

Peer review

The authors investigated the effect of LPA on calcium mobilization, and localization of the transcription factor, NF-κB in the pancreatic cell line, Panc-1. This is an interesting paper. They demonstrated that LPA stimulates calcium mobilization from intracellular stores *via* PTX sensitive G proteins and PLC.

REFERENCES

1 **Eichholtz T**, Jalink K, Fahrenfort I, Moolenaar WH. The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem J* 1993; **291** (Pt 3): 677-680
2 **Gerrard JM**, Robinson P. Lysophosphatidic acid can activate platelets without increasing 32P-labelling of phosphatidic acid. *Biochim Biophys Acta* 1984; **795**: 487-492
3 **Tigyi G**, Miledi R. Lysophosphatidates bound to serum albumin activate membrane currents in Xenopus oocytes and neurite retraction in PC12 pheochromocytoma cells. *J Biol Chem* 1992; **267**: 21360-21367
4 **van Corven EJ**, Groenink A, Jalink K, Eichholtz T, Moolenaar WH. Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. *Cell* 1989; **59**: 45-54
5 **Moolenaar WH**, Kruijer W, Tilly BC, Verlaan I, Bierman AJ, de Laat SW. Growth factor-like action of phosphatidic acid. *Nature* 1986; **323**: 171-173
6 **Jalink K**, Hordijk PL, Moolenaar WH. Growth factor-like effects of lysophosphatidic acid, a novel lipid mediator. *Biochim Biophys Acta* 1994; **1198**: 185-196
7 **Ridley AJ**, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 1992; **70**: 389-399
8 **Moolenaar WH**, Kranenburg O, Postma FR, Zondag GC. Lysophosphatidic acid: G-protein signalling and cellular responses. *Curr Opin Cell Biol* 1997; **9**: 168-173
9 **Kumagai N**, Morii N, Fujisawa K, Nemoto Y, Narumiya S. ADP-ribosylation of rho p21 inhibits lysophosphatidic acid-induced protein tyrosine phosphorylation and phosphatidylinositol 3-kinase activation in cultured Swiss 3T3 cells. *J Biol Chem* 1993; **268**: 24535-24538
10 **Contos JJ**, Ishii I, Chun J. Lysophosphatidic acid receptors. *Mol Pharmacol* 2000; **58**: 1188-1196

11 **Karin M**. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. *Oncogene* 1999; **18**: 6867-6874
12 **Shahrestanifar M**, Fan X, Manning DR. Lysophosphatidic acid activates NF-kappaB in fibroblasts. A requirement for multiple inputs. *J Biol Chem* 1999; **274**: 3828-3833
13 **Goetzl EJ**, Kong Y, Mei B. Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. *J Immunol* 1999; **162**: 2049-2056
14 **Goetzl EJ**, Dolezalova H, Kong Y, Zeng L. Dual mechanisms for lysophospholipid induction of proliferation of human breast carcinoma cells. *Cancer Res* 1999; **59**: 4732-4737
15 **Shida D**, Kitayama J, Yamaguchi H, Okaji Y, Tsuno NH, Watanabe T, Takuwa Y, Nagawa H. Lysophosphatidic acid (LPA) enhances the metastatic potential of human colon carcinoma DLD1 cells through LPA1. *Cancer Res* 2003; **63**: 1706-1711
16 **Raj GV**, Sekula JA, Guo R, Madden JF, Daaka Y. Lysophosphatidic acid promotes survival of androgen-insensitive prostate cancer PC3 cells via activation of NF-kappaB. *Prostate* 2004; **61**: 105-113
17 **Fang X**, Yu S, Bast RC, Liu S, Xu HJ, Hu SX, LaPushin R, Claret FX, Aggarwal BB, Lu Y, Mills GB. Mechanisms for lysophosphatidic acid-induced cytokine production in ovarian cancer cells. *J Biol Chem* 2004; **279**: 9653-9661
18 **An S**, Bleu T, Zheng Y, Goetzl EJ. Recombinant human G protein-coupled lysophosphatidic acid receptors mediate intracellular calcium mobilization. *Mol Pharmacol* 1998; **54**: 881-888
19 **Yuan J**, Slice LW, Gu J, Rozengurt E. Cooperation of Gq, Gi, and G12/13 in protein kinase D activation and phosphorylation induced by lysophosphatidic acid. *J Biol Chem* 2003; **278**: 4882-4891
20 **Contos JJ**, Ishii I, Fukushima N, Kingsbury MA, Ye X, Kawamura S, Brown JH, Chun J. Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2). *Mol Cell Biol* 2002; **22**: 6921-6929
21 **Stahle M**, Veit C, Bachfischer U, Schierling K, Skripczynski B, Hall A, Gierschik P, Giehl K. Mechanisms in LPA-induced tumor cell migration: critical role of phosphorylated ERK. *J Cell Sci* 2003; **116**: 3835-3846
22 **Ryder NM**, Guha S, Hines OJ, Reber HA, Rozengurt E. G protein-coupled receptor signaling in human ductal pancreatic cancer cells: neurotensin responsiveness and mitogenic stimulation. *J Cell Physiol* 2001; **186**: 53-64
23 **Sato T**, Odagiri H, Ikenaga SK, Maruyama M, Sasaki M. Chemosensitivity of human pancreatic carcinoma cells is enhanced by IkappaBalpha super-repressor. *Cancer Sci* 2003; **94**: 467-472
24 **Khanbolooki S**, Nawrocki ST, Arumugam T, Andtbacka R, Pino MS, Kurzrock R, Logsdon CD, Abbruzzese JL, McConkey DJ. Nuclear factor-kappaB maintains TRAIL resistance in human pancreatic cancer cells. *Mol Cancer Ther* 2006; **5**: 2251-2260
25 **Cummings R**, Zhao Y, Jacoby D, Spannhake EW, Ohba M, Garcia JG, Watkins T, He D, Saatian B, Natarajan V. Protein kinase Cdelta mediates lysophosphatidic acid-induced NF-kappaB activation and interleukin-8 secretion in human bronchial epithelial cells. *J Biol Chem* 2004; **279**: 41085-41094
26 **Chen BC**, Lin WW. PKC- and ERK-dependent activation of I kappa B kinase by lipopolysaccharide in macrophages: enhancement by P2Y receptor-mediated CaMK activation. *Br J Pharmacol* 2001; **134**: 1055-1065

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RAPID COMMUNICATION

Comparison of esophageal capsule endoscopy and esophagogastroduodenoscopy for diagnosis of esophageal varices

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Abstract

AIM: To investigate the utility of esophageal capsule endoscopy in the diagnosis and grading of esophageal varices.

METHODS: Cirrhotic patients who were undergoing esophagogastroduodenoscopy (EGD) for variceal screening or surveillance underwent capsule endoscopy. Two separate blinded investigators read each capsule endoscopy for the following results: variceal grade, need for treatment with variceal banding or prophylaxis with beta-blocker therapy, degree of portal hypertensive gastropathy, and gastric varices.

RESULTS: Fifty patients underwent both capsule and EGD. Forty-eight patients had both procedures on the same day, and 2 patients had capsule endoscopy within 72 h of EGD. The accuracy of capsule endoscopy to decide on the need for prophylaxis was 74%, with sensitivity of 63% and specificity of 82%. Inter-rater agreement was moderate (kappa = 0.56). Agreement between EGD and capsule endoscopy on grade of varices was 0.53 (moderate). Inter-rater reliability was good (kappa = 0.77). In diagnosis of portal hypertensive gastropathy, accuracy was 57%, with sensitivity of 96% and specificity of 17%. Two patients had gastric varices seen on EGD, one of which was seen on capsule endoscopy. There were no complications from capsule endoscopy.

CONCLUSION: We conclude that capsule endoscopy has a limited role in deciding which patients would benefit from EGD with banding or beta-blocker therapy. More data is needed to assess accuracy for staging esophageal varices, PHG, and the detection of gastric varices.

Key words: Esophageal varices; Capsule endoscopy; Portal hypertension

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INTRODUCTION

Cirrhosis affects 3.6 out of every 1000 adults in North America. A major cause of cirrhosis-related morbidity and mortality is the development of variceal hemorrhage, a direct consequence of portal hypertension. The reported prevalence of esophageal varices in patients with chronic liver disease varies from 24% to 81%[1-3]. Variceal hemorrhage occurs in 25%-40% of patients with cirrhosis, and is associated with a mortality rate of up to 30%[1,2]. Accurate identification of patients with an increased risk of bleeding allows for primary prophylaxis to prevent variceal bleeding. Prophylactic use of beta-blockers has been shown to decrease the incidence of first variceal bleeding and death in patients with cirrhosis, and is currently the standard of care in patients who are at high risk for variceal hemorrhage[4,5]. Factors predictive of variceal hemorrhage include location of varices, size of varices, appearance of varices, clinical features of the patient, and variceal pressure[6].

Esophagogastroduodenoscopy (EGD) is the standard of care for evaluation of varices. An EGD is currently recommended at diagnosis of cirrhosis, and

then yearly screening for patients with no varices on initial EGD for patients with progression of their liver disease or every two years for those who remain stable[5]. In patients with small varices, endoscopy should be performed every year to assess for a change in size[7].

Currently, there is no universally accepted grading system for varices. Reliability of endoscopy is affected by inter-observer variability[8,9]. The subjective grading, invasiveness, risks of sedation, and cost of EGD has prompted a search for other alternatives. As of yet, no alternative had proven to be as accurate as EGD.

Several pilot studies have been published comparing capsule endoscopy (CE) to EGD for variceal screening. Eisen *et al* studied 32 patients, and found an overall concordance rate of 96.9% for the diagnosis of esophageal varices and 90.6% for the diagnosis of portal hypertensive gastropathy[10]. Lapalus *et al* performed unsedated EGD and capsule endoscopy in 21 patients, with an accuracy of 84.2% for the presence or absence of esophageal varices[11].

Herein, we report the results of a study designed to assess the ability of capsule endoscopy to correctly identify the presence of esophageal varices and related features of portal hypertension in patients undergoing screening or surveillance endoscopy, and to determine the need for treatment or prophylaxis of esophageal varices.

MATERIALS AND METHODS

All patients enrolled were from the patient population of Scripps Clinic, La Jolla, California. Patients were eligible if they were scheduled to undergo EGD for screening or surveillance of esophageal varices. Screening was performed in patients with either biopsy-proven cirrhosis, or biochemical and imaging studies consistent with cirrhosis. Surveillance was performed in patients who had previously been diagnosed with esophageal varices *via* EGD and were repeating the test to assess for progression of varices. Patients who had previously undergone banding of esophageal varices were included in the study if they were stable and had not had a variceal hemorrhage for $\geqslant$ 6 mo. Consecutive patients scheduled for EGD as screening or surveillance of esophageal varices were screened for eligibility to participate. All patients were age > 18 years, able to give informed consent, and hemodynamically stable.

Exclusion criteria included dysphagia, known Zenker's diverticulum, the presence of cardiac pacemaker or other implantable electro-medical devices, pregnancy, or a scheduled MRI within 7 d after capsule ingestion. Patients also were excluded if they had a history of or risk for intestinal obstruction, including any prior abdominal surgery of the gastrointestinal tract other than uncomplicated cholecystectomy or appendectomy.

All patients who consented underwent capsule endoscopy and EGD on the same day or within 72 h. The endoscopies were performed under moderate sedation by three staff hepatologists at Scripps Clinic. The hepatologists were blinded to the results of the capsule endoscopy, but not to the patient's prior history or previous endoscopy findings. Photographs were taken of any pertinent findings at endoscopy and grading of varices was agreed to by all three physicians after unblinding.

EGDs and CEs were both graded by the following scale: F0, no varices; F1, small straight varices; F2, tortuous varices and < 50% of esophageal radius; F3, large and tortuous varices with or without red spots[6,12]. Presence or absence of high risk stigmata, defined as neovascularization or red or white spots was noted separately. Each observer decided whether or not treatment was indicated based on presence of F2 or F3 varices or the presence of high risk stigmata on any size varix. Portal hypertensive gastropathy (PHG) was graded on the following scale: none, mild (mucosal mosaic pattern), moderate (mosaic mucosal pattern with occasional red spots), or severe (mosaic mucosal pattern, extensive red or black spots, active oozing)[13,14]. Portal hypertensive gastropathy was diagnosed on capsule endoscopy *via* photographs of any area of the gastric mucosa as it was not possible to assess the location of the visualized area. The presence or absence of gastric varices was noted separately, as well as other findings unrelated to portal hypertension such as esophagitis, gastritis (defined as erythema or erosions of gastric lining), peptic ulcer disease, or duodenal lesions.

Capsule endoscopy was administered in the following manner in all patients. After imbibing 100 mL of water with 0.6 mL of simethicone, patients lay supine and then ingested the pill with 5 mL of water without raising their head. Any difficulty with ingestion was recorded by the administrator, and patients were instructed not to speak after pill ingestion. After 2 min supine, they were raised to a 30 degree incline. After another 2 min they were raised to 60 degrees, and after 1 min at 60 degrees the patient imbibed a sip of water. They then sat up completely and imbibed another sip of water, at which time they were placed in the left lateral decubitus position in order to improve visualization of the fundus. Three minutes after being placed on their left side the patients were instructed to sit up or walk around for the remaining 12 min of the examination.

Capsule endoscopies were read by two separate investigators, who were blinded to EGD findings, patient medical history, and reading of the other investigator. Both capsule readers had prior experience in endoscopic evaluation and diagnosis of esophageal varices. Prior to the study, both readers underwent training as recommended by the capsule manufacturer, consisting of review of a CD Rom and participation in an online course, which included review of 10 cases of capsule endoscopy. Each CE was read twice by each investigator on two separate occasions at least 60 d apart. Capsule images were evaluated for the presence and grade of esophageal varices, the presence and grade of PHG, the presence of gastric varices, and any other findings. Esophageal transit time and time spent reading each examination was recorded.

One week after capsule ingestion, each patient was contacted by telephone to assess for symptoms of capsule retention. At that time, patient satisfaction was

Table 1 Demographics demographics of 50 patients undergoing esophageal capsule endoscopy and EGD for diagnosis of esophageal varices (n = 50)

	Patient population (%)
Male gender	34 (68)
Average age	58 (range, 25-74)
Average MELD[1]	9.48 (range, 6-23)
Average Child-Pugh	6.8 (range, 5-13)
Race	
Caucasian	40 (80)
Hispanic	6 (12)
African American	3 (6)
Middle Eastern	1 (2)
Etiology of cirrhosis	
Hepatitis C	24 (48)
Hepatitis C and alcohol	7 (14)
Alcohol	6 (12)
Nonalcoholic steatohepatitis	6 (12)
Other[2]	7 (14)

[1]MELD: Model for End Stage Liver Disease; [2]Primary biliary cirrhosis, sarcoidosis, cryptogenic cirrhosis, autoimmune hepatitis, Wilson's disease, idiopathic pulmonary hypertension.

assessed. Patients were asked if they would be willing to undergo CE or EGD again, and which study they preferred.

Statistical analysis was performed to assess sensitivity, specificity, and accuracy of CE versus EGD in determining need for prophylaxis or treatment. A weighted kappa scale was used to determine agreement of variceal grade by CE compared to EGD, as well as inter- and intra-observer agreement[14-17]. Inter-observer agreement was defined as comparing results from Reader 1 to results from Reader 2. Intra-observer agreement measured results from the first read and results from the second read of each reader independent of the other reader. The sample size of 50 was chosen because with these numbers one typically will expect a standard deviation of 0.10 and coefficients of variation of 15% or less.

The study was approved by the local institutional review board.

RESULTS

Fifty-five patients were screened to participate in the study. Five patients were not included: 2 patients refused, 1 patient had a history of an esophageal stricture, and 2 patients had history of surgery on the gastrointestinal tract. Fifty patients successfully underwent EGD and esophageal capsule endoscopy. In most cases, patients underwent CE on the same day as and just prior to EGD. There were two patients who underwent CE on a different day but within 72 h and two patients who underwent CE immediately after EGD. Median esophageal transit time was 249.5 s (range, 1-352 s). The esophageal transit times were as follows: 2 capsules 0-5 s, 15 capsules 5-60 s, and 33 capsules 60-352 s. Five patients (10%) had a mild amount of difficulty swallowing the capsule, and four patients (8%) had a moderate amount of difficulty, one of whom had to swallow it in a sitting position.

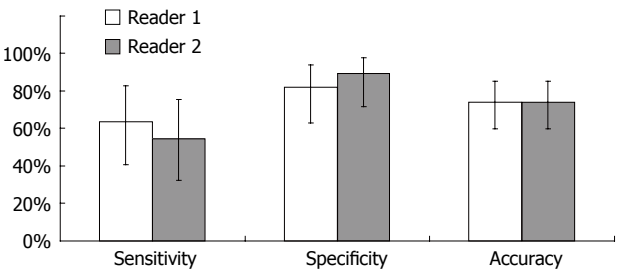

Figure 1 Sensitivity, specificity and accuracy of esophageal capsule endoscopy compared to EGD for two separate blinded investigators. The error bars represent 95% confidence intervals.

Demographics of the patients can be seen in Table 1. Thirteen patients (26%) were undergoing surveillance of varices and had a history of previous variceal banding; the remainder were undergoing screening examinations. The patients who were undergoing surveillance had not been banded for at least 6 mo, and previously had been obliterated. All patients had undergone banding in the past for history of variceal bleeding. Based on EGD findings, prevalence of esophageal varices was 66%: 17 patients had no varices, 16 patients with F1 varices, 15 patients with F2 varices, and 2 patients with F3 varices. 5 patients underwent banding at the time of EGD.

In determining need for prophylaxis using EGD as the gold standard, sensitivity of CE was 63% (95% CI, 0.40-0.83; SD, 0.04), specificity was 82% (95% CI, 0.63-0.94; SD, 0.03), and accuracy was 74% (95% CI, 0.59-0.85; SD 0.04; Figure 1). The accuracy was not improved when patients with prior banding were excluded or when patients with difficulty swallowing the capsule were excluded. Positive predictive value in this population was 73% (95% CI, 0.48-0.91; SD, 0.04) and negative predictive value was 74% (95% CI, 0.55-0.88; SD, 0.04). There was no association between time of esophageal transit of the capsule and accuracy of the results, assessed by splitting the group at the median time of 249 s and comparing the two groups. Inter-rater reliability for need for prophylaxis was 0.56 (moderate agreement). Intra-rater reliability was 0.61 (good) for Reader 1 and 0.41 (moderate) for Reader 2. For grade of varices, agreement between EGD and CE was 0.53 (moderate). Inter-rater reliability for grade of varices was 0.77 (good), and intra-rater reliability was 0.76 (good) for Reader 1 and 0.69 for Reader 2.

Two patients (4%) had gastric varices. One of these patients had gastric varices suspected on CE by Reader 2, and neither of the patients had large esophageal varices requiring primary prophylaxis. It was not possible to gauge the location of the varices based on the capsule photographs.

Forty-five patients (90%) had portal hypertensive gastropathy: 28 patients with mild disease and 17 patients with moderate disease. In determining the presence or absence of PHG, sensitivity was 96% (95% CI, 0.78-0.99) and specificity was 17% (95% CI, 0.05-0.39). Accuracy was 57% (95% CI, 0.41-0.71). Inter-rater reliability for presence of PHG was 0.61 (good).

Seventeen patients (34%) had other findings seen on

EGD. Seven patients had gastritis seen on EGD, two of which were detected by CE. Two patients had Barrett's esophagus; one was detected by Reader 1 and one was detected by Reader 2. Two patients had esophagitis seen on EGD but not on CE. One patient had gastric polyps and one had duodenal polyps seen on EGD, and neither was detected on CE. One patient had an esophageal ring seen on EGD that was also detected on CE by Reader 2. One patient had scarring from prior banding that was seen on EGD but not CE. 11 patients underwent biopsy at time of EGD: 10 to rule out *H pylori* and one for diagnosis of Barrett's esophagus.

There were no complications from either CE or EGD. Thirty-six patients (72%) were satisfied equally with EGD and CE. Thirteen (26%) preferred CE to EGD, and one patient preferred EGD to CE. There were no instances of capsule retention.

DISCUSSION

Complications of portal hypertension remain one of the major causes of morbidity and mortality in patients with cirrhosis. Up to 33% of cirrhotics will experience bleeding from varices, and 70% of these will be plagued with recurrent variceal bleeding[1,2,6]. In 1998, an AASLD single-topic symposium on portal hypertension devised the following current recommendations for variceal screening: EGD at time of diagnosis of cirrhosis, and if no varices were present, on a biyearly basis if liver function is stable, or yearly if liver function worsens, and yearly if small varices were present on initial screening[7]. Numerous studies have demonstrated the efficacy of beta-blocker therapy for reduction of risk of variceal bleeding and related mortality, decreasing the risk of variceal bleeding by 50%[18-20]. Recent data have suggested that variceal banding is also effective as primary prevention of variceal bleeding in patients with high risk varices[18-21]. Despite these recommendations, compliance with screening has been quite poor. Arguedas *et al* in 2001 reported that just 46% of cirrhotic patients underwent variceal screening by EGD prior to referral for liver transplantation, despite having a diagnosis of cirrhosis for a median duration of 3 years[22]. Results of a survey of practicing gastroenterologists suggested an even lower screening rate of 39%[23].

Alternative methods to EGD have been studied for variceal screening, including transnasal endoluminal ultrasound[24], platelet count/spleen diameter ratio[25], multidetector computed tomography esophagography[26], and esophageal capsule endoscopy[10,11,27]. To date, no method has proven accurate enough to replace EGD.

The results of our study are different from the two published pilot studies, showing a lower sensitivity, specificity, and accuracy for esophageal capsule endoscopy. Because there is known variability in grading of varices by EGD[8,9], the accuracy of capsule endoscopy when measured against EGD may be wrong. We attempted to decrease this effect by verification of variceal grade diagnosed at endoscopy after unblinding by all physicians involved in the study, through inspection of photographs. Other possible reasons that our study results may vary include the small size of prior studies compared to ours. Our trial size was still somewhat small, but we balanced that expectation with the recognition that a much larger trial would be needed for confirmation of this as a pilot trial. Other confounders for the data could include the absence of complete industry funding in our study as opposed to the prior ones, and our relative lack of expertise with capsule endoscopy or other technical difficulties.

Concern has been raised regarding the utility of capsule endoscopy in patients who have previously undergone banding of esophageal varices. Patients were included in our study if they had not undergone banding for at least 6 mo. We chose to include these patients because we felt that varices would still be able to be diagnosed at esophageal capsule endoscopy. When patients with previous banding were excluded from analysis, our accuracy did not improve significantly. A total of 5 patients out of 13 who were undergoing surveillance for esophageal varices required repeat banding at the time of EGD. This underscores a limitation of capsule endoscopy: that patients with varices seen at diagnosis may then have to undergo EGD for therapy.

There has been some concern about the mixed results of capsule endoscopy use for evaluation of esophageal pathology, such as varices, Barrett's esophagus, or esophagitis[27-29]. It is thought that the mixed results of capsule endoscopy may have to do with deviations from the standard ingestion procedure recommended by the manufacturer[30]. We note that in our study, all patients were able to successfully swallow the capsule, with only 9 patients having some difficulty, including two patients that needed to lift their heads from the supine position and one patient that had to ingest the pill in the sitting position. We feel that there is little chance these deviations influenced our results. When we looked at patient history of banding, time of esophageal transit, and reader experience/learning curve, none of these factors significantly changed the results of our study. We, therefore, feel that the accuracy reported here may be more reflective of what can be expected with capsule endoscopy use in community gastroenterology practice.

Esophageal capsule endoscopy has been designed specifically to look at the esophagus; there is no way to ensure that full inspection of the gastric mucosa and duodenum will occur, as it would with EGD. When screening for varices, this usually is not an issue. However, as in our study, there are patients who have gastric varices in the absence of significant esophageal varices that would require pharmacologic prophylaxis against bleeding. These patients may be missed if screening was done solely with capsule endoscopy. In addition, capsule endoscopy had poor accuracy for diagnosis of portal hypertensive gastropathy. Capsule endoscopy limits the patient to diagnosis only. In 11 of our patients, biopsies were performed for diagnosis of *H pylori* or Barrett's esophagus. Obviously, these biopsies would not have been able to be performed if capsule endoscopy was the only diagnostic method used.

Given our results for capsule endoscopy, we are uncertain if its routine use can replace EGD at this time as a screening tool. It may be useful for those patients who are unable or unwilling to undergo upper endoscopy, but clinicians need to be cognizant of the possibility of a false negative result. At this time, we would recommend use of esophageal capsule endoscopy only in the setting of a clinical trial.

In conclusion, we feel that capsule endoscopy has a limited role in deciding which patients would benefit from EGD with banding or beta-blocker therapy in early cirrhosis, as well as for determining the specific grade of esophageal varices, PHG, or gastric varices. More data is needed to assess accuracy for staging esophageal varices, PHG, and the detection of gastric varices. Clinicians who choose to employ capsule endoscopy as part of their routine clinical practice should be cognizant of the lower accuracy for esophageal variceal screening.

COMMENTS

Background

Esophageal varices are found in up to 81% of patient with cirrhosis, and results in significant gastrointestinal bleeding in up to half of patients. In order to prevent variceal bleeding, screening is recommended with upper endoscopy every 1-3 years, with prophylaxis given to those patients with large varices. Esophageal capsule endoscopy is a new device designed to image the esophagus in a noninvasive way. The utility of esophageal capsule endoscopy in the diagnosis of esophageal varices is not known.

Research frontiers

To date, two pilot studies have been published regarding the use of esophageal capsule endoscopy for the diagnosis of esophageal varices. These initial studies were performed in 32 and 21 patients, respectively, and showed high concordance and accuracy for the diagnosis of esophageal varices with capsule endoscopy (96.9% and 84.2%, respectively).

Innovations and breakthroughs

In this publication, 50 patients underwent upper endoscopy and esophageal capsule endoscopy. The capsule endoscopies were independently read by two blinded investigators. The accuracy of capsule endoscopy for diagnosis of esophageal varices was found to be 74% in determining the need for prophylaxis based on the presence of large varices. The sensitivity was 63% and the specificity was 82%. Inter-rater reliability was moderate for determining the need for prophylaxis. Intra-rater reliability was moderate for one reader and good for the other reader. 34% of patients studied had other findings seen at upper endoscopy that were not reliably diagnosed with capsule endoscopy, including gastric varices, gastric and duodenal polyps, esophagitis, and Barrett's esophagus. Accuracy for diagnosis of portal hypertensive gastropathy was poor at only 57%.

Applications

Currently, the use of capsule endoscopy for variceal screening cannot be routinely recommended. Refinements to the capsule procedure may improve the accuracy in the future. Further studies are needed to verify these results.

Peer review

This paper details the use of esophageal capsule endoscopy for the diagnosis of esophageal varices. Two pilots studies suggested that capsule endoscopy may be useful for detection of large varices. In this largest cohort to date, we found that capsule endoscopy has a poor sensitivity in detecting large varices requiring prophylactic therapy. In addition, there is also poor inter- and intra-observer agreement when using this method for grading esophageal varices. Finally, since three quarters of all patients do not prefer one method over the other, it appears that capsule endoscopy would have a limited role in diagnosis of esophageal varices.

REFERENCES

1 **Merli M**, Nicolini G, Angeloni S, Rinaldi V, De Santis A, Merkel C, Attili AF, Riggio O. Incidence and natural history of small esophageal varices in cirrhotic patients. *J Hepatol* 2003; **38**: 266-272

2 **Sanyal AJ**, Fontana RJ, Di Bisceglie AM, Everhart JE, Doherty MC, Everson GT, Donovan JA, Malet PF, Mehta S, Sheikh MY, Reid AE, Ghany MG, Gretch DR, Halt-C Trial Group. The prevalence and risk factors associated with esophageal varices in subjects with hepatitis C and advanced fibrosis. *Gastrointest Endosc* 2006; **64**: 855-864

3 **Zaman A**, Hapke R, Flora K, Rosen H, Benner K. Prevalence of upper and lower gastrointestinal tract findings in liver transplant candidates undergoing screening endoscopic evaluation. *Am J Gastroenterol* 1999; **94**: 895-899

4 **de la Mora-Levy JG**, Baron TH. Endoscopic management of the liver transplant patient. *Liver Transpl* 2005; **11**: 1007-1021

5 **Qureshi W**, Adler DG, Davila R, Egan J, Hirota W, Leighton J, Rajan E, Zuckerman MJ, Fanelli R, Wheeler-Harbaugh J, Baron TH, Faigel DO. ASGE Guideline: the role of endoscopy in the management of variceal hemorrhage, updated July 2005. *Gastrointest Endosc* 2005; **62**: 651-655

6 **Prediction of the first variceal hemorrhage in patients with cirrhosis of the liver and esophageal varices**. A prospective multicenter study. The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices. *N Engl J Med* 1988; **319**: 983-989

7 **Grace ND**, Groszmann RJ, Garcia-Tsao G, Burroughs AK, Pagliaro L, Makuch RW, Bosch J, Stiegmann GV, Henderson JM, de Franchis R, Wagner JL, Conn HO, Rodes J. Portal hypertension and variceal bleeding: an AASLD single topic symposium. *Hepatology* 1998; **28**: 868-880

8 **Bendtsen F**, Skovgaard LT, Sorensen TI, Matzen P. Agreement among multiple observers on endoscopic diagnosis of esophageal varices before bleeding. *Hepatology* 1990; **11**: 341-347

9 **Reliability of endoscopy in the assessment of variceal features**. The Italian Liver Cirrhosis Project. *J Hepatol* 1987; **4**: 93-98

10 **Eisen GM**, Eliakim R, Zaman A, Schwartz J, Faigel D, Rondonotti E, Villa F, Weizman E, Yassin K, deFranchis R. The accuracy of PillCam ESO capsule endoscopy versus conventional upper endoscopy for the diagnosis of esophageal varices: a prospective three-center pilot study. *Endoscopy* 2006; **38**: 31-35

11 **Lapalus MG**, Dumortier J, Fumex F, Roman S, Lot M, Prost B, Mion F, Ponchon T. Esophageal capsule endoscopy versus esophagogastroduodenoscopy for evaluating portal hypertension: a prospective comparative study of performance and tolerance. *Endoscopy* 2006; **38**: 36-41

12 **Idezuki Y**. General rules for recording endoscopic findings of esophagogastric varices (1991). Japanese Society for Portal Hypertension. *World J Surg* 1995; **19**: 420-422; discussion 423

13 **Thuluvath PJ**, Yoo HY. Portal Hypertensive gastropathy. *Am J Gastroenterol* 2002; **97**: 2973-2978

14 **Stewart CA**, Sanyal AJ. Grading portal gastropathy: validation of a gastropathy scoring system. *Am J Gastroenterol* 2003; **98**: 1758-1765

15 **Cohen J**. Weighted kappa: nominal scale agreement with provision for scaled disagreement or partial credit. *Psychol Bull* 1968; **70**: 213-220

16 **Fleiss JL**, Levin BA, Levin B, Paik MC. Statistical Methods for Rates and Proportions. 3rd ed. New York: John Wiley, 1981: 384

17 **Landis JR**, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977; **33**: 159-174

18 **Imperiale TF**, Chalasani N. A meta-analysis of endoscopic variceal ligation for primary prophylaxis of esophageal variceal bleeding. *Hepatology* 2001; **33**: 802-807

19 **Schepke M**, Kleber G, Nurnberg D, Willert J, Koch L, Veltzke-Schlieker W, Hellerbrand C, Kuth J, Schanz S, Kahl S, Fleig WE, Sauerbruch T. Ligation versus propranolol for the primary prophylaxis of variceal bleeding in cirrhosis. *Hepatology* 2004; **40**: 65-72

20 **Lui HF**, Stanley AJ, Forrest EH, Jalan R, Hislop WS, Mills PR, Finlayson ND, Macgilchrist AJ, Hayes PC. Primary prophylaxis of variceal hemorrhage: a randomized controlled trial comparing band ligation, propranolol, and isosorbide mononitrate. *Gastroenterology* 2002; **123**: 735-744
21 **Khuroo MS**, Khuroo NS, Farahat KL, Khuroo YS, Sofi AA, Dahab ST. Meta-analysis: endoscopic variceal ligation for primary prophylaxis of oesophageal variceal bleeding. *Aliment Pharmacol Ther* 2005; **21**: 347-361
22 **Arguedas MR**, McGuire BM, Fallon MB, Abrams GA. The use of screening and preventive therapies for gastroesophageal varices in patients referred for evaluation of orthotopic liver transplantation. *Am J Gastroenterol* 2001; **96**: 833-837
23 **Hapke R**, Zaman A, Benner KG, Flora K, Rosen H. Prevention of first variceal hemorrhage-a survey of community practice patterns. *Hepatology* 1997; **26**: A30 (abstract)
24 **Kane L**, Kahaleh M, Shami VM, Caldwell SH, Berg CL, Abdrabbo KM, Yoshida CM, Arseneau KO, Yeaton P. Comparison of the grading of esophageal varices by transnasal endoluminal ultrasound and esophagogastroduo denoscopy. *Clin Gastroenterol Hepatol* 2005; **3**: 806-810
25 **Giannini EG**, Zaman A, Kreil A, Floreani A, Dulbecco P, Testa E, Sohaey R, Verhey P, Peck-Radosavljevic M, Mansi C, Savarino V, Testa R. Platelet count/spleen diameter ratio for the noninvasive diagnosis of esophageal varices: results of a multicenter, prospective, validation study. *Am J Gastroenterol* 2006; **101**: 2511-2519
26 **Kim SH**, Kim YJ, Lee JM, Choi KD, Chung YJ, Han JK, Lee JY, Lee MW, Han CJ, Choi JI, Shin KS, Choi BI. Esophageal varices in patients with cirrhosis: multidetector CT esophagography--comparison with endoscopy. *Radiology* 2007; **242**: 759-768
27 **Sanchez-Yague A**, Caunedo-Alvarez A, Garcia-Montes JM, Romero-Vazquez J, Pellicer-Bautista FJ, Herrerias-Gutierrez JM. Esophageal capsule endoscopy in patients refusing conventional endoscopy for the study of suspected esophageal pathology. *Eur J Gastroenterol Hepatol* 2006; **18**: 977-983
28 **Eliakim R**, Yassin K, Shlomi I, Suissa A, Eisen GM. A novel diagnostic tool for detecting oesophageal pathology: the PillCam oesophageal video capsule. *Aliment Pharmacol Ther* 2004; **20**: 1083-1089
29 **Eliakim R**, Sharma VK, Yassin K, Adler SN, Jacob H, Cave DR, Sachdev R, Mitty RD, Hartmann D, Schilling D, Riemann JF, Bar-Meir S, Bardan E, Fennerty B, Eisen G, Faigel D, Lewis BS, Fleischer DE. A prospective study of the diagnostic accuracy of PillCam ESO esophageal capsule endoscopy versus conventional upper endoscopy in patients with chronic gastroesophageal reflux diseases. *J Clin Gastroenterol* 2005; **39**: 572-578
30 **Gralnek IM**, Rabinovitz R, Afik D, Eliakim R. A simplified ingestion procedure for esophageal capsule endoscopy: initial evaluation in healthy volunteers. *Endoscopy* 2006; **38**: 913-918

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RAPID COMMUNICATION

Association between *calcium sensing receptor* gene polymorphisms and chronic pancreatitis in a US population: Role of *serine protease inhibitor Kazal 1type* and alcohol

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Abstract

AIM: To test the hypothesis that calcium sensing receptor (*CASR*) polymorphisms are associated with chronic pancreatitis (CP), and to determine whether *serine protease inhibitor Kazal 1type* (*SPINK1*) N34S or alcohol are necessary co-factors in its etiology.

METHODS: Initially, 115 subjects with pancreatitis and 66 controls were evaluated, of whom 57 patients and 21 controls were predetermined to carry the high-risk *SPINK1* N34S polymorphism. We sequenced *CASR* gene exons 2, 3, 4, 5 and 7, areas containing the majority of reported polymorphisms and novel mutations. Based on the initial results, we added 223 patients and 239 controls to analyze three common nonsynonymous single nucleotide polymorphisms (SNPs) in exon 7 (A986S, R990G, and Q1011E).

RESULTS: The *CASR* exon 7 R990G polymorphism was significantly associated with CP (OR, 2.01; 95% CI, 1.12-3.59; P = 0.015). The association between *CASR* R990G and CP was stronger in subjects who reported moderate or heavy alcohol consumption (OR, 3.12; 95% CI, 1.14-9.13; P = 0.018). There was no association between the various *CASR* genotypes and *SPINK1* N34S in pancreatitis. None of the novel *CASR* polymorphisms reported from Germany and India was detected.

CONCLUSION: Our United States-based study confirmed an association of *CASR* and CP and for the first time demonstrated that *CASR* R990G is a significant risk factor for CP. We also conclude that the risk of CP with *CASR* R990G is increased in subjects with moderate to heavy alcohol consumption.

Key words: Calcium sensing receptor; *Serine protease inhibitor Kazal 1type*; Chronic pancreatitis; Alcohol

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INTRODUCTION

Chronic pancreatitis (CP) is a debilitating, inflammatory disease of the pancreas, characterized by progressive organ destruction and fibrosis. CP results in profound exocrine and endocrine insufficiency and, in many cases, intractable, chronic pain. As a complex disorder, CP can develop from a variety of etiologies with multiple pathological pathways[1]. For several years, alcohol abuse has been considered the most likely causative agent for CP in Western countries, although etiologies including toxic, metabolic (hypercalcemia, hyperlipidemia), genetic mutations, autoimmune, and duct obstruction, have also been implicated[2,3].

Consistent experimental evidence links elevated acinar cell calcium levels with acute pancreatitis in association with premature trypsinogen activation to trypsin[4]. Recurrent acute pancreatitis (RAP), as illustrated in patients with hereditary and sporadic pancreatitis, can lead to CP[5-7]. Hypercalcemia itself has been associated with the development and complications of CP[2]. Recent studies from Germany and India have reported that novel *calcium sensing receptor (CASR)* gene mutations in combination with the presence of *serine protease inhibitor Kazal 1type (SPINK1)* N34S increased the risk of CP[8-10]. The *SPINK1* N34S "high-risk haplotype" is strongly associated with CP, but only a limited portion of mutation carriers develop CP during their life time, suggesting that additional factors are necessary to develop this complex disorder[11,12].

CASR is a member of the G-protein-coupled receptor (GPCR) superfamily[13]. *CASR* plays an important role in calcium homeostasis, as is reflected in its expression by cells of the parathyroid gland and renal tubules that are involved in calcium metabolism. *CASR* has been identified in both human pancreatic acinar and ductal cells, as well as in various non-exocrine cells[14], although its functional significance in the pancreas has not been determined.

The human *CASR* gene is located on chromosome 3q 13.3-21[15,16]. *CASR* possesses a coding region of 3234 base pairs (bp) which is contained within 6 of the seven exons that make up the gene. One hundred and twelve functional mutations (40 activating and 72 inactivating) have been described in the *CASR* mutation database related to familial hypocalciuric hypercalcemia (FHH), neonatal severe primary hyperparathyroidism (NSHPT), and autosomal dominant hypocalcemia (ADH) families as well as in *de-novo* disease[17]. In addition, single activating or inactivating *CASR* mutations may cause hypercalcemic or hypocalcemic disorders[18,19].

We hypothesized that *CASR* polymorphisms are associated with the development of CP and that *SPINK1* N34S mutations or alcohol may be important co-factors in its etiology. We tested this hypothesis by evaluating subjects with RAP, CP and healthy controls with known *SPINK1* genotypes and alcohol intake for common and novel *CASR* polymorphisms in exons 2, 3, 4, 5 and 7.

MATERIALS AND METHODS

Study population

Subjects were recruited from the North American Pancreatic Study2 (NAPS2). The NAPS2 study is a multicenter, molecular epidemiology study designed to evaluate the genetic and environmental factors predisposing to recurrent acute pancreatitis (RAP) and CP. Detailed description of methods are presented elsewhere[20]. The subjects were stratified into alcohol categories based on self-reported average number of drinks consumed per week during the period of heaviest lifetime drinking. Alcohol categories were defined based on the drinking pattern as: (1) abstainers: no alcohol use or < 20 drinks in lifetime; (2) light drinkers: ⩽ 3 drinks/week; (3) moderate drinkers: 4-7 drinks/week for females; 4-14 drinks/week for males; (4) heavy drinkers; 8-34 drinks/week for females; 15-34 drinks/week for males; (5) very heavy drinkers: ⩾ 35 drinks/week for both males and females. For analysis, alcohol drinking categories were combined into 3 groups based on their risk for causing CP: (1) abstainers and light drinkers were considered very low risk, (2) moderate and heavy drinkers were considered moderate risk, and (3) very heavy drinkers were considered substantial risk[20].

One hundred and fifteen affected individuals and 66 controls were selected initially from four sites of the NAPS2 cohort. These subjects were selected based on the presence or absence of *SPINK1* N34S mutations, of which 57 patients and 21 controls were determined by previous genetic analysis to carry the high-risk *SPINK1* mutation. From the twenty site NAPS2 consortium, 219 affected subjects and 239 controls were later screened for the three common nonsynonymous single nucleotide polymorphisms (SNPs) seen in the coding region of the intracellular *CASR* tail in exon 7 which appeared to be the region of interest. These were A986S (rs # 1801725), R990G (rs # 1042636), and Q1011E (rs # 1801726).

DNA preparation and mutation analysis

Genomic DNA was extracted from whole blood as described[20]. PCR primers were designed for *CASR* gene exons 2, 3, 4, 5 and 7, which contains most of the commonly seen activating and inactivating mutations as well as the novel mutations found in Germany and India (Table 1). Exons 4 and 7 were lengthy and thus were divided into 2 and 4 fragments respectively.

PCR was performed in a total volume of 25 μL; 200 nmol of forward and reverse primer, 200 μmol of dNTP and 1 × PCR Buffer Ⅱ (ABI, CA) with 10 ng of DNA. Amplification settings were 95℃ for 12 min × 1 cycle, 95℃ for 30 s, annealing temperature (Table 1) × 20 s and 72℃ × 20 s for 35 cycles and 72℃ for 2 min × 1 cycle. Annealing temperatures and magnesium concentrations for different primers are shown in Table 1. PCR amplification products were purified with exonuclease Ⅰ (NEB, Beverley, MA) and shrimp alkaline phosphatase (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's recommendations. Cycle sequencing was performed using the ABI Prism Big Dye Terminator Sequencing Kit v3.1 diluted 1:8 (ABI, Foster City, CA) using the appropriate PCR primers. Products from the sequencing reaction were purified by ethanol EDTA precipitation. Sequence products were run on

Table 1 Polymerase chain reaction primer pairs, magnesium concentration and annealing temperatures used for genetic analysis of the *CASR* gene

Scanning region	Forward and reverse primer sequences	$MgCl_2$ (mmol/L)	Annealing temperature (℃)
Exon 2	5′-ACCACCCACATTACAAGTC-3′ 5′-GCTTTTCTCCAACCACTCAG-3′	2.5	55
Exon 3	5′-ATGAAGCCAGAGAGTAGTAAC-3′ 5′-TAAACCGTATGGCTATTGGG-3′	2.5	58
Exon 4a	5′-GCTTTTCCTTACCCTTTCTTTCATC-3′ 5′-ATCACCTCTACCACATGCTG-3′	2	58
Exon 4b	5′-CAGATCTTGAGCCCCTCATC-3′ 5′-GCAGCCCAACTCTGCTTTAT-3′	2	59
Exon 5	5′-TGGGGCTTGTACTCATTCTT-3′ 5′-CTGGTTTTCTGATGGACAGC-3′	1.5	59
Exon 7a	5′-CACACAATAACTCACTCTTCAC-3′ 5′-CAGAGGAAAACCAGCAGGAAC-3′	2.0	61
Exon 7b	5′-AAAACCAACCGTGTCCTCCTG-3′ 5′-ATGGCAATCACCTCTACGGC-3′	1.0	53
Exon 7c	5′-GCTCATCTTCTTCATCGTCTGG-3′ 5′-CGTATCGCTGCTTTTCTGGG-3′	1.0	58
Exon 7d	5′-CCCAGCAAGAGCAGCAG-3′ 5′-ACAACTCTTCAGGGTCCTCC-3′	1.0	58

Table 2 Participant characteristics

Demographic	CP (n = 219)	RAP (n = 115)	Controls (n = 305)
Age, mean (SD)	45.3 (18.1)	46.1 (16.2)	54.7 (14.5)
Race, % White	91	91	94
Sex (M/F)	125/94	49/66	121/184
Alcohol drinking pattern (%)			
Abstainers	45 (22.5)	30 (27)	72 (25)
Light	38 (19)	26 (23)	83 (28)
Moderate	34 (17)	22 (20)	56 (19)
Heavy	39 (19.5)	20 (18)	58 (20)
Very heavy	44 (22)	13 (12)	23 (8)

CP: Chronic pancreatitis; RAP: Recurrent acute pancreatitis; SD: Standard deviation. Abstainers: No alcohol use or < 20 drinks in lifetime; Light: < 3 drinks/week; Moderate: 4-7 drinks/week for females, 4-14 drinks/week for males; Heavy; 8-34 drinks/week for females, 15-34 drinks/week for males; Very heavy: > 35 drinks/week.

an ABI Prism 3730 Genetic Analyzer and sequence data were analyzed using Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI)[5,21].

Statistical analysis

Genotype frequencies were assessed for Hardy-Weinberg equilibrium. The frequencies of genotypes among cases and controls were compared using Chi square test or the Fisher's exact test when appropriate. Odds ratio (OR) and 95% confidence intervals (95% CI) for genotypes were calculated using an autosomal dominant model. For all statistical comparisons, $P < 0.05$ was considered significant.

RESULTS

Subject demographics and alcohol drinking patterns are given in Table 2. The proportion of subjects reporting a moderate or heavy alcohol drinking pattern was similar between patients and controls. Of the 334 patients with pancreatitis, 219 (66%) had CP and 115 (34%) had RAP.

The initial study consisted of 115 patients (CP = 82 and RAP = 33) and 66 controls, of which 57 patients (CP = 47 and RAP = 10) and 21 controls carried the *SPINK1* N34S high risk haplotype. Of the 58 patients without *SPINK1* N34S, 35 were diagnosed with CP and 23 had RAP.

The genotype frequencies were found to be in Hardy-Weinberg equilibrium. The R990G polymorphism (AGG→ GGG transition) in exon 7 of the *CASR* gene, the G allele was more common among CP patients (n = 35) than controls (n = 45), but only in subjects without *SPINK1* N34S. In comparing CP patients (n = 47) and controls (n = 21) with *SPINK1* N34S, there was a non-significant trend towards an increased occurrence of the G allele in patients (OR, 4.03; 95% CI, 0.48-190.8, P = 0.255). One limitation of this study was the small number of *SPINK1* N34S subjects for comparison; Therefore, caution must be exercised before this association is either accepted or rejected.

From the 112 mutations reported previously, the following three mutations--E191E, Y440C and A746A were each observed once in CP patients with *SPINK1* N34S. Another mutation, P748P, was identified in two CP patients without *SPINK1* N34S. Recently identified novel *CASR* mutations from Germany and India seen in exons 3 (P163R), 4 (L173P, F391F, I425S, D433H), 5 (V477A) and 7 (E870E, R896E)[8-10] were not observed in either patients or controls. Two intronic polymorphisms 493-94 C>T and 493-134 T>C included in exon 4 amplicon occurred with similar frequency in CP patients and controls, both with and without *SPINK1* N34S polymorphisms.

Secondarily, 219 patients (137 CP and 82 RAP) and 239 controls from the NAPS2 study who did not carry *SPINK1* N34S were analyzed to test the association of *CASR* A990G and CP. This ancillary analysis confirmed that the R990G was significantly associated with CP, as shown in Table 3 (OR, 2.01; 95% CI, 1.12-3.59; P = 0.015). The frequencies of R990G among RAP patients and controls, with and without *SPINK1* N34S were similar. There was

Table 3 Genotype analysis of *CASR* R990G polymorphism in patients and controls

	Patients	Controls	P^1	OR (95% CI)
CP patients *vs* controls without *SPINK1* N34S (%)				
AA	140 (82)	255 (90)		
AG	31 (18)	28 (10)		
GG	1 (1)	1 (0.5)		
AA *vs* AG/GG			0.015	2.01 (1.12-3.59)
RAP patients *vs* controls without *SPINK1* N34S (%)				
AA	93 (89)	255 (90)		
AG	10 (9)	28 (10)		
GG	2 (2)	1 (0.5)		
AA *vs* AG/GG			0.712	1.28 (0.67-2.47)
SPINK1 N34S positive CP patients *vs* controls (%)				
AA	39 (83)	20 (95)		
AG	8 (17)	1 (5)		
GG	0	0		
AA *vs* AG/GG			0.255	4.1 (0.48-35.14)

CP: Chronic pancreatitis; RAP: Recurrent acute pancreatitis. *SPINK1*: Serine protease Kazal type 1 gene. [1]Fisher exact test.

Table 4 *CASR* genotype comparison for R990G polymorphism in CP patients and controls with similar alcohol drinking patterns

	Patients	Controls	P^1	OR (95% CI)
A/L Alcohol CP patients *vs* controls (%)				
AA	69 (83)	136 (88)		
AG	13 (16)	18 (11)		
GG	1 (1)	1 (1)		
AA *vs* AG/GG			0.332	1.45 (0.69-3.07)
M/H Alcohol CP patients *vs* controls (%)				
AA	59 (81)	106 (93)		
AG	14 (19)	8 (7)		
GG	0	0		
AA *vs* AG/GG			0.018	3.12 (1.14-9.13)
VH Alcohol CP patients *vs* controls (%)				
AA	37 (84)	21 (91)		
AG	7 (16)	2 (9)		
GG	0	0		
AA *vs* AG/GG			0.708	1.99 (0.38-0.45)

A: Abstainer; L: Light; M: Moderate; H: Heavy; VH: Very heavy. Alcohol categories: Abstainers: No alcohol use or < 20 drinks in lifetime; Light drinkers: < 3 drinks/week; Moderate drinkers: 4-7 drinks/week for females, 4-14 drinks/week for males; Heavy drinkers: 8-34 drinks/week for females, 15-34 drinks/week for males; Very heavy drinkers: > 35 drinks/week. [1]Fisher's exact test.

no difference in A986S and Q1011E polymorphisms among RAP and CP patients, and controls.

To determine if the risk was modified with alcohol use we compared *CASR* R990G genotypes in subjects with moderate and heavy alcohol drinking pattern. CP was strongly associated with the *CASR* R990G in moderate and heavy alcohol drinkers, as is demonstrated in Table 4 (OR, 3.12; 95% CI, 1.14-9.13; $P = 0.018$). No association was observed with this particular polymorphism in abstainers or in subjects with self-reported light or very heavy alcohol drinking patterns.

DISCUSSION

In the past, CP was commonly attributed to heavy alcohol consumption. More recent studies, however, suggest there is also a strong genetic basis for this illness[22]. Growing knowledge of complex gene-environment interactions has provided fundamental insight into the pathophysiological mechanisms that result in fibrotic destruction of the pancreas[11,23-25]. Studies from Germany and India have recently identified 8 novel *CASR* mutations that were associated with *SPINK1* N34S in idiopathic and tropical CP subjects. Our study did not detect these novel *CASR* mutations. However, we were able to demonstrated and verify that *CASR* R990G confers significant risk for developing of CP especially when linked to moderate and heavy alcohol consumption.

Three common nonsynonymous SNPs are located in the region coding the intracellular tail of *CASR*[26] and play an important role in cellular signal transduction that alters serum ionized calcium level[27,28]. Previously, it was reported that individuals carrying the 990 variant G allele may experience very mild decrease in serum ionized calcium levels from 4.92 mg/dL to 4.84 mg/dL[28]. Although serum ionized calcium levels

alter the cytosolic calcium ion concentrations in acinar cells in a concentration-dependent manner, and may alter the risk of acute pancreatitis[29,30], the *CASR* R990G allele associated with increased risk of CP should slightly reduce the risk of acute pancreatitis. Furthermore, the magnitude of change in serum calcium levels due to *CASR* R990G alone is small, and it is difficult to imagine that this small change would, by itself, significantly alter the risk of acute pancreatitis. Indeed, our data suggests that *CASR* R990G is associated with CP rather than RAP. Our speculation is that *CASR* R990G might induce direct changes in the acinar and ductal cells that increase the risk for CP. However, the mechanism remains unknown.

Interestingly, while 55%-80% of pancreatitis cases may be attributed to alcohol abuse, less than 5% of heavy alcohol users develop pancreatitis[31]. Alcohol abuse may not be the sole risk for the development of CP[32]; rather alcoholic CP is likely the result of an interaction of several co-factors[2]. It has been demonstrated that chronic alcohol consumption accelerates fibrosis in response to cerulein-induced CP in rats[33]. Alcohol metabolites in pancreatic acinar cells induce persistent cytosolic Ca^{2+} signals in a concentration-dependent manner and depolarize mitochondria.

The discovery and characterization of a genetic cause of hereditary pancreatitis generated renewed interest in a possible genetic predisposition to alcoholic CP[34]. Several CP-related gene mutations have been described previously with *CFTR*, *PRSS1*, *SPINK1* and others[35]. Our study also demonstrates the association of *CASR* R990G with CP, especially with moderate and heavy alcohol consumption. The presence of *CASR* R990G alone doubled the risk of developing CP, while in those individuals reporting moderate and heavy alcohol consumption, the risk was increased by 3-fold. Our hypothesis for testing *CASR* R990G in subjects with moderate and heavy drinking patterns is that this group represented a "threshold" alcohol pancreatitis risk group in which the addition of another risk factor would increase the overall risk of developing CP. The risk of CP in subjects with *CASR* R990G but with minimal or no alcohol consumption would be lower, while very heavy drinkers would be at high risk, regardless of the *CASR* genotype. Our experimental findings support this hypothesis.

The novel *CASR* gene mutations that were identified in German and Indian populations appeared to be closely associated with the *SPINK1* N34S haplotype. We did not detect these, or other novel *CASR* mutations, and our study was not powered to demonstrate an interaction between *SPINK1* N34S and *CASR* R990G. On the other hand, it was not clear whether or not the German and Indian studies tested for an effect of alcohol in a "threshold" dose range. However, both studies suggest that the overall effect of *CASR* polymorphisms are relatively small, and become clinically significant in the presence of additional risk factors in an additive or multiplicative way. This is consistent with current concepts that CP is a complex syndrome.

The present study confirmed the association of *CASR* genetic variants with CP. Our genotyping results in a US population were different from those reported from Germany and India. *CASR* R990G significantly increased the risk of developing CP and this effect was enhanced in subjects who consumed alcohol in a moderate to heavy dose range. Certain polymorphisms in the *CASR* gene may be considered risk factors for the development of CP, especially within the context of alcohol consumption. The relationship with *SPINK1* mutations warrants further study.

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COMMENTS

Background

Chronic pancreatitis is a highly morbid, complex disease whose development depends on the combination of genetic and environmental factors. Elucidating the genetic links to this illness is critical in diagnosis, treatment and risk assessment.

Research frontiers

This study adds another gene to the growing number of genetic and other factors that confers increased risk of chronic pancreatitis. As new factors continue to be identified and confirmed, the emphasis will turn to integrating these risks, using systems approaches, as described in reference #1.

Innovations and breakthroughs

This study is one of the first to consider the complexity of gene-environment and gene-gene interactive paradigms by evaluating alcohol consumption and *serine protease inhibitor Kazal 1type (SPINK1)* N34S variants with *calcium sensing receptor (CASR)* polymorphisms. The confirmation of *CASR* genetic variants as risk factors for chronic pancreatitis strengthens the importance of dysfunctional calcium regulating genes in the etiology of pancreatitis.

Application

With the inclusion of associated *CASR* polymorphisms in comprehensive evaluation of selected patients, we may improve the accuracy of overall pancreatitis risk prediction and may be able to provide a target for preventive approaches and possible treatment options.

Peer review

Our peer reviewers noted this brief manuscript to be well-developed and well-written. They felt that the abstract was clear and the hypothesis being tested and methodology were sound and well presented.

REFERENCES

1 **Aoun E**, Chang CC, Greer JB, Papachristou GI, Barmada MM, Whitcomb DC. Pathways to injury in chronic pancreatitis: decoding the role of the high-risk SPINK1 N34S haplotype

using meta-analysis. *PLoS ONE* 2008; **3**: e2003
2 **Etemad B**, Whitcomb DC. Chronic pancreatitis: diagnosis, classification, and new genetic developments. *Gastroenterology* 2001; **120**: 682-707
3 **Ahmed SA**, Wray C, Rilo HL, Choe KA, Gelrud A, Howington JA, Lowy AM, Matthews JB. Chronic pancreatitis: recent advances and ongoing challenges. *Curr Probl Surg* 2006; **43**: 127-238
4 **Ward JB**, Petersen OH, Jenkins SA, Sutton R. Is an elevated concentration of acinar cytosolic free ionised calcium the trigger for acute pancreatitis? *Lancet* 1995; **346**: 1016-1019
5 **Whitcomb DC**, Gorry MC, Preston RA, Furey W, Sossenheimer MJ, Ulrich CD, Martin SP, Gates LK Jr, Amann ST, Toskes PP, Liddle R, McGrath K, Uomo G, Post JC, Ehrlich GD. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nat Genet* 1996; **14**: 141-145
6 **Gorry MC**, Gabbaizedeh D, Furey W, Gates LK Jr, Preston RA, Aston CE, Zhang Y, Ulrich C, Ehrlich GD, Whitcomb DC. Mutations in the cationic trypsinogen gene are associated with recurrent acute and chronic pancreatitis. *Gastroenterology* 1997; **113**: 1063-1068
7 **Aoun E**, Slivka A, Papachristou DJ, Gleeson FC, Whitcomb DC, Papachristou GI. Rapid evolution from the first episode of acute pancreatitis to chronic pancreatitis in human subjects. *JOP* 2007; **8**: 573-578
8 **Felderbauer P**, Hoffmann P, Klein W, Bulut K, Ansorge N, Epplen JT, Schmitz F, Schmidt WE. Identification of a novel calcium-sensing receptor gene mutation causing familial hypocalciuric hypercalcemia by single-strand conformation polymorphism analysis. *Exp Clin Endocrinol Diabetes* 2005; **113**: 31-34
9 **Felderbauer P**, Klein W, Bulut K, Ansorge N, Dekomien G, Werner I, Epplen JT, Schmitz F, Schmidt WE. Mutations in the calcium-sensing receptor: a new genetic risk factor for chronic pancreatitis? *Scand J Gastroenterol* 2006; **41**: 343-348
10 **Murugaian EE**, Premkumar RM, Radhakrishnan L, Vallath B. Novel mutations in the calcium sensing receptor gene in tropical chronic pancreatitis in India. *Scand J Gastroenterol* 2008; **43:** 117-121
11 **Muller-Pillasch F**, Menke A, Yamaguchi H, Elsasser HP, Bachem M, Adler G, Gress TM. TGFbeta and the extracellular matrix in pancreatitis. *Hepatogastroenterology* 1999; **46**: 2751-2756
12 **Whitcomb DC**. How to think about SPINK and pancreatitis. *Am J Gastroenterol* 2002; **97**: 1085-1088
13 **Brown EM**, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J, Hebert SC. Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature* 1993; **366**: 575-580
14 **Bruce JI**, Yang X, Ferguson CJ, Elliott AC, Steward MC, Case RM, Riccardi D. Molecular and functional identification of a Ca2+ (polyvalent cation)-sensing receptor in rat pancreas. *J Biol Chem* 1999; **274**: 20561-20568
15 **Janicic N**, Soliman E, Pausova Z, Seldin MF, Riviere M, Szpirer J, Szpirer C, Hendy GN. Mapping of the calcium-sensing receptor gene (CASR) to human chromosome 3q13.3-21 by fluorescence in situ hybridization, and localization to rat chromosome 11 and mouse chromosome 16. *Mamm Genome* 1995; **6**: 798-801
16 **Aida K**, Koishi S, Tawata M, Onaya T. Molecular cloning of a putative Ca(2+)-sensing receptor cDNA from human kidney. *Biochem Biophys Res Commun* 1995; **214**: 524-529
17 **Pidasheva S**, D'Souza-Li L, Canaff L, Cole DE, Hendy GN. CASRdb: calcium-sensing receptor locus-specific database for mutations causing familial (benign) hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism, and autosomal dominant hypocalcemia. *Hum Mutat* 2004; **24**: 107-111
18 **Brown EM**, Pollak M, Hebert SC. The extracellular calcium-sensing receptor: its role in health and disease. *Annu Rev Med* 1998; **49**: 15-29
19 **Hendy GN**, D'Souza-Li L, Yang B, Canaff L, Cole DE. Mutations of the calcium-sensing receptor (CASR) in familial hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism, and autosomal dominant hypocalcemia. *Hum Mutat* 2000; **16**: 281-296
20 **Whitcomb DC**, Yadav D, Slivka Aea. Multicenter approach to recurrent acute and chronic pancreatitis in the United States: the North American Pancreatitis Study 2 (NAPS2). *Pancreatology* 2008; **8:** 520-531
21 **Pfutzer RH**, Barmada MM, Brunskill AP, Finch R, Hart PS, Neoptolemos J, Furey WF, Whitcomb DC. SPINK1/PSTI polymorphisms act as disease modifiers in familial and idiopathic chronic pancreatitis. *Gastroenterology* 2000; **119**: 615-623
22 **Witt H**. Gene mutations in children with chronic pancreatitis. *Pancreatology* 2001; **1**: 432-438
23 **Van Laethem JL**, Robberecht P, Resibois A, Deviere J. Transforming growth factor beta promotes development of fibrosis after repeated courses of acute pancreatitis in mice. *Gastroenterology* 1996; **110**: 576-582
24 **Bachem MG**, Schneider E, Gross H, Weidenbach H, Schmid RM, Menke A, Siech M, Beger H, Grunert A, Adler G. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology* 1998; **115**: 421-432
25 **Apte MV**, Wilson JS. Stellate cell activation in alcoholic pancreatitis. *Pancreas* 2003; **27**: 316-320
26 **Heath H 3rd**, Odelberg S, Jackson CE, Teh BT, Hayward N, Larsson C, Buist NR, Krapcho KJ, Hung BC, Capuano IV, Garrett JE, Leppert MF. Clustered inactivating mutations and benign polymorphisms of the calcium receptor gene in familial benign hypocalciuric hypercalcemia suggest receptor functional domains. *J Clin Endocrinol Metab* 1996; **81**: 1312-1317
27 **Cole DE**, Peltekova VD, Rubin LA, Hawker GA, Vieth R, Liew CC, Hwang DM, Evrovski J, Hendy GN. A986S polymorphism of the calcium-sensing receptor and circulating calcium concentrations. *Lancet* 1999; **353**: 112-115
28 **Scillitani A**, Guarnieri V, De Geronimo S, Muscarella LA, Battista C, D'Agruma L, Bertoldo F, Florio C, Minisola S, Hendy GN, Cole DE. Blood ionized calcium is associated with clustered polymorphisms in the carboxyl-terminal tail of the calcium-sensing receptor. *J Clin Endocrinol Metab* 2004; **89**: 5634-5638
29 **Krims PE**, Pandol SJ. Free cytosolic calcium and secretagogue-stimulated initial pancreatic exocrine secretion. *Pancreas* 1988; **3**: 383-390
30 **Sutton R**, Criddle D, Raraty MG, Tepikin A, Neoptolemos JP, Petersen OH. Signal transduction, calcium and acute pancreatitis. *Pancreatology* 2003; **3**: 497-505
31 **Dani R**, Penna FJ, Nogueira CE. Etiology of chronic calcifying pancreatitis in Brazil: a report of 329 consecutive cases. *Int J Pancreatol* 1986; **1**: 399-406
32 **Lankisch PG**, Lowenfels AB, Maisonneuve P. What is the risk of alcoholic pancreatitis in heavy drinkers? *Pancreas* 2002; **25**: 411-412
33 **Deng X**, Wang L, Elm MS, Gabazadeh D, Diorio GJ, Eagon PK, Whitcomb DC. Chronic alcohol consumption accelerates fibrosis in response to cerulein-induced pancreatitis in rats. *Am J Pathol* 2005; **166**: 93-106
34 **Hanck C**, Schneider A, Whitcomb DC. Genetic polymorphisms in alcoholic pancreatitis. *Best Pract Res Clin Gastroenterol* 2003; **17**: 613-623
35 **Schneider A**, Pfutzer R, Whitcomb DC. Genetics and pancreatic disease. *Curr Opin Gastroenterol* 2002; **18**: 545-551

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RAPID COMMUNICATION

Folic acid supplementation inhibits recurrence of colorectal adenomas: A randomized chemoprevention trial

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Abstract

AIM: To determine whether folic acid supplementation will reduce the recurrence of colorectal adenomas, the precursors of colorectal cancer, we performed a double-blind placebo-controlled trial in patients with adenomatous polyps.

METHODS: In the current double-blind, placebo-controlled trial at this VA Medical Center, patients with colorectal adenomas were randomly assigned to receive either a daily 5 mg dose of folic acid or a matched identical placebo for 3 years. All polyps were removed at baseline colonoscopy and each patient had a follow up colonoscopy at 3 years. The primary endpoint was a reduction in the number of recurrent adenomas at 3 years.

RESULTS: Of 137 subjects, who were eligible after confirmation of polyp histology and run-in period to conform compliance, 94 completed the study; 49 in folic acid group and 45 in placebo group. Recurrence of adenomas at 3-year was compared between the two groups. The mean number of recurrent polyps at 3-year was 0.36 (SD, 0.69) for folic acid treated patients compared to 0.82 (SD, 1.17) for placebo treated subjects, resulting in a 3-fold increase in polyp recurrence in the placebo group. Patients below 70 years of age and those with left-sided colonic adenomas or advanced adenomas responded better to folic acid supplementation.

CONCLUSION: High dose folic acid supplementation is associated with a significant reduction in the recurrence of colonic adenomas suggesting that folic acid may be an effective chemopreventive agent for colorectal neoplasia.

Key words: Folic acid; Adenoma; Colorectal cancer

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INTRODUCTION

Colorectal cancer is the second most common cancer in the United States[1]. Although the etiology of this disease is related to genetic susceptibility, dietary factors such as vitamins and micronutrients are thought to influence carcinogenesis[2]. Considerable interest has recently been focused on the water soluble vitamin folic acid. Although the specific mechanism(s) by which folate deficiency enhances colorectal carcinogenesis have not been fully elucidated, it has been hypothesized that aberrations in DNA methylation may contribute to abnormalities in DNA synthesis and genomic instability[3].

Several clinical trials have noted an inverse relationship between dietary folic acid and the development of colorectal cancer[4-7]. A folate deficient diet is thought to increase the risk of colonic neoplasia[8-11], whereas supplementation of this nutrient may be chemopreventive[12-15]. However, the timing of folate supplementation may be particularly important since folate intervention after the establishment of microscopic neoplastic foci in the colorectal mucosa may promote

rather than suppress colorectal carcinogenesis[16].

Accumulating data from murine studies have also supported a role for folic acid in the prevention of colon carcinogenesis. Folate deficient rats demonstrate an increased susceptibility to dimethylhydrazine induced colonic neoplasia as compared to folate replete animals[10]. In a similar model, folate supplementation protected against the development of colonic neoplastic lesions in a dose dependent manner[17]. We have previously demonstrated that folic acid supplementation can reduce the age-related susceptibility of murine colorectal mucosa to a colonic carcinogen[18]. In the azoxymethane-induced colon cancer rat model, supplemental folic acid has also been shown to decrease the formation of aberrant crypt foci, which are considered to be precursors of colorectal adenomas and carcinoma[19,20]. Additionally, *in vitro* studies have further demonstrated that supplemental folic acid greatly inhibits proliferation of colon cancer cells[21,22]. Although these studies suggest a chemopreventive role for folic acid in colorectal cancer, to the best of our knowledge, no conclusive long-term clinical trials have been performed to evaluate the efficacy of folic acid in preventing the recurrence of colorectal adenomas. The current 3-year placebo-controlled clinical trial was, therefore, undertaken to test the hypothesis that folic acid will inhibit the recurrence of colorectal adenomas.

MATERIALS AND METHODS

Objectives

The primary objective of this chemopreventive trial is to determine if supplementation of folic acid for 3 years will inhibit the recurrence of colorectal adenomas. The study was initiated in December, 1998 with a 2-year patient accrual followed by a 3-year treatment with folic acid (5 mg/d) or placebo. The study was completed in June, 2005. The study protocol was approved by the Human Investigation Committee of Wayne State University. All subjects provided written informed consent.

Study subjects and treatment

Eligible subjects were male or female, from the age of 18-80 years. However, the youngest subject enrolled in this clinical trial was 44 years of age. All subjects underwent a colonoscopy for colon polyps noted on screening flexible sigmoidoscopy or as routine surveillance for a history of colon polyps at the Detroit VA Medical Center. Prior to colonoscopy, potential subjects agreed in advance to participate if they were found to have at least one adenoma (tubular, tubulovillous, villous) > 0.5 cm, and had no exclusionary factors including hyperplastic histology of the index polyp. The histology of all polyps was examined by a pathologist blinded to the sample coding.

At study entry, all patients completed a lifestyle questionnaire. Nutritional assessment was evaluated by a registered dietitian using a Block Dietary Data System for California, Berkley. Nutrient intakes were computed according to the composition values from the U.S. Department of Agriculture[23], supplemented with other sources[24].

Eligible participants underwent a complete colonoscopy and had all adenomas removed at colonoscopy (with at least one adenoma > 0.5 cm). They were then randomized in a double-blind trial to receive either a 5 mg folic acid tablet (Stanley Pharmaceutical, Toronto, Canada) or one identical placebo tablet (sucrose/fructose base) daily per oral with breakfast for 3 years. Compliance was monitored by both pill count and telephone contact. Patients were seen or contacted by telephone every 90 d by the study coordinator to obtain pill counts, assess adverse events and to renew a 90 d supply of study medication. Patients were required to take $\geqslant$ 90% of their prescribed study treatment. At the end of 3 years, a repeat colonoscopy was performed, and all identified polyps were removed endoscopically. Serum and RBC folate concentrations were monitored at baseline and every 6 mo. During the course of the trial all adverse events including deaths were reported to the Institutional Review Board (IRB).

Choice of folic acid dose

A 5 mg dose of folic acid was chosen on the basis of the previous observations that diets high in folate protect against the development of colorectal neoplasia. Although lower doses of folic acid (0.4-1 mg) resulted in a reduced relative risk of neoplasia, the risk reduction did not achieve statistical significance[12,14]. Kim *et al* noted a significant increase in colonic mucosal and systemic folate concentrations in patients who were treated for 1 year with 5 mg folic acid[25]. Folate supplementation, even at a dose of 15 mg/d, has been rarely associated with gastrointestinal or CNS adverse effects[26]. In addition, the high prevalence of dietary supplementation of folic acid (up to 1 mg/d) in the general population would have been a confounding variable.

Exclusion criteria

Subjects were excluded if they had any of the following criteria: severe co-morbid conditions, such as severe heart disease, cancer, or other diseases causing organ dysfunction or contraindications for colonoscopy and polypectomy. Subjects with gastrointestinal disorders that affect absorption or metabolism of folic acid, B12 deficiency, and hereditary predisposition to colorectal cancer were excluded. In addition, pregnant or nursing mothers were excluded. Sexually active females agreed to use an effective method of birth control. Patients who drank more than 2 alcoholic drinks daily or who were regularly ingesting or anticipating chronic therapy with vitamin, mineral or any other nutritional supplement, steroids and non-steroidal anti-inflammatory drugs (excluding cardiopreventive aspirin doses), antineoplastic agents or folate were also excluded. Patients were asked if they had a family history of familial colorectal cancer syndrome. This question was asked to exclude obvious known history of FAP or HNPCC.

Placebo run-in

Subjects were supplied with a known number of placebo

Table 1 Baseline characteristics of the subjects

Characteristics	Folate group (n = 80)	Placebo group (n = 97)	P
Age (yr)	60.36 ± 10.34	62.64 ± 9.59	NS
Sex (male, %)	93	92	NS
Race			
African American	48%	50%	NS
Caucasian	51%	49%	
Other	1%	1%	
BMI (kg/m^2)	31.62 ± 4.68	29.84 ± 5.71	NS
Dietary intake			
Total calories	2069.58 ± 902.9	1823.53 ± 741.12	NS
Protein (g/d)	79.57 ± 30.07	74.31 ± 36.33	NS
Fat (g/d)	88.89 ± 52.04	75.2 ± 38.38	NS
Carbohydrate (g/d)	237.29 ± 129.32	206.48 ± 86.37	NS
Fiber (g/d)	7.28 ± 5.84	8.51 ± 7.93	NS
Folate (μg/d)	184.45 ± 231.7	162.64 ± 140.23	NS
Calcium (mg/d)	577.14 ± 433.68	569.69 ± 353.75	NS
Aspirin users (≤ 325 mg/d)	24%	24%	NS
Number with advanced polyp (%)	59	53	NS
Adenomas per patient	2.34 ± 1.46	2.06 ± 1.38	NS
Total polyps per patient including hyperplastic polyps	2.88 ± 1.73	2.87 ± 2.21	NS
Current smokers	16 (35%)	19 (39%)	NS
Serum folic acid (ng/mL)	14.53 ± 19.51	11.35 ± 6.65	NS
RBC folate (ng/mL)	446.57 ± 164.81	477.82 ± 148.76	NS
Serum vit B12 (pg/mL)	472.97 ± 456.10	393.02 ± 190.93	NS
Serum calcium (mg/mL)	9.31 ± 0.48	9.33 ± 0.37	NS

NS: Not significant. Advance adenoma: ≥ 2 adenomas, large (> 1 cm) or adenoma with villous component or high grade dysplasia. Number of patients in placebo and folate group represents those who completed the baseline colonoscopy and satisfied the criteria for enrollment. Ninety-four subjects completed the 3-year study.

tablets to be taken daily during breakfast for 4 wk. Those who had taken ≥ 90% of their tablets were randomized.

Randomization and stratification

Participants were randomized to the folic acid or placebo group using a stratified randomization block scheme. There were 3 stratification factors: number of adenomas (1, 2-5 and ≥ 6), size of the largest adenoma (≤ 1 cm, >1 cm) and history of polyps (no, yes). Block randomization was used in a block size of 8 to ensure that at no time during the study would there be a large imbalance between the intervention and control groups. Subject assignment was made in advance and recorded in sealed envelops, numbered consecutively.

Statistical analysis

The statistical analyses were all performed using the Statistical Package for Social Sciences (SPSS, version 8.0; 1997, Chicago, IL). All t-tests were two sided. Initially, the two treatment groups were compared across demographic information using independent t-tests for continuous data and Chi-Square analyses for categorical information. Treatment efficacy was assessed between intervention groups using independent t-tests across classifications of polyp morphology, lateralization, and age grouping. Logistic regression was utilized to assess the incidence of recurring polyps three years post-removal for individuals taking folic acid versus those taking placebo. A contingency table was computed *via* Chi-Square analysis, and Odds Ratios were computed *via* logistic regression analysis.

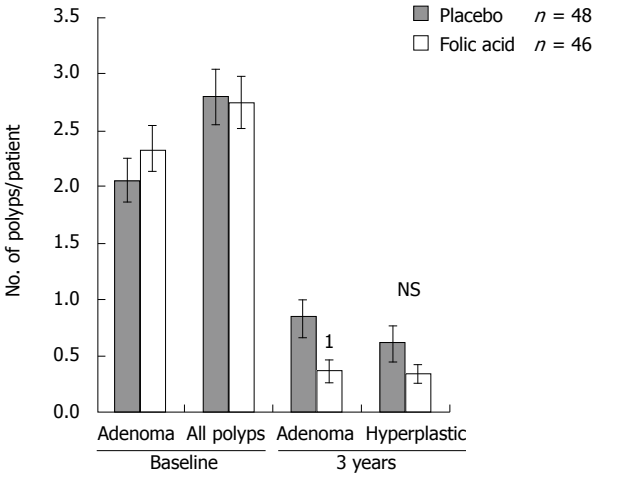

Figure 1 Number of adenomas *versus* treatment. Histograms showing the number of adenomas or all types of polyps in folic acid and placebo-treated groups at baseline and 3 years after treatment. [1]P = 0.02514, compared to the placebo-treated group. Each histogram represents the mean ± SD.

RESULTS

One hundred and thirty seven patients fulfilled the eligibility criteria. Ninety four completed the 3-year follow up colonoscopy and were included in this analysis. There were 43 subjects that dropped out from this study; of which 28 died from various causes unrelated to colon cancer and 15 subjects had geographic relocation precluding further participation. Of those who did not complete the study, there were no statistically significant differences (age, BMI, sex, NSAID/multivitamin, baseline adenoma, RBC folate, deaths) between those assigned to receive folic acid or placebo. Forty nine of the subjects who completed the 3-year follow-up received supplemental folic acid and 45 were given placebo tablets. At post-randomization, there was no statistical difference in the serum levels of folic acid between the two groups (Table 1). Demographic data and other baseline parameters were also comparable between these two groups (Table 1). At the 3-year follow-up colonoscopy, patients in the folic acid group showed a significantly lower number of adenomas per patient (0.36 ± 0.69) with a 64% lower risk ratio, compared to the placebo group (0.82 ± 1.17; odds ratio, 2.77; t = -2.26, P = 0.02514, 95% CI, 0.06-0.84; Chi Square = 11.2, P = 0.00142; Figure 1). The recurrence of adenoma at the 3-year follow-up was twice as high in the placebo group, compared to the folic acid group. There was no significant difference in the recurrence of hyperplastic polyps between the groups (folic acid: 0.44 ± 0.89, placebo: 0.51 ± 0.94; P = 0.74; 95% CI, 0.31-0.43).

Folic acid supplementation caused a significant reduction (P = 0.02335) in the recurrence of adenomas in patients with advanced adenoma [large (> 1 cm)

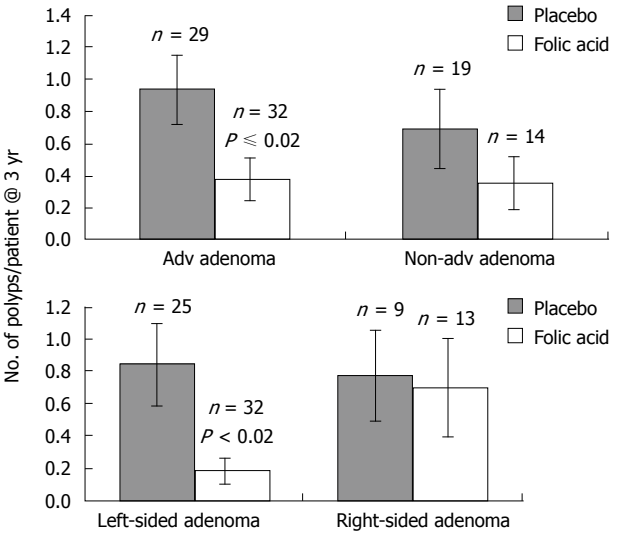

Figure 2 Polyp characteristics and response to treatment. Recurrence of advanced [large (> 1 cm) and polyps with villous component] or non-advanced adenomas (upper panel) as well as right or left-sided adenomas (lower panel) following 3 years of treatment with a high dose of folic acid. The numbers of subjects as well as the levels of significance between the two groups are shown.

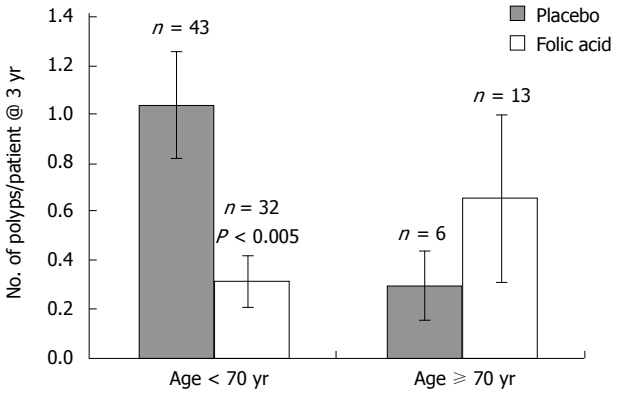

Figure 3 The effect of age on the response to treatment. Recurrence of adenomas in response to 3 years of folic acid treatment in patients over or below 70 years of age in shown. The number of subjects in each group as well as the levels of significance is shown in the figure.

adenoma or adenoma with villous component or high grade dysplasia], compared to the placebo-treated controls (Figure 2, upper panel). Those with non-advanced adenomas also showed a reduction in the recurrence of adenomas with folic acid, compared to placebo controls, but this was not statistically significant (Figure 2, upper panel). On further stratifications, it was noted that subjects with left-sided polyps had a significantly lower ($P = 0.01964$) recurrence of adenomas than those with right-sided polyps in response to folic acid supplementation, when compared with the corresponding placebo-treated controls (Figure 2, lower panel).

Since colorectal cancer is an age-related disease, the data were analyzed to determine the age-related differences in responsiveness to folic acid. We observed that the younger subjects responded better than older subjects in that the recurrence of adenomas was significantly lower ($P = 0.00496$) in younger patients, compared to older patients (Figure 3). This response was maintained until 70 years of age (Figure 3). However, patients older than 70 years of age failed to respond to folic acid supplementation demonstrating a higher recurrence rate of polyps as compared to the placebo group. This difference was not statistically significant (Figure 3). There were more deaths in the folic acid group, compared to the placebo-treated group, but this difference was not statistically significant (19 in folic acid *vs* 9 in placebo, $P > 0.1$).

DISCUSSION

Despite recent advances in medicine, the mortality from colorectal cancer, a leading cause of death in the USA and other Western countries, still remains unacceptably high. Therefore, the search for strategies to prevent the development and progression of colorectal cancer has greatly intensified. Chemoprevention offers a viable option to block neoplastic inception or delay disease progression. Since colorectal cancer is an age-related disease, typically diagnosed after the age of 50, any delay in the onset and subsequent progression of this disease through the use of dietary agents is likely to have significant health benefits. Folic acid has recently emerged as a major contender in the repertoire of promising colorectal cancer prevention agents. A number of animal, as well as a few case controlled human studies, strongly support folic acid as a potentially efficacious chemopreventive agent with a negligible toxicity profile[3]. However, there have been no systematic conclusive studies to examine the effect of supplemental folic acid on recurrence of adenomas in the colon.

Our data, for the first time, show that the daily consumption of a high dose of folic acid over a period of 3 years prevents the recurrence of colorectal adenomas. This reduction could not be attributed to differences in diet or lifestyle. The patients completed a detailed lifestyle questionnaire and nutritional assessment with both study groups demonstrating statistically similar caloric, fiber, fat and protein intake as well as similar baseline BMI, folate, B12 and calcium status. Additionally, the groups were similar with regard to aspirin use and the number and type of adenoma at baseline. Most patients were male which is consistent with the Veterans Affairs based population. Interestingly, patients who had large adenomas or adenomas with a villous component (referred to as advanced adenomas) responded better to high dose folate supplementation, as evidenced by the significantly reduced number of recurrent adenomatous polyps. A similar phenomenon was also observed among patients with left-sided adenomas and those who were less than 70 years of age. Although the reasons for this are not fully understood, it is plausible that the increased responsiveness of these subjects could be a result of greater tissue accumulation of folic acid due to a better active folate transport system. The basis for this inference comes from the observations by Mennan *et al* which suggest that mucosal folate levels may be a determinant factor

in the development of adenomas[27]. They demonstrated that the levels of folate in adenoma, carcinoma as well as normal appearing adjacent mucosa are lower than the corresponding polyp-free controls[27]. Future studies analyzing folate levels in adjacent tissue near recurrent adenomas need to be completed.

Although several clinical trials have suggested a role for folic acid in the prevention of colorectal adenomas, there are no prospective controlled trials addressing this issue at the dose of 5 mg[5-8]. It has also been demonstrated that supplementation of a high dose of folic acid in animals with colonic neoplasia may accelerate the progression of carcinogenesis[16]. A more recent human study showed that supplemental folic acid may not reduce the incidence of colorectal adenomas and in some cases may actually increase the risk[28]. Although the reasons for these controversial issues are not fully understood, one possibility could be attributed to the dual modulatory effect of folic acid on carcinogenesis. It has been demonstrated that the timing and the dose of folate intervention has a promoting effect on the progression of established neoplasms, while it could have a chemopreventive effect if given in premalignant conditions. Data from our clinical trial clearly supports a chemopreventive role of folic acid since supplementation of this vitamin for 3 years inhibits the recurrence of colonic adenomas. More importantly, none of the patients in the folate treatment group were found to have histologically aggressive adenomas or carcinoma at final endoscopy.

The mechanisms by which folic acid exerts its chemopreventive role in colorectal carcinogenesis are becoming increasingly understood. Since folic acid plays a key role in DNA methylation and cellular homeostasis, folate deficiency may result in a variety of cellular consequences including misincorporation of uracil for thymidine during DNA synthesis resulting in an increased spontaneous mutation as well as chromosomal abnormalities and errors in DNA synthesis[29-33]. The restoration of DNA methylation status in patients with colorectal neoplasms treated with supraphysiological doses of folic acid lends further support to the hypothesis. In a recent study, we examined the changes in mutational status of APC, DCC and p53 genes in macroscopically normal appearing rectal mucosa at baseline and after 1 year of treatment with either folic acid or placebo[34]. We have observed that folate supplementation prevented the loss of heterozygosis (LOH) of the DCC gene in 5 out of 5 patients who demonstrated baseline heterozygosis, whereas 2 out of 4 placebo treated patients with baseline heterozygosis demonstrated complete allelic loss. Mucosal protein levels of DCC were also reduced in 70% of placebo treated patients compared to only 10% of folate treated patients[34]. Cell culture studies have further demonstrated that supplemental folic acid and its metabolite 5-methyltetrahydrofolate (5-MTF) inhibit EGF-receptor (EGFR) promoter activity in colon cancer HCT-116 cells by enhancing methylation[35]. Since EGFR is known to play a critical role in the development and progression of a wide variety of epithelial cancers, including colorectal cancer[36,37], the inhibition of basal as well as serum-stimulated EGFR promoter activity by folic acid and 5-MTF suggests that these changes may partly contribute to specific inhibition of growth-related processes in colorectal neoplasia. Supplemental folic acid may also attenuate the downstream events of EGFR signal transduction pathways that are critically involved in modulating growth-related processes. We have observed that in polypectomized patients, supplemental folic acid for 1 year leads to a decreased nuclear translocation of β-catenin[38], which interacts with the T-cell factor 4 (TCF-4) transcription factor to induce expression of specific target genes, including cyclin D1, VEGF and c-myc, which promote cell growth and proliferation[39-42].

The dose of folic acid supplementation may be important when considering the differing effects of supplementation. This has been more explored in the cardiovascular literature in attempting to modulate homocysteine levels where the VISP study showed greater efficacy at higher doses in lowering homocysteine levels[43]. A cogent example of this was the recently published large scale study interventional study of over 1000 men and women who were randomized to receive either 1mg folic acid or placebo. The endpoints were similar to our study, but the 3 year follow up data were very different in that no effect was seen for the dose used[28]. Of interest, there was no effect of gender in that study which may have important implications for our study in terms of applicability to the general population. The timing of supplementation may also be important[44].

In summary, daily consumption of a high dose of folic acid over 3 years prevents the recurrence of colorectal adenomas. Patients below 70 years of age and those with left-sided colonic adenomas or advanced adenomas responded better to folic acid supplementation. We conclude that folic acid is an effective chemopreventive agent for colorectal adenomas, and more specifically for that category of adenomas which are believed to possess the highest risk of cancer progression.

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COMMENTS

Background

Colorectal cancer is one of the major causes of cancer related deaths. In the US and the other developed countries, 50% of the subjects diagnosed with colon cancer die. Therefore, there is a need to prevent the development and progression of colon cancer using chemopreventive agents. Water soluble vitamins, such as folic acid, have shown to have chemopreventive potential for colon cancer. Aim of this investigation was to determine whether folic acid supplementation will reduce the recurrence of colorectal polyps, the precursors of colorectal cancer, we performed a double-blind placebo-controlled trial in patients with polyps.

Research frontiers

Several clinical trials have noted an inverse relationship between dietary folic acid and the development of colorectal cancer. A folate deficient diet is thought to increase the risk of colonic neoplasia, whereas supplementation of this nutrient may be chemopreventive. However, the timing of folate supplementation may be particularly important since folate intervention, after the establishment of microscopic neoplastic foci in the colorectal mucosa, may promote rather than suppress colorectal carcinogenesis. A similar approach using aspirin and similar non-steroidal anti-inflammatory agents have shown promising activity in prevention of colon cancer after resection of colon polyps.

Innovations and breakthroughs

This is a large randomized, single institution, double-blind placebo controlled trial demonstrating the efficacy of folic acid in secondary chemoprevention of colorectal cancer. This is the only study examining high dose supplementation over a period of three years further establishing safety and efficacy of large dose of folic acid. It should also be noted that the present study is the only study of its kind specifically targeting the US veteran population.

Applications

Daily consumption of a high dose of folic acid over 3 years prevents the recurrence of colorectal adenomas. Particularly, patients below 70 years of age and those with left-sided colonic adenomas or advanced adenomas responded better to folic acid supplementation. We conclude that folic acid is an effective chemopreventive agent for colorectal adenomas, and more specifically for that category of adenomas which are believed to possess the highest risk of cancer progression.

Peer review

This is an important study which, for the first time, demonstrates that daily consumption of a high dose folic acid over a prolonged period of time leads to a significant reduction in the recurrence of colonic adenomas. The results suggest that folic acid may be an effective chemopreventive agent for colorectal neoplasia.

REFERENCES

1 **Bond JH**. Colorectal cancer update. Prevention, screening, treatment, and surveillance for high-risk groups. *Med Clin North Am* 2000; **84**: 1163-1182, viii

2 **Giovannucci E**, Stampfer MJ, Colditz GA, Rimm EB, Trichopoulos D, Rosner BA, Speizer FE, Willett WC. Folate, methionine, and alcohol intake and risk of colorectal adenoma. *J Natl Cancer Inst* 1993; **85**: 875-884

3 **Majumdar AP**, Kodali U, Jaszewski R. Chemopreventive role of folic acid in colorectal cancer. *Front Biosci* 2004; **9**: 2725-2732

4 **Lashner BA**, Heidenreich PA, Su GL, Kane SV, Hanauer SB. Effect of folate supplementation on the incidence of dysplasia and cancer in chronic ulcerative colitis. A case-control study. *Gastroenterology* 1989; **97**: 255-259

5 **Freudenheim JL**, Graham S, Marshall JR, Haughey BP, Cholewinski S, Wilkinson G. Folate intake and carcinogenesis of the colon and rectum. *Int J Epidemiol* 1991; **20**: 368-374

6 **Benito E**, Stiggelbout A, Bosch FX, Obrador A, Kaldor J, Mulet M, Munoz N. Nutritional factors in colorectal cancer risk: a case-control study in Majorca. *Int J Cancer* 1991; **49**: 161-167

7 **Meyer F**, White E. Alcohol and nutrients in relation to colon cancer in middle-aged adults. *Am J Epidemiol* 1993; **138**: 225-236

8 **Paspatis GA**, Kalafatis E, Oros L, Xourgias V, Koutsioumpa P, Karamanolis DG. Folate status and adenomatous colonic polyps. A colonoscopically controlled study. *Dis Colon Rectum* 1995; **38**: 64-67; discussion 67-68

9 **Lashner BA**. Red blood cell folate is associated with the development of dysplasia and cancer in ulcerative colitis. *J Cancer Res Clin Oncol* 1993; **119**: 549-554

10 **Cravo ML**, Mason JB, Dayal Y, Hutchinson M, Smith D, Selhub J, Rosenberg IH. Folate deficiency enhances the development of colonic neoplasia in dimethylhydrazine-treated rats. *Cancer Res* 1992; **52**: 5002-5006

11 **Baron JA**, Sandler RS, Haile RW, Mandel JS, Mott LA, Greenberg ER. Folate intake, alcohol consumption, cigarette smoking, and risk of colorectal adenomas. *J Natl Cancer Inst* 1998; **90**: 57-62

12 **Paspatis GA**, Karamanolis DG. Folate supplementation and adenomatous colonic polyps. *Dis Colon Rectum* 1994; **37**: 1340-1341

13 **Song J**, Medline A, Mason JB, Gallinger S, Kim YI. Effects of dietary folate on intestinal tumorigenesis in the apcMin mouse. *Cancer Res* 2000; **60**: 5434-5440

14 **Lashner BA**, Provencher KS, Seidner DL, Knesebeck A, Brzezinski A. The effect of folic acid supplementation on the risk for cancer or dysplasia in ulcerative colitis. *Gastroenterology* 1997; **112**: 29-32

15 **Riddell RH**, Goldman H, Ransohoff DF, Appelman HD, Fenoglio CM, Haggitt RC, Ahren C, Correa P, Hamilton SR, Morson BC. Dysplasia in inflammatory bowel disease: standardized classification with provisional clinical applications. *Hum Pathol* 1983; **14**: 931-968

16 **Kim YI**. Role of folate in colon cancer development and progression. *J Nutr* 2003; **133**: 3731S-3739S

17 **Kim YI**, Salomon RN, Graeme-Cook F, Choi SW, Smith DE, Dallal GE, Mason JB. Dietary folate protects against the development of macroscopic colonic neoplasia in a dose responsive manner in rats. *Gut* 1996; **39**: 732-740

18 **Nensey YM**, Arlow FL, Majumdar AP. Aging. Increased responsiveness of colorectal mucosa to carcinogen stimulation and protective role of folic acid. *Dig Dis Sci* 1995; **40**: 396-401

19 **Wargovich MJ**, Jimenez A, McKee K, Steele VE, Velasco M, Woods J, Price R, Gray K, Kelloff GJ. Efficacy of potential chemopreventive agents on rat colon aberrant crypt formation and progression. *Carcinogenesis* 2000; **21**: 1149-1155

20 **Pretlow TP**, Barrow BJ, Ashton WS, O'Riordan MA, Pretlow TG, Jurcisek JA, Stellato TA. Aberrant crypts: putative preneoplastic foci in human colonic mucosa. *Cancer Res* 1991; **51**: 1564-1567

21 **Jaszewski R**, Khan A, Sarkar FH, Kucuk O, Tobi M, Zagnoon A, Dhar R, Kinzie J, Majumdar AP. Folic acid inhibition of EGFR-mediated proliferation in human colon cancer cell lines. *Am J Physiol* 1999; **277**: C1142-C1148

22 **Akoglu B**, Faust D, Milovic V, Stein J. Folate and chemoprevention of colorectal cancer: Is 5-methyl-tetrahydrofolate an active antiproliferative agent in folate-treated colon-cancer cells? *Nutrition* 2001; **17**: 652-653

23 **US Department of Agriculture**. 1989. Composition of foods-raw, processed and prepared, 1963-1988. In: Agricultural Handbook NO. 8 Series. Washington (DC): Department of Agriculture. US Govt Print Off

24 **Rimm EB**, Giovannucci EL, Stampfer MJ, Colditz GA, Litin LB, Willett WC. Reproducibility and validity of an expanded self-administered semiquantitative food frequency questionnaire among male health professionals. *Am J Epidemiol* 1992; **135**: 1114-1126; discussion 1127-1136

25 **Kim YI**, Fawaz K, Knox T. The effect of folate supplementation on systemic and colonic mucosal folate concentrations: A randomized double-blind placebo-controlled pilot study. *Gastroenterology* 1997; **112**: A592

26 **Butterworth CE Jr**, Tamura T. Folic acid safety and toxicity: a brief review. *Am J Clin Nutr* 1989; **50**: 353-358

27 **Meenan J**, O'Hallinan E, Scott J, Weir DG. Epithelial cell folate depletion occurs in neoplastic but not adjacent normal colon mucosa. *Gastroenterology* 1997; **112**: 1163-1168

28 **Cole BF**, Baron JA, Sandler RS, Haile RW, Ahnen DJ, Bresalier RS, McKeown-Eyssen G, Summers RW, Rothstein RI, Burke CA, Snover DC, Church TR, Allen JI, Robertson DJ, Beck GJ, Bond JH, Byers T, Mandel JS, Mott LA, Pearson LH, Barry EL, Rees JR, Marcon N, Saibil F, Ueland PM, Greenberg ER. Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. *JAMA* 2007; **297**: 2351-2359

29 **Hagmar L**, Bonassi S, Stromberg U, Brogger A, Knudsen LE, Norppa H, Reuterwall C. Chromosomal aberrations in lymphocytes predict human cancer: a report from the European Study Group on Cytogenetic Biomarkers and Health (ESCH). *Cancer Res* 1998; **58**: 4117-4121

30 **Bonassi S**, Hagmar L, Stromberg U, Montagud AH, Tinnerberg H, Forni A, Heikkila P, Wanders S, Wilhardt P, Hansteen IL, Knudsen LE, Norppa H. Chromosomal aberrations in lymphocytes predict human cancer independently of exposure to carcinogens. European Study Group on Cytogenetic Biomarkers and Health. *Cancer Res* 2000; **60**: 1619-1625

31 **Jacob RA**. Folate, DNA methylation, and gene expression: factors of nature and nurture. *Am J Clin Nutr* 2000; **72**: 903-904

32 **Jacob RA**, Gretz DM, Taylor PC, James SJ, Pogribny IP, Miller BJ, Henning SM, Swendseid ME. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. *J Nutr* 1998; **128**: 1204-1212

33 **Pogribny IP**, Miller BJ, James SJ. Alterations in hepatic p53 gene methylation patterns during tumor progression with folate/methyl deficiency in the rat. *Cancer Lett* 1997; **115**: 31-38

34 **Nagothu KK**, Jaszewski R, Moragoda L, Rishi AK, Finkenauer R, Tobi M, Naumoff JA, Dhar R, Ehrinpreis M, Kucuk O, Majumdar AP. Folic acid mediated attenuation of loss of heterozygosity of DCC tumor suppressor gene in the colonic mucosa of patients with colorectal adenomas. *Cancer Detect Prev* 2003; **27**: 297-304

35 **Nagothu KK**, Rishi AK, Jaszewski R, Kucuk O, Majumdar AP. Folic acid-mediated inhibition of serum-induced activation of EGFR promoter in colon cancer cells. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G541-G546

36 **Grunwald V**, Hidalgo M. Development of the epidermal growth factor receptor inhibitor OSI-774. *Semin Oncol* 2003; **30**: 23-31

37 **Yarden Y**. The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. *Eur J Cancer* 2001; **37** Suppl 4: S3-S8

38 **Jaszewski R**, Millar B, Hatfield JS, Nogothu K, Finkenauer R, Rishi AK, Naumoff JA, Kucuk O, Axelrod BN, Majumdar AP. Folic acid reduces nuclear translocation of beta-catenin in rectal mucosal crypts of patients with colorectal adenomas. *Cancer Lett* 2004; **206**: 27-33

39 **Zhang X**, Gaspard JP, Chung DC. Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. *Cancer Res* 2001; **61**: 6050-6054

40 **Tetsu O**, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 1999; **398**: 422-426

41 **Easwaran V**, Lee SH, Inge L, Guo L, Goldbeck C, Garrett E, Wiesmann M, Garcia PD, Fuller JH, Chan V, Randazzo F, Gundel R, Warren RS, Escobedo J, Aukerman SL, Taylor RN, Fantl WJ. beta-Catenin regulates vascular endothelial growth factor expression in colon cancer. *Cancer Res* 2003; **63**: 3145-3153

42 **He TC**, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. *Science* 1998; **281**: 1509-1512

43 **Toole JF**, Malinow MR, Chambless LE, Spence JD, Pettigrew LC, Howard VJ, Sides EG, Wang CH, Stampfer M. Lowering homocysteine in patients with ischemic stroke to prevent recurrent stroke, myocardial infarction, and death: the Vitamin Intervention for Stroke Prevention (VISP) randomized controlled trial. *JAMA* 2004; **291**: 565-575

44 **Ulrich CM**, Potter JD. Folate and cancer--timing is everything. *JAMA* 2007; **297**: 2408-2409

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RAPID COMMUNICATION

CT colonography after incomplete colonoscopy in subjects with positive faecal occult blood test

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Abstract

AIM: To report our experience with computed tomography colonography (CTC) systematically performed in subjects with positive faecal occult blood test (FOBT) and an incomplete colonoscopy in the setting of a population-based screening for colorectal cancer (CRC).
METHODS: From April 2006 to April 2007, 43 290 individuals (age range 50-70) who adhered to the regional screening program for the prevention of CRC underwent immunochemical FOBT. FOBT was positive in 1882 subjects (4.3%). 1463 (77.7%) of these subjects underwent colonoscopy, 903 performed in a single center. Of 903 colonoscopies 65 (7.2%) were incomplete. Forty-two of these subjects underwent CTC. CTC was performed with a 16-MDCT scanner after standard bowel prep (polyethylene glycole) in both supine and prone position. Subjects whose CTC showed polyps or masses were referred to the endoscopist for repeat colonoscopy under sedation or underwent surgery. Per-lesion and per-segment positive predictive values (PPV) were calculated.
RESULTS: Twenty-one (50%) of 42 CTCs showed polyps or masses. Fifty-five of these subjects underwent a repeat colonoscopy, whereas 2 subjects underwent surgery for colonic masses of indeterminate nature. Four subjects refused further examinations. CTC correctly identified 2 colonic masses and 20 polyps. PPV for masses or polyps greater than 9 mm was of 87.5%. Per-lesion and per-segment PPV were, respectively, 83.3% and 83.3% for polyps greater or equal to 10 mm, and 77.8% and 85.7% for polyps of 6-9 mm.
CONCLUSION: In the context of a screening program for CRC based on FOBT, CTC shows high per-segment and per-lesion PPV for colonic masses and polyps greater than 9 mm. Therefore, CTC has the potential to become a useful technique for evaluation of the non visualized part of the colon after incomplete colonoscopy.

Key words: Computed tomography colonography; Virtual colonoscopy; Incomplete colonoscopy; Positive faecal occult blood test; Colorectal cancer screening

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INTRODUCTION

Randomized clinical trials have demonstrated that screening with faecal occult blood test (FOBT) reduces mortality for colorectal cancer (CRC)[1]. Accordingly, population-based screening with FOBT is currently recommended by the European community health associations and has been applied in many countries, including Italy[2].

Subjects with a positive FOBT are usually examined with a total colonoscopy which allows removal of polyps and histological diagnosis of the lesions. However, colonoscopy can be incomplete due to several reasons, including intolerance to the procedure, adhesions from previous surgery, redundant colon and the presence of stenosis. The reported rates of incomplete colonoscopy from various studies carried out in U.S. and Europe over the past 15 years range from 4% to 25%[3,4].

In order to complete evaluation of the colon, radiological examinations can be performed such as double contrast barium enema (DCBE)[5] and computed tomography colonography (CTC). In particular, several studies have shown that CTC is a valuable tool to evaluate the proximal colon after incomplete colonoscopy[6-10], and the American Gastroenterologists Association (AGA) recognized that CTC is indicated for adults with failed colonoscopy[11].

We report the results of CTC systematically performed in subjects with positive FOBT and incomplete colonoscopy in the context of a population-based screening programme for CRC with FOBT.

MATERIALS AND METHODS

Subjects

This prospective study was approved by our institutional review board; informed consent was obtained in all subjects. A population-based screening program for CRC has been active in the Tuscany Region, Italy, since 1998. The screening protocol is directed to all subjects aged 50-70 living in the regional area who are invited via mail every second year to perform immunochemical FOBT. Subjects with negative FOBT are notified of their result by mail and advised to repeat screening after two years. Subjects with positive test are invited to perform colonoscopy[12].

From April 2006 to April 2007, 43 290 asymptomatic individuals aged 50-70 years attended the FOBT-based Florence District screening program and were tested. FOBT was positive in 1882 (4.3%) subjects. These subjects were invited to undergo colonoscopy assessment: 1463 (77.7%) subjects underwent colonoscopy and 419 refused. 903 colonoscopies were performed in a single center by two experienced endoscopists.

According to the screening protocol, colonoscopy was performed without sedation. 838 (92.8%) colonoscopies were complete (i.e. the caecum was reached) and 65 (7.2%) were incomplete. The levels at which colonoscopy was interrupted were the sigmoid colon in 33 subjects, the descending colon in 21, the transverse colon in 8 and the ascending colon in 3. According to the endoscopist's report, presumptive reasons for incomplete colonoscopy were dolichocolon (14.8%), diverticular disease (23.1%), adherences due to previous abdominal surgery (16.4%) or intolerance to the procedure (12.1%).

Forty-two of these 65 subjects (17 males, 25 females; mean age 60.7 years; age range 51-70) agreed to complete colonic examination with CTC and constitute of the base for this report.

CTC was performed within 6 wk after incomplete colonoscopy (mean interval 16 d). In those subjects in whom endoscopic polyp removal was performed during the incomplete colonoscopy, CTC was delayed for at least 1 mo after polypectomy.

CTC technique

All subjects underwent a standard bowel preparation for CTC with 4 L of a polyethylene glycole solution (Isocolan; Giuliani, Milan, Italy) administered the day before the procedure and a low residue diet for 3 d. All subjects received intravenously 30 mg of scopolamine butylbromide (Buscopan; Boehringer Ingelheim, Florence, Italy) before air insufflation, in order to improve colonic distension[13].

The subjects were placed on the right lateral decubitus and a 24 Fr rubber catheter, Foley type, with a small retention balloon (10 mL) was inserted into the rectum. After catheter positioning the patient was turned in supine position and colonic distension was obtained with manual insufflation of room air. Air was administered from an enema bag connected to the rectal tube with a maximum capacity of 2 L. Insufflation was performed by gently squeezing the enema bag during 3 to 5 min up to subject tolerance.

Both supine and prone CT scans were obtained in all subjects. Colonic distension was evaluated with an anterior-posterior scout view in both supine and prone position, and additional air was inflated using a manual bulb if distension was unsatisfactory. In one subject, unable to stay prone because of abdominal pain, a right lateral decubitus acquisition was obtained instead of the prone scan. Intravenous contrast medium was not used.

CTC was performed with a 16-MDCT scanner (Sensation 16; Siemens, Erlangen, Germany) using a detector configuration of 16 mm × 0.75 mm, 120 kVp, 50 effective mAs, tube rotation time of 500 ms and a pitch of 1.25. Data were reconstructed using a slice thickness of 1 mm with a reconstruction increment of 0.7 mm (30% overlap). For each acquisition $CTDI_{vol}$ was 4.15 mGy with a calculated equivalent dose of 3.5 mSv for females and 2.7 mSv for males (CT Patient Dosimetry Calculator, ImPACT; measures executed on MonteCarlo Phantom).

CTC evaluation

The images of each study were transferred to a workstation equipped with CTC dedicated software (Syngo; Siemens, Erlangen, Germany). The software provides axial, multi-planar reformatted (MPR), endoluminal surface-shaded images and double-contrast-like reconstructions of the colon. All studies were interpreted on the workstation by two readers, one experienced gastrointestinal radiologist and one radiology resident, by consensus.

Preliminarily, the degree of colonic distension was evaluated on axial images. The colon was divided into six segments: caecum, ascending, transverse, descending, sigmoid and rectum (the different segments were evaluated both in supine and prone acquisitions). Distension for each segment was graded on a scale from 0 to 3, in

which a grade of 0 indicated complete collapse and a grade of 3 optimal distension[13]. The least-distended section of any individual segment was used to assign the overall distention score for that segment. Colonic distension was deemed clinically adequate if all segments had a score of 2 or 3 at least in supine or prone acquisition.

Moreover, we assessed the adequacy of preparation by evaluating the proportion of colonic segments containing residual faecal matter or fluid for each subject (no specific attempt was made to rank the amount of fluid or stool).

CTCs were evaluated with a primary 3D approach, using 2D for problem solving. In all cases, endoluminal navigation was performed from rectum to caecum and backwards for both supine and prone acquisitions. Then axial images were examined with an abdominal window (level 40 HU, width 350 HU), in order to discover areas of colonic wall thickening, and to look for extra-colonic findings.

All lesions detected at CTC were localized according to their segmental location in the colon. Each lesion was measured taking account of its maximum diameter on 2D images viewed with a bone window (level 400 HU, width 2000 HU).

Subjects management

All subjects with CTC showing polyps were referred to the endoscopist to repeat colonoscopy under sedation. Also subjects with colonic masses were referred to the endoscopist who evaluated in agreement with the subject the opportunity of a repeat colonoscopy or a surgical consult.

The results of repeat colonoscopy and/or the pathological findings on surgical specimens were used as a gold standard for CTC performance assessment. Lesions were measured by open biopsy forceps at endoscopy and with ruler for pathological specimens.

CTC findings were classified as true-positive or false-positive results. A true-positive lesion at CTC was defined as a lesion that was confirmed at repeat colonoscopy or at surgery, a lesion that was in the same or an adjacent colonic segment, and a lesion for which the size correlated within 50% of the diameter. A lesion was defined as false positive if the lesion reported at CTC was not detected at repeat colonoscopy, was not in the same or an adjacent colonic segment or there was more than a 50% discrepancy in the lesion diameter. Endoscopic evidence of polyps or masses at repeat colonoscopy not detected at CTC was assumed as a false negative result for CTC.

Descriptive statistics were used to calculate per-lesion and per-segment positive predictive values (PPV) for polyps equal or greater than 10 mm, for polyps of 6-9 mm and for smaller lesions (< 6 mm). In per-segment analysis the colon was divided into six segments (caecum, ascending, transverse, descending, sigmoid and rectum) and the segments examined by initial colonoscopies were excluded from the evaluation.

Subjects with a negative CTC did not undergo further examination and were scheduled for standard follow-up according to the screening protocol[12].

Table 1 CTC results for polyps

	True positive	False positive	False negative	Per-lesion PPV (%)
Polyps < 6 mm	8	4	2	66.7
Polyps 6-9 mm	7	2	0	77.8
Polyps ⩾ 10 mm	5	1	0	83.3
Total	20	7	2	

RESULTS

Complete colonic distension was obtained in 36 (85.7%) of 42 subjects. Considering both supine and prone acquisitions, the rectum in one patient and the sigmoid colon in 5 patients were not adequately distended. Incomplete distension was mainly due to advanced diverticular disease. The mean overall bowel distension scores were 2.75 ± 0.37 for supine position and 2.84 ± 0.23 for prone position. Either fluid and faecal residua, or inadequate distension precluded evaluation of 14 (5.6%) of 252 colonic segments [rectum (n = 1), sigmoid colon (n = 10), descending colon (n = 3)].

Twenty one (50%) of 42 CTCs showed polyps or colonic masses of indeterminate nature. Fifteen patients with polyps at CTC underwent repeat colonoscopy under sedation. Two patients with colonic masses of indeterminate nature were referred to surgical consult and underwent colectomy. Four patients with CTC findings of polyps smaller than 6 mm did not undergo repeat colonoscopy because of medical problems or refusal.

All repeat colonoscopic examinations were performed within a mean of 34 d after CTC (range 15 d to 6 mo) and were complete. No complications occurred after CTC or diagnostic and operative colonoscopy.

CTC correctly identified 20 polyps in segments not visualized at initial colonoscopy, and gave 7 false positive and 2 false negative results (Table 1). All polyps were endoscopically removed and histology was obtained (Figure 1). Of 20 polyps 11 were adenomas (2 tubulovillous adenomas with high-grade dysplasia, 6 tubulo-villous adenomas, 3 tubular adenomas) and 9 hyperplastic or inflammatory polyps. No cancers were observed. The two false negative polyps not identified at CTC included one hyperplastic polyp and one tubular adenoma, both smaller than 6 mm. A total of 5 advanced adenomas were found at CTC and histologically proved.

CTC correctly depicted two colonic masses of indeterminate nature at the level of the proximal sigmoid colon, which were found to be advanced diverticular disease complicated by stenosis at surgery (Figure 2). None of these patients had endoscopically visualized masses or adenomatous polyps at initial colonoscopy.

Per-segment analysis was performed on patients who completed repeat colonoscopy or underwent surgery (n = 17). On a total of 102 colonic segments, 26 segments examined by initial colonoscopies were excluded, and the analysis was based on the remaining 76 segments.

CTC showed a PPV for masses or polyps greater than 9 mm of 87.5%. Per-lesion and per-segment PPV

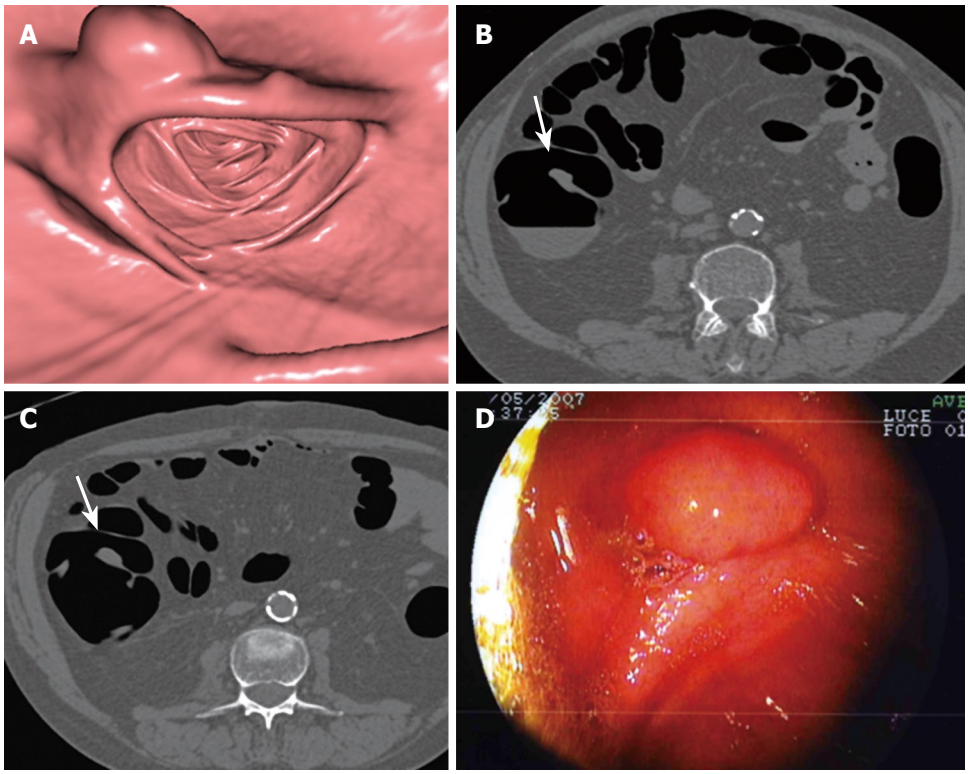

Figure 1 Adenomatous polyp of 13 mm of the ascending colon in a 61-year-old female with initial colonoscopy interrupted at the descending colon for severe discomfort. **A**: Endoluminal CT image of the ascending colon shows 13 mm sessile polyp lying on a fold; **B** and **C**: Axial CT images acquired in supine and prone position show the polypoid lesion (arrow) on a fold; **D**: Sessile polyp of 13 mm of the ascending colon found at repeat colonoscopy. Histology evaluation revealed adenomatous polyp.

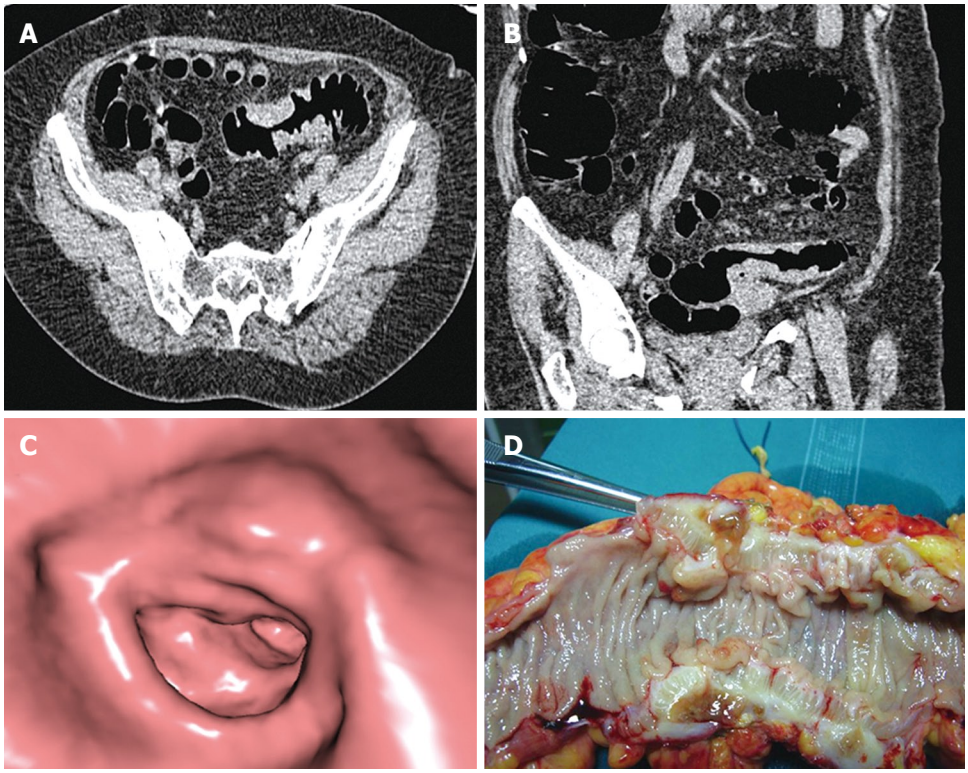

Figure 2 Stenosing mass of the proximal sigmoid colon in a 69-year-old female with initial colonoscopy interrupted at the distal sigmoid colon for diverticular disease. **A**: Axial CT image acquired in prone decubitus shows a stenosing lesion of the proximal sigmoid colon with CT findings suspicious for malignancy: eccentric wall thickening, "shoulder sign", absence of pericolonic fat stranding; **B**: Coronal oblique multiplanar reformation shows the lesion in the sigmoid colon; **C**: Endoluminal CT image shows the passage from the normal colonic wall to the stenosis; **D**: Surgical specimen from left hemicolectomy shows a stenosing lesion of about 5 cm in the proximal sigmoid colon with marked wall thickening due to advanced diverticular disease confirmed at histological evaluation.

were respectively 83.3% and 83.3% for polyps greater or equal to 10 mm, 77.8% and 85.7% for polyps of 6-9 mm, 66.7% and 50% for polyps smaller than 6 mm.

Diverticular disease was found in 20 subjects (47.6%) and 5 of these subjects (11.9%) showed signs of chronic diverticulitis such as diffuse wall thickening and pericolic fat stranding.

Major extra-colonic findings included aneurysm of the abdominal aorta (n = 1), renal masses (n = 2), hepatic focal lesion other than cystic (n = 1), splenomegaly (n = 1) and pulmonary nodules (n = 2).

DISCUSSION

Due to its natural history CRC is an ideal candidate for screening[14]. In fact, most CRC originate from pre-existing adenomatous polyps that, in 10 to 15 years, undergo malignant transformation[15]. Likelihood for malignant

transformation is not the same for all adenomas. In particular adenomas equal or greater than 10 mm (advanced adenomas) tend to become malignant after an average of 5.5 years, whereas it is estimated that less than 1% of adenomas smaller than 10 mm contain a cancer[14]. Thus, advanced adenoma is a precancerous lesion and should be considered the main target of a screening test for CRC.

In the majority of screening programmes, subjects with positive FOBT are invited to undergo colonoscopy, which can be performed with or without sedation. Colonoscopies without sedation can be incomplete in up to 25% of the cases[4] and in our series incomplete colonoscopies were 7.2%. Before adoption of CTC in such cases, we previously performed DCBE.

Several studies showed that DCBE has a low accuracy in detecting colonic neoplasms, with sensitivity for adenomas greater than 9 mm in the range of 45%-50%[16]. CTC is more accurate in detecting colorectal neoplasms as shown in some meta-analyses in which the performance of CTC *versus* optical colonoscopy revealed sensitivity in the range of 85%-90% and specificity of about 95% for polyps greater than 9 mm[17,18].

We evaluated the performance of CTC after incomplete colonoscopy in the setting of a large population based screening program with FOBT. In this context, CTC showed its potential for diagnostic assessment, identifying 2 colonic masses, 5 advanced adenomas and 6 smaller adenomas. CTC gave 7 false positive results which led to unnecessary repeat colonoscopy. One false positive was a polypoid lesion of 14 mm of the ascending colon that was visible only on the supine dataset. Four false positive results were for polyps smaller than 6 mm which should not be reported according to current recommendations[19].

The possibility that diverticular disease simulates with colonic masses is well known, as is the fact that the differential diagnosis with cancer can be difficult with CT[20]. In our series, CTC detected two colonic masses of the proximal sigmoid colon which showed CT features suspicious for malignancy, but were demonstrated by pathology to be due to diverticular disease in absence of any malignancy. In both cases, initial colonoscopy was interrupted at distal sigmoid colon because of advanced diverticular disease. In the two cases, the endoscopist and the subject decided not to perform a repeat colonoscopy and the subjects were referred to the surgeon to undergo colectomy.

Colonic distension and cleansing were adequate for an accurate examination in the majority of our cases. In some cases, colonic collapse or repletion by fluid or faeces precluded evaluation of the rectum, sigmoid or descending colon. This limitation can be partially overcome by the fact that lower colonic segments are usually examined at initial colonoscopy. Indeed, segments examined by initial colonoscopies were excluded from per-segment analysis.

Almost 50% of 42 patients of our study had diverticular disease which represented an obstacle to complete conventional colonoscopy. Our series showed that diverticular disease did not seriously compromise colonic distension and evaluation of the proximal colon at CTC.

Previous studies on CTC after incomplete colonoscopy have been conducted[6-10]. These studies were inhomogeneous regarding the patients' selection, because they included asymptomatic as well as symptomatic subjects. Our results in terms of PPV, acquired in a selected group of screening subjects, were comparable with those obtained in the largest study on CTC after incomplete colonoscopy conducted by Copel *et al*[10]. They reported per-lesion PPV of 91.7% for masses, of 70% for polyps of 10 mm or greater, and of 30.4% for polyps of 6-9 mm[10]. In our small group of subjects, we considered, altogether masses and polyps greater than 9 mm obtaining a similar result (PPV of 87.5%). Our better results for medium sized polyps (6-9 mm) with a per-lesion PPV value of 77.8% might be due to thinner collimation for CT scanning and double reading of the examinations we utilize.

We observed a significant number of false positive results which led to unnecessary repeat colonoscopy. The use of faecal tagging should reduce the number of false positive, enabling a better distinction between polyps and faecal residues, as showed in a series of CTC after incomplete colonoscopies[9].

Our study had limitations. First, it was carried out on a small series of subjects. Second, repeat colonoscopy was conducted with segmental blinding, and this could have increased the number of false positive results of the CTC. Third, since subjects with negative CTC did not undergo further examinations, we could not evaluate sensitivity and specificity of CTC with respect to optical colonoscopy.

In conclusion, in the context of a population-based screening program for CRC based on FOBT, CTC showed a high per-segment and per-lesion PPV for colonic masses and polyps greater than 9 mm. Therefore, CTC has the potential to become a useful technique for evaluation of the non-visualized part of the colon after incomplete colonoscopy and should replace DCBE.

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COMMENTS

Background

Colorectal cancer (CRC) is a relevant neoplastic disease for its high incidence and mortality. Due to its natural history CRC is suitable for screening. Screening with faecal occult blood test (FOBT) reduces mortality from CRC. Subjects with positive FOBT are usually examined by colonoscopy which can be incomplete.

Research frontiers

Computed tomography colonography (CTC) is a non-invasive imaging technique with a high sensitivity and specificity in the diagnosis of colonic cancer and polyps equal or greater than 10 mm, which are the target for screening. Therefore, it might represent a second step examination before colonoscopy to examine subjects with positive FOBT.

Innovations and breakthroughs

This study on CTC after incomplete colonoscopy was conducted in the frame of a population based screening program. Previous reports on this topic were car-

ried out in heterogeneous samples of symptomatic and asymptomatic subjects or patients with known colonic pathology.

Application

In the context of a screening program with FOBT, CTC has high positive predictive value (PPV) for colonic masses or polyps equal or greater than 10 mm and should replace double contrast barium enema (DCBE) for evaluation of the non visualized part of the colon after incomplete colonoscopy.

Terminology

CTC is a thin slice CT scan of the abdomen after adequate bowel preparation and colon insufflation in which data are reconstructed providing axial, multiplanar, and endoluminal views (virtual colonoscopy), in order to visualize colonic wall. Colonoscopy is the more accurate technique to evaluate colonic internal surface and it is performed passing a flexible tube with fiber optic through the anus. FOBT is a chemical test that can detect tiny traces of blood in the stool that may indicate the presence of CRC.

Peer review

This paper shows the usefulness of CTC after insufficient colonoscopy in order to detect colorectal lesions. It was conducted as a part of population-based screening programme of CRC. It's an interesting study.

REFERENCES

1 **Hardcastle JD**, Chamberlain JO, Robinson MH, Moss SM, Amar SS, Balfour TW, James PD, Mangham CM. Randomised controlled trial of faecal-occult-blood screening for colorectal cancer. *Lancet* 1996; **348**: 1472-1477

2 **Recommendations on cancer screening in the European union**. Advisory Committee on Cancer Prevention. *Eur J Cancer* 2000; **36**: 1473-1478

3 **Anderson ML**, Heigh RI, McCoy GA, Parent K, Muhm JR, McKee GS, Eversman WG, Collins JM. Accuracy of assessment of the extent of examination by experienced colonoscopists. *Gastrointest Endosc* 1992; **38**: 560-563

4 **Marshall JB**, Barthel JS. The frequency of total colonoscopy and terminal ileal intubation in the 1990s. *Gastrointest Endosc* 1993; **39**: 518-520

5 **Chong A**, Shah JN, Levine MS, Rubesin SE, Laufer I, Ginsberg GG, Long WB, Kochman ML. Diagnostic yield of barium enema examination after incomplete colonoscopy. *Radiology* 2002; **223**: 620-624

6 **Macari M**, Berman P, Dicker M, Milano A, Megibow AJ. Usefulness of CT colonography in patients with incomplete colonoscopy. *AJR Am J Roentgenol* 1999; **173**: 561-564

7 **Morrin MM**, Kruskal JB, Farrell RJ, Goldberg SN, McGee JB, Raptopoulos V. Endoluminal CT colonography after an incomplete endoscopic colonoscopy. *AJR Am J Roentgenol* 1999; **172**: 913-918

8 **Neri E**, Giusti P, Battolla L, Vagli P, Boraschi P, Lencioni R, Caramella D, Bartolozzi C. Colorectal cancer: role of CT colonography in preoperative evaluation after incomplete colonoscopy. *Radiology* 2002; **223**: 615-619

9 **Gryspeerdt S**, Lefere P, Herman M, Deman R, Rutgeerts L, Ghillebert G, Baert F, Baekelandt M, Van Holsbeeck B. CT colonography with fecal tagging after incomplete colonoscopy. *Eur Radiol* 2005; **15**: 1192-1202

10 **Copel L**, Sosna J, Kruskal JB, Raptopoulos V, Farrell RJ, Morrin MM. CT colonography in 546 patients with incomplete colonoscopy. *Radiology* 2007; **244**: 471-478

11 **Rockey DC**, Barish M, Brill JV, Cash BD, Fletcher JG, Sharma P, Wani S, Wiersema MJ, Peterson LE, Conte J. Standards for gastroenterologists for performing and interpreting diagnostic computed tomographic colonography. *Gastroenterology* 2007; **133**: 1005-1024

12 **Grazzini G**, Castiglione G, Ciabattoni C, Franceschini F, Giorgi D, Gozzi S, Mantellini P, Lopane P, Perco M, Rubeca T, Salvadori P, Visioli CB, Zappa M. Colorectal cancer screening programme by faecal occult blood test in Tuscany: first round results. *Eur J Cancer Prev* 2004; **13**: 19-26

13 **Taylor SA**, Halligan S, Goh V, Morley S, Bassett P, Atkin W, Bartram CI. Optimizing colonic distention for multi-detector row CT colonography: effect of hyoscine butylbromide and rectal balloon catheter. *Radiology* 2003; **229**: 99-108

14 **Winawer SJ**, Fletcher RH, Miller L, Godlee F, Stolar MH, Mulrow CD, Woolf SH, Glick SN, Ganiats TG, Bond JH, Rosen L, Zapka JG, Olsen SJ, Giardiello FM, Sisk JE, Van Antwerp R, Brown-Davis C, Marciniak DA, Mayer RJ. Colorectal cancer screening: clinical guidelines and rationale. *Gastroenterology* 1997; **112**: 594-642

15 **Muto T**, Bussey HJ, Morson BC. The evolution of cancer of the colon and rectum. *Cancer* 1975; **36**: 2251-2270

16 **Winawer SJ**, Stewart ET, Zauber AG, Bond JH, Ansel H, Waye JD, Hall D, Hamlin JA, Schapiro M, O'Brien MJ, Sternberg SS, Gottlieb LS. A comparison of colonoscopy and double-contrast barium enema for surveillance after polypectomy. National Polyp Study Work Group. *N Engl J Med* 2000; **342**: 1766-1772

17 **Halligan S**, Altman DG, Taylor SA, Mallett S, Deeks JJ, Bartram CI, Atkin W. CT colonography in the detection of colorectal polyps and cancer: systematic review, meta-analysis, and proposed minimum data set for study level reporting. *Radiology* 2005; **237**: 893-904

18 **Mulhall BP**, Veerappan GR, Jackson JL. Meta-analysis: computed tomographic colonography. *Ann Intern Med* 2005; **142**: 635-650

19 **Zalis ME**, Barish MA, Choi JR, Dachman AH, Fenlon HM, Ferrucci JT, Glick SN, Laghi A, Macari M, McFarland EG, Morrin MM, Pickhardt PJ, Soto J, Yee J. CT colonography reporting and data system: a consensus proposal. *Radiology* 2005; **236**: 3-9

20 **Chintapalli KN**, Chopra S, Ghiatas AA, Esola CC, Fields SF, Dodd GD 3rd. Diverticulitis versus colon cancer: differentiation with helical CT findings. *Radiology* 1999; **210**: 429-435

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RAPID COMMUNICATION

Development of hepatorenal syndrome in bile duct ligated rats

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Abstract

AIM: To evaluate in bile duct ligated rats whether there were progressive alterations of renal function without changes in histopathology.

METHODS: Male Wistar rats were submitted to sham-surgery or bile duct ligation (BDL) and divided according to the post-procedure time (2, 4 and 6-wk). To determine renal function parameters, rats were placed in metabolic cages and, at the end of the experiment, blood and urine samples were obtained. Histology and hydroxyproline content were analyzed in liver and renal tissue.

RESULTS: Rats with 2 wk of BDL increased free water clearance (P = 0.02), reduced urinary osmolality (P = 0.03) and serum creatinine (P = 0.01) in comparison to the sham group. In contrast, rats at 6 wk of BDL showed features of HRS, including significant increase in serum creatinine and reductions in creatinine clearance, water excretion and urinary sodium concentration. Rats with 4 wk of BDL exhibited an intermediate stage of renal dysfunction. Progressive hepatic fibrosis according to post-procedure time was confirmed by histology. The increased levels of liver hydroxyproline contrasted with the absence of structural changes in the kidney, as assessed by histology and unchanged hydroxyproline content in renal tissue.

CONCLUSION: Our data show that BDL produced progressive renal dysfunction without structural changes in the kidney, characterizing HRS. The present model will be useful to understand the pathophysiology of HRS.

Key words: Hepatorenal syndrome; Bile duct ligation; Renal function; Renin angiotensin system

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INTRODUCTION

Hepatorenal syndrome (HRS) has been defined as a progressive renal failure that occurs in patients with chronic liver disease and advanced hepatic failure in

the absence of any apparent clinical cause for renal insufficiency[1,2]. HRS represents the final stage of a process that gradually reduces the renal blood flow and the glomerular filtration rate (GFR) due to a marked renal vasoconstriction[1-4]. Despite the severity of renal failure, no significant histological abnormalities are found in the kidneys.

There are many experimental models to induce hepatic fibrosis[5]. However, none of them has been evaluated systematically as a model of hepatorenal syndrome. The two most frequently used experimental models of liver disease are the administration carbon tetrachloride, and the common bile duct ligation (BDL)[6]. The main advantage of BDL is to allow the study of renal function alterations in a short period of time with lower mortality rates than the administration of carbon tetrachloride[6]. In addition, this model mimics clinical conditions characterized by obstructive jaundice, such as biliary atresia and choledocal cysts[5,6]. In this study, we aimed to systematically evaluate renal function parameters, renal histology and tissue hydroxyproline content at different time-points of BDL.

MATERIALS AND METHODS

Animals and experimental design

Male Wistar rats weighing 220 to 300 g were maintained under temperature controlled conditions with an artificial 12-h light-dark cycle, and were allowed standard chow and water ad libitum. Hepatic fibrosis was induced by BDL. Briefly, the animals were anesthetized with intraperitoneal administration of 2.5% tribromoethanol (1 mL/100 g). A 1.5 cm midline incision was made and the common bile duct was located, double ligated with 4-0 silk and sectioned as previously described[7]. Our Ethics Committee approved all animal procedures.

Experimental protocol

Animals were randomized into the following groups: sham-operated and those that underwent BDL. Sham-operated rats (n = 17) underwent a midline incision and manipulation of the bile duct without ligation and were evaluated at various times following sham-surgery: 2-wk (n = 5), 4-wk (n = 7), and 6-wk (n = 5). Bile duct ligated rats were also evaluated at the same post-procedure times: 2-wk (n = 8), 4-wk (n = 7) and 6-wk (n = 7). Three days before blood sampling, all rats were placed in metabolic cages to measure urinary volume, water and food intake. At the end of the experiment, animals were weighed and blood samples were collected by decapitation to determine renal function parameters. Liver and renal tissue fragments were also obtained for histology and hydroxyproline determination.

Biochemical parameters

Serum and urinary levels of creatinine (Jaffe method) were measured using Katal Kit and a semi-automatic analyzer BIO 2000. Urinary and serum osmolality were determined using a freezing point osmometer (Fiske Osmometer, Fiske Ass. Inc., MA, USA). Serum and urinary levels of sodium and potassium were measured by flame photometry (Corning 400, Corning Inc., NY, USA).

Hydroxyproline determination

Fragments (200 mg) of liver and renal tissue were removed for hydroxyproline determination as an indirect measure of tissue collagen content, as described by Reddy & Enwemeka[8]. Briefly, tissue fragments were homogenized in saline 0.9%, frozen and lyophilized. The assay was performed with 40 mg of the lyophilized tissue that was subjected to alkaline hydrolysis in 300 μL plus 75 μL NaOH 10 mol/L at 120℃ for 20 min. An aliquot of 50 μL of the hydrolysed tissue was added to 450 μL of chloramine T oxidizing reagent (Chloramine T 0.056 mol/L, n-propanol 10% in acetate/citrate buffer pH 6.5) and allowed to react for 20 min. A hydroxyproline standard curve with the highest concentration of 400 μg was prepared likewise. Colour was developed by the addition of 500 μL of the Ehrlich reagent (p-dimethylaminebenzaldehyde, 1 mol/L) diluted in n-propanol/perchloric acid, 2:1 v/v). The samples then were centrifuged for 1500 g for 10 min at 4℃. An aliquot of 200 μL of the supernatant was transferred to 96-well plates and the absorbance was read at 550 nm.

Five-micrometer sections of formalin-fixed and paraffin-embedded right liver lobes and kidney slices were processed routinely with hematoxylin-eosin, Masson's trichrome and ammoniac silver of Gomori. A single pathologist, blinded to experimental protocol, analyzed all liver and kidney fragments using light microscopy. The degree of liver fibrosis was measured based on the semi-quantitative scoring described by Ishak *et al*[9].

Statistical analysis

The data are expressed as mean ± SD. Analysis of variance followed by Student Newman Keuls test was used to compare the differences between groups. Values of $P < 0.05$ were considered significant.

RESULTS

Morphological studies

Because there did not appear to be any difference in the many variables studied in the sham group at two, four and 6 wk after sham operation, results from all the sham-operated groups were pooled for ease of presentation.

Hepatic fibrosis progressed over the time after BDL. Based on Ishak's score, the following score values for each group of BDL rats were obtained: sham operated rats scored 0 (normal hepatic architecture), 2-wk rats scored 3 (fibrous expansion of portal tracts with occasional portal-to-portal bridging), 4-wk rats scored 4 (fibrous expansion of portal tracts with marked portal-to-portal and portal-to-central bridging) and, at 6-wk, definite cirrhosis occurred (Score 6). In sharp contrast, as shown on Figure 1, no alterations in renal histology were observed in any bile duct ligated rats when compared to sham-operated animals.

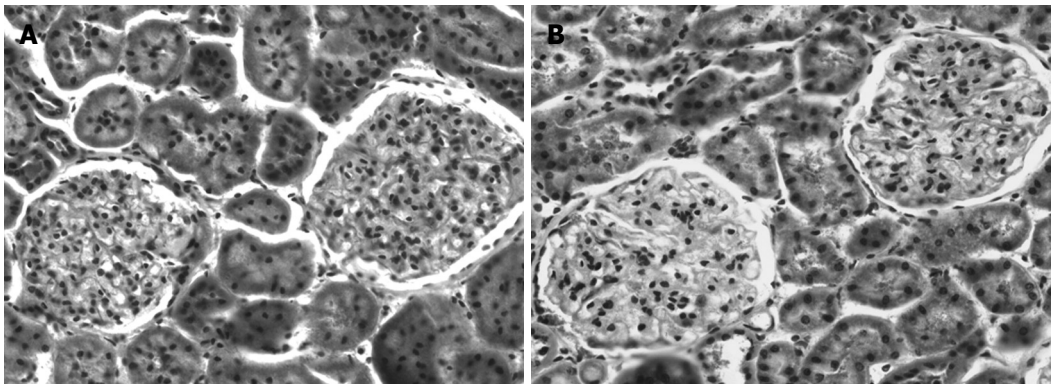

Figure 1 Representative micrographs of the renal slices from bile duct ligated (BDL) and sham operated rats (HE, x 100). **A**: Sham operated rat with normal kidney; **B**: BDL rat at 6-wk also showing the absence of kidney histological alterations.

Hydroxyproline determination

The progression of collagen deposition in liver tissue was also confirmed by the measurement of tissue hydroxyproline content at different time-points after bile duct ligation. Sham-operated rats represented the basal values of hydroxyproline content from a normal liver (235 ± 45 μg/mg of liver tissue). As expected, according to the time after BDL, hydroxyproline content progressively increased in liver tissue, reaching values significantly higher than the control group in all time-points (2 wk: 540 ± 60 μg/mg; 4 wk: 863 ± 57 μg/mg; 6 wk: 1735 ± 73 μg/mg; $P = 0.0001$ for all comparisons, Figure 2A). The highest amount of liver hydroxyproline was detected in animals at 6 wk of BDL, indicating the significant degree of liver fibrosis (Figure 2A). Hydroxyproline content in renal tissue remained unchanged in sham-operated animals (288 ± 31 μg/mg of kidney tissue) as well as in all groups of bile duct ligated rats (2 wk: 298 ± 55 μg/mg; 4 wk: 300 ± 73 μg/mg; 6 wk: 294 ± 39 μg/mg; $P > 0.05$ for all comparisons, Figure 2B). The results obtained with histological analysis and tissue hydroxyproline determinations showed the absence of structural changes in renal tissue during the development of liver fibrosis.

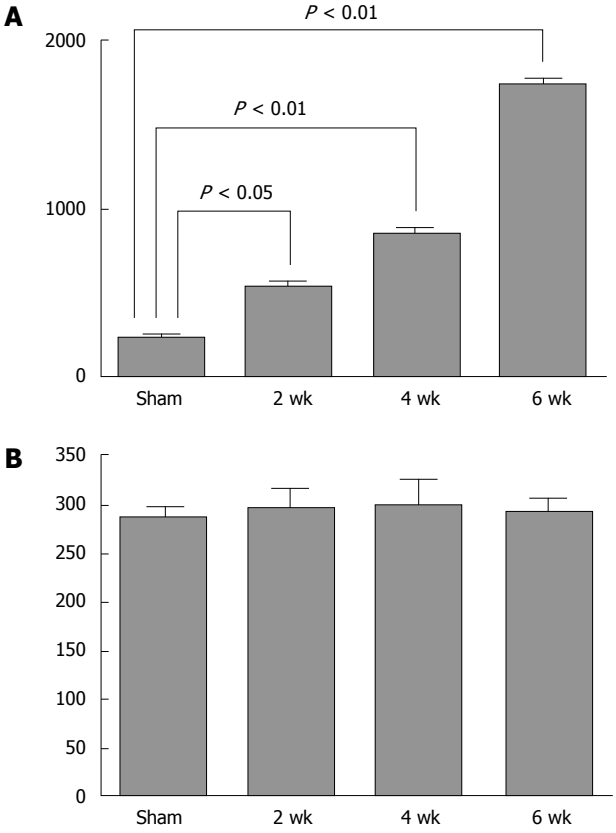

Figure 2 Hydroxyproline determinations in the liver and renal tissue from bile duct ligated and sham operated rats. **A**: Hydroxyproline content in the liver tissue of sham operated rats (sham), and animals with 2-wk, 4-wk and 6-wk of bile duct ligation; **B**: Hydroxyproline content in renal tissue of sham operated rats (sham), and animals with 2 wk, 4 wk and 6 wk of bile duct ligation.

Renal function parameters

Despite the well-preserved renal structure, important changes in renal function were clearly evidenced in bile duct ligated rats, as shown in Table 1 and Figure 3. As shown in Table 1, the 24-h urinary volume was significantly higher in animals with 4 and 6 wk of BDL compared to sham group. On the other hand, the 24-h urinary volume of 2 wk animals did not differ from sham group. Despite having the same urinary volume as sham-operated animals, rats with 2 wk of BDL exhibited an attempt to compensate the hydroelectrolyte imbalance produced by hepatic dysfunction. These animals significantly increased water excretion (Figure 3A, $P = 0.02$), and reduced the urinary osmolality ($P = 0.03$) and serum creatinine levels ($P = 0.01$) in comparison to sham-operated rats (Table 1). An elevation in potassium excretion was also observed. However, the fractional excretion of this ion was unchanged in comparison with the sham group. Rats with 4 wk of BDL presented a progression in renal dysfunction as shown by a significant increase in serum creatinine ($P = 0.01$) and a reduction in urinary sodium concentration ($P = 0.02$) when compared to sham-operated animals (Table 1). Rats with 6 wk of BDL clearly developed hepatorenal syndrome as revealed by a complete deterioration in renal compensatory mechanisms. These animals presented high levels of serum creatinine, a pronounced decrease in creatinine clearance (Figure 3B, $P = 0.01$), and an important impairment in water excretion (Figure 3A, $P = 0.02$) when compared to sham operated and 2 wk of BDL animals ($P < 0.05$ for all comparisons, Table 1). Rats with 4 and 6 wk of BDL also presented dilutional hyponatremia and an elevation of fractional excretion of potassium when compared to sham group and animals with 2-wk of BDL ($P < 0.05$ for both comparisons, Table 1). It

Table 1 Renal function parameters in sham-operated (Sham) and bile duct ligated rats at 2-wk, 4-wk and 6-wk (mean ± SD)

	Sham (n = 17)	2-wk (n = 8)	4-wk (n = 7)	6-wk (n = 7)
Urinary volume (mL/24 h)	12 ± 0.5	14.2 ± 1.0	19.4 ± 1.7[a]	20.4 ± 2.8[a]
Serum creatinine (mg/dL)	0.60 ± 0.10	0.28 ± 0.05[a]	1.21 ± 0.25[a]	2.50 ± 0.40[a]
Creatinine clearance (mL/min)	1.14 ± 0.19	1.31 ± 0.11	0.97 ± 0.43	0.47 ± 0.25[a]
Serum osmolality (mOsm/kg)	292 ± 2	289 ± 4	280 ± 14[a]	282 ± 3[a]
Urinary osmolality (mOsm/kg)	2147 ± 115	1578 ± 76[a]	1499 ± 117[a]	1745 ± 73[a]
Osmolal clearance (mL/min)	0.061 ± 0.003	0.049 ± 0.002	0.071 ± 0.009	0.088 ± 0.013
Free water clearance (mL/min)	-0.052 ± 0.003	-0.040 ± 0.002[a]	-0.056 ± 0.008	-0.074 ± 0.011[a]
Serum [Na^+] (mEq/L)	137 ± 1	138 ± 3	125 ± 3[a]	126 ± 2[a]
Urinary [Na^+] (mEq/L)	111 ± 13	102 ± 10	57 ± 15[a]	50 ± 15[a]
Na^+ excreted (mEq)	1.47 ± 0.15	1.59 ± 0.19	1.20 ± 0.33	1.09 ± 0.34
Fractional Na^+ excreted (%)	0.65 ± 0.17	0.63 ± 0.13	0.91 ± 0.40	1.72 ± 0.61
Serum [K^+] (mEq/L)	4.5 ± 0.3	5.1 ± 0.4	4.0 ± 0.5	4.3 ± 0.1
Urinary [K^+] (mEq/L)	281 ± 12	269 ± 5	199 ± 21[a]	180 ± 27[a]
K^+ excreted (mEq)	3.35 ± 0.17	4.12 ± 0.31[a]	3.65 ± 0.56	3.38 ± 0.39
Fractional K^+ excreted (%)	38 ± 6	43 ± 8	> 100[a]	> 100[a]

[Na^+], sodium concentration; [K^+], potassium concentration. [a]$P < 0.05$ *vs* sham group.

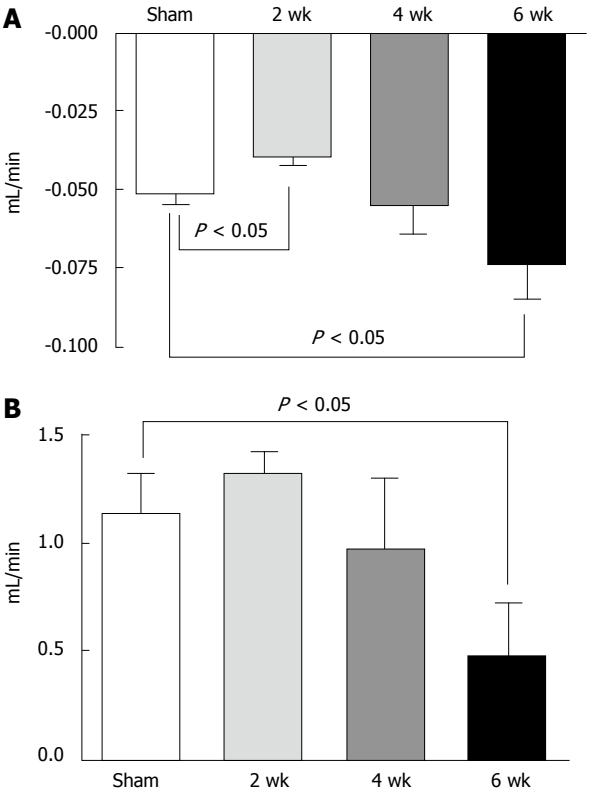

Figure 3 Free water and creatinine clearance in bile duct ligated (BDL) and sham operated rats. **A**: Free water clearance of sham operated rats (sham), and animals with 2-wk, 4-wk and 6 of bile duct ligation; **B**: Creatinine clearance of sham operated rats (sham), and animals with 2 wk, 4 wk and 6 wk of bile duct ligation.

should be pointed out that body weights were similar in all groups at the beginning of the experimental protocol and no differences were observed in water and food intake (data not shown). No ascites was observed in the rats at 2-wk after BDL. In contrast, animals at 4 wk and 6 wk clearly exhibited ascites, also indicating the presence of water retention.

DISCUSSION

This study supports the concept that the progression of hepatic damage promotes the manifestation of HRS. Indeed, the duration of BDL was positively correlated with renal function disarrangement without alterations in renal histology.

Animals at 2 wk of BDL seemed to be in a compensated state of hepatic injury, without ascites and alterations in water balance. These rats exhibited well-preserved renal function, suggesting that the homeostatic compensatory mechanisms remained intact at this moment of hepatic damage. Of note, serum creatinine was reduced in this group even when compared to sham operated animals. The creatinine clearance was slightly higher than in sham group, but significantly increased when compared to rats at 6 wk of BDL. These animals were also able to excrete water by increasing free water clearance. For this reason, serum osmolality remained at normal range and urinary osmolality was reduced when compared to sham. It has been reported that, at early stages of hepatic injury, as observed in animals with 2 wk of BDL, renal compensatory mechanisms against fluid retention still remain operating[1,3,4]. However, the progression of the process culminates in a non-compensated state by compromising the negative feedback loops of different regulatory systems[1,3,4]. Consistent with this, 4 wk after BDL, the rats already presented ascites, changes in water balance and an initial disturbance in renal function, revealed by sodium retention and an increase of the serum creatinine levels. After 6 wk of BDL, the hepatic damage evolved into a non-compensated stage with features of HRS[1,3,4], including reduction in creatinine clearance and an evident fluid retention associated with significant reductions of the serum osmolality and of the free water clearance.

Clinical studies have attempted to delineate the natural history of cirrhotic patients with ascites with

respect to the development of HRS. Factors predictive for the development of HRS include intense urinary sodium retention, dilutional hyponatremia, and increased activity of systemic vasoconstrictors[10]. These features were clearly evidenced in our bile duct ligated rats, mostly at 6 wk. However, some characteristics of bile duct ligated rats are not typically observed in HRS seen in clinical medicine. While patients with HRS are usually oliguric[1,3], our bile duct ligated animals at 4 wk and 6 wk increased the urinary volume when compared to the sham-operated group. It should be mentioned that, despite the elevation in the urinary volume, these animals still presented water and sodium retention, according to the observed reductions in free water clearance and in urinary sodium concentration. The so-called polyuria was not enough to excrete the whole amount of water and sodium retained by rats with 4 wk and 6 wk of BDL. Indeed, most investigators have used the BDL model to study pathological sodium retention[6,11], which occurs in liver disease. Another possible explanation for this apparently elevated urinary volume is the well-known effect of circulating bile acids in kidney function during obstructive jaundice[12,13]. Acute cholaemia may cause volume depletion by increasing urinary salt loss, which, in turn, may aggravate the direct nephrotoxicity of circulating bile compounds[12]. *In vitro* addition of bile acids or bilirubin at concentrations comparable to those found in the plasma of BDL rats, to a mixture of reactive enzymes strongly inhibited most, particularly mitochondrial oxidative phosphorylation[13]. Thus, high concentrations of these substances in the blood may explain the development of renal failure during liver disease, and its reversibility when liver function returns to normal[13]. It also should be noted that the normal kidney histology and the unchanged levels of renal hydroxyproline content also favors the existence of HRS, a syndrome characterized by functional rather than structural disarrangement of the kidneys in presence of progressive liver disease[1,4]. Despite the differences in renal parameters in HRS in our system and in HRS observed in humans, this model seems to be very useful to evaluate the progression of renal dysfunction in hepatic diseases, since BDL rats are normally able to maintain a residual diuresis, probably allowing their long-lasting survival[6].

The pathophysiology of HRS is still poorly understood. Hypoperfusion of the kidney due to active renal vasoconstrictors has been considered the hallmark of HRS[1-4]. In this context, Ozdogan and co-workers[14] conducted an elegant study that evidenced the role of endothelin-1, a potent vasoconstrictor, in an experimental model of HRS, which was induced by endotoxin administration to carbon tetrachloride-treated rats. In addition, the renin-angiotensin system (RAS) and the sympathetic nervous system, some of the major systems with a vasoconstrictor effect in the renal circulation, have been suggested as potential mediators of renal vasoconstriction in HRS[4,11,15]. During hepatic damage, systemic vasodilation and hyperdynamic circulation have been observed[16], which in turn promote an increase in sympathetic nervous activity, plasma renin activity (PRA), angiotensin Ⅱ and aldosterone levels, especially in the presence of HRS[3,4]. It is well known that angiotensin Ⅱ is one of the most powerful regulators of sodium excretion, operating through extrarenal as well as intrarenal mechanisms[17-19]. Some authors believe that, at the early stages of hepatic injury, the renal effects of angiotenisin Ⅱ represent a compensatory mechanism against the drop in organ perfusion pressure[3,4]. However, the development of renal impairment leading to HRS would occur as a result of an uncontrolled activation of systemic vasoconstrictor factors such as angiotensin Ⅱ, sympathetic nervous system, endothelin and others that could not be counteracted by vasodilators such as nitric oxide, prostaglandins, bradykinin and maybe angiotensin-(1-7)[3,4].

In this regard, we recently have shown that bile duct ligated rats presented different profiles of circulating RAS expression according to the progress of hepatic damage[20]. At early stages (1 wk and 2 wk of BDL), animals exhibited an elevation of angiotensin Ⅱ and angiotensin-(1-7) levels, without concomitant changes in PRA and angiotensin Ⅰ. With the progression of liver fibrosis (4 wk and 6 wk of BDL), RAS profile changed toward an overall enhancement of the PRA and the circulating levels of angiotensin Ⅰ, angiotensin Ⅱ and angiotensin-(1-7)[20]. According to these data[20], we hypothesize that not only angiotensin Ⅱ, but also angiotensin-(1-7) may possibly participate in the regulation of renal blood flow, glomerular filtration, and tubular transport in liver diseases. However, we still do not know how angiotensin-(1-7) could affect renal function in BDL rats. It has been clearly demonstrated that angiotensin-(1-7) also exerts complex renal actions[19,21,22]. Our group and others detected *in vivo* and *in vitro* antidiuretic effects of angiotensin-(1-7) by increasing fluid reabsorption[23-26]. These renal actions could contribute to sodium and water retention observed in bile duct ligated rats. In contrast, other studies showed that angiotensin-(1-7) has natriuretic and diuretic effects by inhibiting sodium reabsorption[27,28]. In addition, angiotensin-(1-7) seems to be involved in renal hemodynamic regulation by opposing the vasoconstrictive effects of angiotensin Ⅱ in glomerular vessels[29,30]. However, it is difficult to know if the changes in the components of the RAS preceded or were caused by the decline in renal function. The liver, or maybe also the kidney, could produce angiotensin peptides, which, in turn, act either as systemic hormones or as locally generated factors. Accordingly, Paizis *et al*[31] detected an up-regulation of angiotensin converting enzyme 2 (ACE2), the main enzyme responsible for angiotensin-(1-7) synthesis[22], in liver tissue from cirrhotic patients and bile duct ligated rats. Herath *et al*[32] showed increased expression of angiotensin-(1-7) receptor, the Mas receptor[33], in experimental billiary fibrosis, suggesting a role for ACE2-angiotensin-(1-7)-Mas axis in liver injury.

Finally, the overall state of sodium and water

balance and the effect of many circulating and/or local regulators may influence the direction of the observed renal actions in bile duct ligated rats. Further studies are necessary to clarify the mechanisms involved in the development of HRS in experimental cirrhosis. However, our data indicate that BDL emerges as a good model for the study of HRS.

COMMENTS

Background

Hepatorenal syndrome (HRS) has been defined as a progressive renal failure that occurs in patients with chronic liver disease and advanced hepatic failure in the absence of any apparent clinical cause for renal insufficiency. HRS corresponds to a functional alteration without histological changes in kidney tissue. There are many experimental models to induce hepatic fibrosis. However, none of them has been systematically evaluated as a model of hepatorenal syndrome.

Research frontiers

In this study, we aimed to systematically evaluate in bile duct ligated rats at different post-procedure time-points, whether there were alterations of renal function without changes in histopathology.

Innovations and breakthroughs

Renal dysfunction without histological changes occurred according to the duration of bile duct ligation (BDL) in the absence of any additional treatment. Animals at 2 wk of BDL exhibited a well-preserved renal function, suggesting that the renal homeostatic compensatory mechanisms remained intact at this moment of hepatic damage. However, the progression of the process culminates in a non-compensated state, as already shown by rats at 4 wk of BDL with ascites, changes in water balance, sodium retention and increased serum creatinine levels. After 6 wk of BDL, features of hepatorenal syndrome (HRS) became evident, including reduction in creatinine clearance and fluid retention without alterations in renal histology and renal tissue collagen content. Our data showed that BDL seems to be a helpful model for the study of HRS, since it mimics clinical conditions characterized by obstructive jaundice, such as biliary atresia and choledochal cysts.

Applications

The mechanisms for the renal changes observed in BDL animals remain unclear; however, this study indicates that BDL emerges as a good model for further studies of HRS and its treatment.

Peer review

This study is a well-designed experimental work, which tries to define an experimental model for hepatorenal syndrome.

REFERENCES

1 **Bataller R**, Sort P, Gines P, Arroyo V. Hepatorenal syndrome: definition, pathophysiology, clinical features and management. *Kidney Int Suppl* 1998; **66**: S47-S53
2 **Cardenas A**, Gines P. Hepatorenal syndrome. *Clin Liver Dis* 2006; **10**: 371-385, ix-x
3 **Arroyo V**, Jimenez W. Complications of cirrhosis. II. Renal and circulatory dysfunction. Lights and shadows in an important clinical problem. *J Hepatol* 2000; **32**: 157-170
4 **Gentilini P**, Vizzutti F, Gentilini A, Zipoli M, Foschi M, Romanelli RG. Update on ascites and hepatorenal syndrome. *Dig Liver Dis* 2002; **34**: 592-605
5 **Oberti F**, Vuillemin E, Fort J, Cales P. [Experimental models of portal hypertension] *Gastroenterol Clin Biol* 2000; **24**: 896-901
6 **Poo JL**, Estanes A, Pedraza-Chaverri J, Cruz C, Perez C, Huberman A, Uribe M. [Chronology of portal hypertension, decreased sodium excretion, and activation of the renin-angiotensin system in experimental biliary cirrhosis] *Rev Invest Clin* 1997; **49**: 15-23
7 **Kountouras J**, Billing BH, Scheuer PJ. Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. *Br J Exp Pathol* 1984; **65**: 305-311
8 **Reddy GK**, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. *Clin Biochem* 1996; **29**: 225-229
9 **Ishak K**, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, Desmet V, Korb G, MacSween RN. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; **22**: 696-699
10 **Cardenas A**, Gines P. Therapy insight: Management of hepatorenal syndrome. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 338-348
11 **Jonassen TE**, Brond L, Torp M, Graebe M, Nielsen S, Skott O, Marcussen N, Christensen S. Effects of renal denervation on tubular sodium handling in rats with CBL-induced liver cirrhosis. *Am J Physiol Renal Physiol* 2003; **284**: F555-F563
12 **Alon U**, Berant M, Mordechovitz D, Better OS. The effect of intrarenal infusion of bile on kidney function in the dog. *Clin Sci* (Lond) 1982; **62**: 431-433
13 **Israeli BA**, Bogin E. Biochemical changes in liver, kidney and blood associated with common bile duct ligation. *Clin Chim Acta* 1986; **160**: 211-221
14 **Ozdogan O**, Goren M, Ratip S, Giral A, Moini H, Enc F, Birsel S, Berkman K, Tozun N. Role of endothelin-1 in a cirrhotic rat model with endotoxin induced acute renal failure. *Hepatol Res* 2002; **24**: 114
15 **Aliaga L**, Zozoya JM, Omar M, Mediavilla JD, Prieto J. Interrelationships between systemic hemodynamics, urinary sodium excretion, and renin-angiotensin system in cirrhosis. *Acta Gastroenterol Belg* 1995; **58**: 213-221
16 **Blendis L**, Wong F. The hyperdynamic circulation in cirrhosis: an overview. *Pharmacol Ther* 2001; **89**: 221-231
17 **Kim S**, Iwao H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* 2000; **52**: 11-34
18 **Ichihara A**, Kobori H, Nishiyama A, Navar LG. Renal renin-angiotensin system. *Contrib Nephrol* 2004; **143**: 117-130
19 **Souza Dos Santos RA**, Passaglio KT, Pesquero JB, Bader M, Simoes E Silva AC. Interactions between angiotensin-(1-7), kinins, and angiotensin II in kidney and blood vessels. *Hypertension* 2001; **38**: 660-664
20 **Pereira RM**, Dos Santos RA, Teixeira MM, Leite VH, Costa LP, da Costa Dias FL, Barcelos LS, Collares GB, Simoes e Silva AC. The renin-angiotensin system in a rat model of hepatic fibrosis: evidence for a protective role of Angiotensin-(1-7). *J Hepatol* 2007; **46**: 674-681
21 **Simoes e Silva AC**, Pinheiro SV, Pereira RM, Ferreira AJ, Santos RA. The therapeutic potential of Angiotensin-(1-7) as a novel Renin-Angiotensin System mediator. *Mini Rev Med Chem* 2006; **6**: 603-609
22 **Chappell MC**, Modrall JG, Diz DI, Ferrario CM. Novel aspects of the renal renin-angiotensin system: angiotensin-(1-7), ACE2 and blood pressure regulation. *Contrib Nephrol* 2004; **143**: 77-89
23 **Santos RA**, Simoes e Silva AC, Magaldi AJ, Khosla MC, Cesar KR, Passaglio KT, Baracho NC. Evidence for a physiological role of angiotensin-(1-7) in the control of hydroelectrolyte balance. *Hypertension* 1996; **27**: 875-884
24 **Garcia NH**, Garvin JL. Angiotensin 1-7 has a biphasic effect on fluid absorption in the proximal straight tubule. *J Am Soc Nephrol* 1994; **5**: 1133-1138
25 **Vallon V**, Richter K, Heyne N, Osswald H. Effect of intratubular application of angiotensin 1-7 on nephron function. *Kidney Blood Press Res* 1997; **20**: 233-239
26 **Magaldi AJ**, Cesar KR, de Araujo M, Simoes e Silva AC, Santos RA. Angiotensin-(1-7) stimulates water transport in rat inner medullary collecting duct: evidence for involvement of vasopressin V2 receptors. *Pflugers Arch* 2003; **447**: 223-230
27 **Andreatta-van Leyen S**, Romero MF, Khosla MC, Douglas JG. Modulation of phospholipase A2 activity and sodium transport by angiotensin-(1-7). *Kidney Int* 1993; **44**: 932-936
28 **Handa RK**. Angiotensin-(1-7) can interact with the rat

proximal tubule AT(4) receptor system. *Am J Physiol* 1999; **277**: F75-F83

29 **Ren Y**, Garvin JL, Carretero OA. Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. *Hypertension* 2002; **39**: 799-802

30 **Sampaio WO**, Nascimento AA, Santos RA. Systemic and regional hemodynamic effects of angiotensin-(1-7) in rats. *Am J Physiol Heart Circ Physiol* 2003; **284**: H1985-H1994

31 **Paizis G**, Tikellis C, Cooper ME, Schembri JM, Lew RA, Smith AI, Shaw T, Warner FJ, Zuilli A, Burrell LM, Angus PW. Chronic liver injury in rats and humans upregulates the novel enzyme angiotensin converting enzyme 2. *Gut* 2005; **54**: 1790-1796

32 **Herath CB**, Warner FJ, Lubel JS, Dean RG, Jia Z, Lew RA, Smith AI, Burrell LM, Angus PW. Upregulation of hepatic angiotensin-converting enzyme 2 (ACE2) and angiotensin-(1-7) levels in experimental biliary fibrosis. *J Hepatol* 2007; **47**: 387-395

33 **Santos RA**, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci USA* 2003; **100**: 8258-8263

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RAPID COMMUNICATION

Effects of microalgae chlorella species crude extracts on intestinal adaptation in experimental short bowel syndrome

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Abstract

AIM: To evaluate the effects of chlorella crude extract (CCE) on intestinal adaptation in rats subjected to short bowel syndrome (SBS).

METHODS: Wistar rats weighing 230-260 g were used in the study. After anesthesia a 75% small bowel resection was performed. Rats were randomized and divided into groups. Control group (n = 10): where 5% dextrose was given through a gastrostomy tube, Enteral nutrition (EN) group (n = 10): Isocaloric and isonitrogen EN (Alitraq, Abbott, USA), study group (n = 10): CCE was administrated through a gastrostomy tube. Rats were sacrificed on the fifteenth postoperative day and blood and tissue samples were taken. Histopathologic evaluation, intestinal mucosal protein and DNA levels, intestinal proliferation and apoptosis were determined in intestinal tissues, and total protein, albumin and citrulline levels in blood were studied.

RESULTS: In rats receiving CCE, villus lengthening, crypt depth, mucosal DNA and protein levels, intestinal proliferation, and serum citrulline, protein and albumin levels were found to be significantly higher than those in control group. Apoptosis in CCE treated rats was significantly reduced when compared to EN group rats.

CONCLUSION: CCE has beneficial effects on intestinal adaptation in experimental SBS.

Key words: Short-bowel syndrome; Intestinal adaptation; Chlorella; Nutrition; Microalgae

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Kerem M, Salman B, Pasaoglu H, Bedirli A, Alper M, Katircioglu H, Atici T, Perçin EF, Ofluoglu E. Effects of microalgae chlorella species crude extracts on intestinal adaptation in experimental short bowel syndrome. *World J Gastroenterol* 2008; 14(28): 4512-4517 Available from: URL: http://www.wjgnet.com/1007-9327/14/4512.asp DOI: http://dx.doi.org/10.3748/wjg.14.4512

INTRODUCTION

Short bowel syndrome (SBS) is a clinical condition characterized by diarrhea, dehydration, electrolyte imbalance, malabsorption, and progressive malnutrition related to a wide resection of the small intestine[1-3]. In the pediatric population, necrotizing enterocolitis, gastroschisis, omphalocele, intestinal atresia and Hirschsprung disease, and in the adults mesenteric vascular occlusion, inflammatory bowel disease (IBD) and malignancies, are the most common reasons for performing extensive resection of the intestine[2]. Retaining intestinal autonomy depends on the length of remaining intestine and the adaptive capacity of the intestinal remnant. Some compensatory changes occur after massive intestinal resection in order to maintain adequate digestion. Restoration of the absorptive surface area and functional capacity result in morphologic and functional improvement. Structural adaptation after

intestinal resection involves intestinal dilatation and elongation, villus lengthening, and increasing crypt cell proliferation. These changes result in a marked increase in the intestinal absorptive surface area[4]. The functional adaptation mechanism in the remnant intestine is still not entirely understood. Regulation of intestinal adaptation is an extremely complicated process influenced by many factors[5]. Some of these factors are nutrients, gastrointestinal secretions, hormones, and a variety of polypeptides stimulating growth capability[6]. The most important therapeutic objectives in the management of SBS are maintenance of the patient's calorie intake and nutritional status. Optimal intestinal rehabilitation should enhance the intestinal adaptation and shorten the period of intestinal recovery[7,8]; Yet, today no such optimal therapy exists. However, some enteral nutrition (EN) products are used for energy supports in order to reduce demand for total parenteral nutrition (TPN). The prospective, randomised and double blind clinical study of Byrne and colleagues[9] showed that glutamine, growth factor and optimum diet could reduce the length of TPN support. O'Dwyer and colleagues[10] emphasized that glutamine enriched TPN significantly improved the mucosal recovery and adaptation. New treatment alternatives to the current ones are still under research in experimental and clinical studies.

Chlorella is a species of green algae that grows in fresh water. The name Chlorella is taken from the Greek word meaning "small, fresh green", it contains the highest level of chlorophyll in the world when compared with all other nutrients. It has been consumed as a food source for centuries mainly in Japan and other Far East countries, and suggestion of its healing properties has enhanced consumption[11,12]. Biotechnological processing for single cell protein production is the most emphasized area of chlorella studies. Because of high protein ingredients, chlorella was considered to be a protein source in the beginning, but later it was seen as a "functional nutrient" first in Japan then Europe and America, and today it is accepted that chlorella is rich in nutritional ingredients[11,12]. Active ingredients of chlorella are: 61.6% protein, 12.5% fat, 13.7% carbohydrate, trace elements (Al, Ze, P, Ca, Mg, Mn, Ni, Se), vitamins (carotene, beta-carotene, thiamine, B_1, B_2, B_6, C, D, E, K), nucleic acids (RNA and DNA), and various enzymes[12,13]. In our previous research, we showed that feeding with chlorella crude extract (CCE) has beneficial effects on malnourished rats which had undergone colon anastomosis[13].

In the current study, we aimed to evaluate the efficacy of chlorella extract which is rich in amino acids, beta carotene, and trace elements on intestinal adaptation in SBS.

MATERIALS AND METHODS

All procedures were conducted according to recommendations of the Animal Research Committee at Gazi University in Ankara, Turkey. Wistar rats weighing 230-260 g were used in the study. The rats were maintained at 23℃ in a 12 h light dark cycle, with free access to water and standard rat chow for a week. Eight hours prior to the start of experiments, rats were deprived of food while drinking water was available ad libitum. Animals were anesthetized by an intramuscular injection of 40 mg/kg ketamine (Ketalar®, Parke Davis, Eczacıbasi, Istanbul, Turkey) and 5 mg/kg xylazine (Rompum®, Bayer AG, Leverkusen, Germany). Rats underwent central venous catheterization by inserting the catheter into the right external jugular vein, and were operated on under sterile conditions. A gastrostomy tube was placed for enteral feeding before the 75% small bowel resection. A 3 cm midline laparotomy was performed and intestinal transections were done 15 cm above the ileocecal junction and 5 cm from the duodenojejunal transition. Interrupted sutures of 7-0 PDS (Ethicon®, USA) were used for end to end bowel anastomosis. The venous catheter and the gastrostomy tube were tunneled subcutaneously through the dorsal cervical area, and attached with special apparatus (Swivel 56-1308; Harvard Apparatus, USA) which has a port and equipped with a spring system just beneath the skin. Before the closure of the abdominal cavity, 3 mL saline was administered intraperitoneally for the fluid resuscitation.

During the postoperative 3 d, rats received 60 kcal non-protein energy and 0.414 g nitrogen total paranteral nutrition (TPN). After postoperative day 4, rats were randomized and divided into the groups below: (1) Sham group: Laparotomy was performed; (2) Control group: 5% dextrose 12 mL/24 h was given to the rats by the gastrostomy tube with the infusion pump; (3) EN group: isocaloric (60 kcal/d) and isonitrogen (0.686 g/d) Alitraq (Abbott, USA) given to the rats through the gastrostomy tube with the infusion pump. Since, Alitraq is the EN product which has the highest amount of glutamine, and research clearly shows that glutamine has beneficial effect in SBS, Alitraq was used for enteral feeding in this study; (4) Study group: rats received CCE (60 kcal/d) through the gastrostomy tube by the infusion pump. In the sham, both EN and study group rats were not allowed to eat solid food, but were free to drink water.

Rats were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentothal before laparotomy. The length of the small bowel was measured from the Treitz to the caecum. After withdrawal of blood samples from vena cava, rats were sacrificed by bleeding. Intestinal resection was quickly performed, and specimens were washed with cold saline. Histopathologic samples were taken from both the jejunal and ileal side of the anastomosis, and the rest of it was weighed.

Preparation of the algae extract, its ingredients and its use

Culturing and growth conditions: Collection and isolation of microalgae were made in compliance with Rippka *et al*[14]. Microalgae were obtained from GUMACC (Gazi University Microalgae Culture Collection) *Chlorella sp.* C1 were expanded in number by culture in BG11 nutrition medium (blue-green medium 11) at less than 3000 lux light intensity, under illumination for 16 h

Table 1 Total biochemical, histopathology and DNA results

	Sham	Control	EN	CCE	ANOVA	Sheffe's
Weight loss (g)	0 ± 0	52 ± 8	34 ± 6	24 ± 5	< 0.001	a, c, e
Width of jejunum (cm)	0.84 ± 0.1	0.48 ± 0.4	0.62 ± 0.3	0.78 ± 0.1	< 0.05	c, e, g
Length of jejunum villi (mm)	0.74 ± 0.22	0.38 ± 0.18	0.54 ± 0.12	0.76 ± 0.11	< 0.05	c, e, g
Depth of jejunum crypt (mm)	0.51 ± 0.13	0.32 ± 0.13	0.52 ± 0.10	0.68 ± 0.08	< 0.05	c, e, g
Number of jejunal mitosis (*n*)	11.2 ± 2.1	4.2 ± 2.1	13.4 ± 4.3	22.8 ± 5.2	< 0.001	c, e, g
Width of ileum (cm)	0.84 ± 0.1	0.48 ± 0.4	0.62 ± 0.3	0.78 ± 0.1	< 0.05	c, e, g
Length of ileum villi (mm)	0.74 ± 0.22	0.38 ± 0.18	0.54 ± 0.12	0.56 ± 0.11	< 0.05	a, c
Depth of ileum crypt (mm)	0.61 ± 0.10	0.36 ± 0.13	0.58 ± 0.19	0.64 ± 0.08	< 0.05	c, e, g
Number of ileum mitosis (*n*)	11.2 ± 2.1	4.2 ± 2.1	13.4 ± 4.3	22.8 ± 5.2	< 0.001	c, e, g
Total protein (mg/dL)	6.8 ± 1.8	4.3 ± 1.3	6.1 ± 1.4	6.6 ± 2.0	< 0.05	c, g
Albumin (mg /dL)	2.2 ± 0.2	1.1 ± 0.3	1.9 ± 0.1	2.0 ± 0.3	< 0.05	c, g
Serum citrulline (micromol/L)	72.2 ± 11.2	34.2 ± 6.2	52.3 ± 7.9	68.8 ± 9.8	< 0.001	c, g
Mucosal DNA (ng/μL)	622 ± 48	318 ± 32	716 ± 61	898 ± 182	< 0.001	c, g
Mucosal protein (mg/mL)	16.2 ± 3.8	6.2 ± 2.6	12.9 ± 3.8	15.3 ± 4.5	< 0.001	c, g
Cell proliferation index Jejunum	310 ± 27	550 ± 40	710 ± 50	850 ± 55	< 0.001	a, c, g
(BrdU (+) cells/ 10 crypts) Ileum	280 ± 32	510 ± 35	730 ± 45	970 ± 65	< 0.001	a, c, g
Apoptosis index Jejunum	12 ± 2.1	25 ± 3.2	18 ± 3.1	14 ± 2.1	< 0.001	a, c, g
(Apoptotic cell/1000 cell) Ileum	13 ± 2.3	29 ± 2.5	21 ± 4.1	16 ± 3.7	< 0.001	a, c, g

[a]$P < 0.05$, Sham group *vs* other groups; [c]$P < 0.05$, CCE and EN and control group; [e]$P < 0.05$, CCE *vs* EN group; [g]$P < 0.05$, control *vs* other groups.

and under darkness for 8 h. Algae were harvested after approximately a 15-d production period.

Preparation of the extracts: Algal mass from an axenic exponential culture of the microalgae strains grown in BG11 were separated from the culture medium by centrifugation and pellets were dried at 60℃ for 24 h. Methanol extracts were prepared according to the methods of Khan *et al*[15] and Vlachos *et al*[16], from dry algal mass (ratio 1:15 g/mL) extracted throughout 24 h. After separating the extraction phase, all of the extracts were preserved at 4℃. The *chlorella sp.* extract was resuspended at 1 g/mL in 0.9% sterile saline.

Ingredients of algae extract and its use: The dose of algae extract used was 50 g/kg BW/d[13,15,16]. The suspension was given *via* oral gavage three times a day in equal doses (each dose was less than 5 mL).

Biochemical analysis

Serum protein, albumin and blood sugar levels were analyzed from blood samples. The mucosal layers of the intestinal samples were brushed with slides and then weighed. After a homogenization process, mucosal DNA and protein levels were evaluated by the techniques of Chomczynski as described previously[17].

Serum citrulline levels

Serum citrulline levels were measured by the tandem mass spectrophotometric technique with isotope embedded amino acid standards. The results were expressed as mmol/L.

Histopathology

Jejunal and ileal tissue samples were fixed in a 10% solution of formaldehyde, and embedded in paraffin wax from which 3-μm-thick sections were mounted on slides. The sections were stained with hematoxylin-eosin (HE), and mucosal widening, villus length and crypt depth were evaluated.

Enterocyte proliferation and apoptosis

Crypt cell proliferation was determined using 5-bromodeoxyuridine (BrdU). Twelve hours before sacrifice, 100 mg/kg BrdU was given intraperitoneally to the rats. Sections were stained with anti-BrdU antibodies. Every 10 crypts stained with positive BrdU were calculated as a proliferation index. The TUNEL technique was used for the determination of apoptotic cells.

Statistical analysis

All values were expressed as mean ± SE and the results were compared by analysis of variance (one-way ANOVA) and Scheffe's post hoc analysis. $P < 0.05$ was considered statistically significant. Statistical evaluation was carried out using SPSS 11.5 software (SPSS, Chicago, IL, USA).

RESULTS

Three rats died during the creation of the SBS, and they were replaced with new ones.

Weight lost

All rats subjected to SBS lost significant weight when compared with their orginal weight before the experiment. In the EN and study (CCE) groups, rats lost less weight than the control group ($P < 0.05$). When they were compared with each other, it was observed that in the CRE group rats lost significantly less weight than the EN group ($P < 0.001$, Table 1).

Histopathologic evaluation

In the control group, mucosal widening, villous length, crypt depth and amount of mitosis markedly decreased

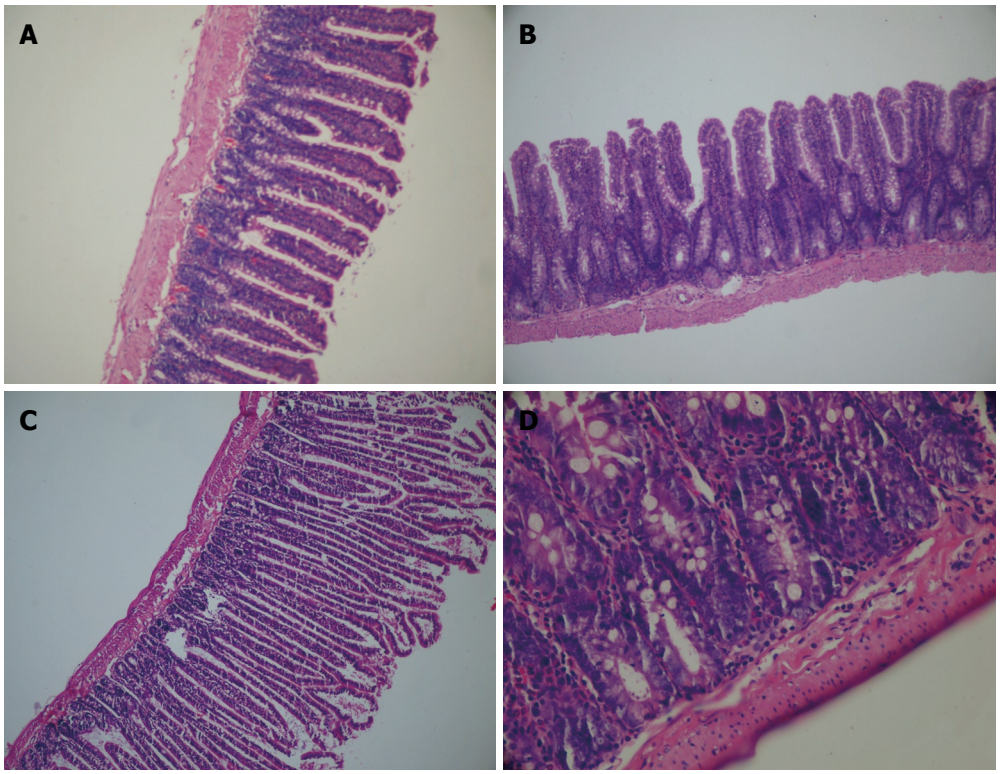

Figure 1 Villi and crypts in the ileum of rats which underwent SBS (HE, x 100): Control group (**A**) and EN group (**B**); **C**: Significant elongation of villi in CCE group (İleum, HE, x 100); **D**: Significant mitotic figures in the crypts in the CCE group (İleum, HE, x 400).

when compared to sham, EN and CCE groups ($P < 0.05$). Jejunal and ileal mitosis number in the EN and CCE groups were significantly higher than those at the control group ($P < 0.05$). The amount of mitosis in both segments of intestine in the CCE group was markedly higher than those in the EN group. Villus length and mucosal widening were not significantly different between the EN and CCE groups, whereas crypt depth was remarkably increased in the CCE group over the others ($P < 0.05$, Table 1, Figure 1).

Mucosal DNA levels

Mucosal DNA levels significantly decreased in the control group when compared to the other groups. The mucosal DNA levels for EN and CCE groups were found to be remarkably higher than the sham and control groups. Moreover, the same parameters significantly increased in the CCE group when compared to the EN group ($P < 0.05$, Table 1).

Mucosal protein levels

Ileal mucosal protein levels were significantly higher in the CCE group than all other groups ($P < 0.05$). The mucosal protein levels of the control group were markedly reduced when compared the other groups ($P < 0.05$). There was no difference between the EN and CCE groups ($P < 0.05$, Table 1).

Cell proliferation index

The jejunal and ileal cell proliferation indexes were significantly higher in the CRE group than all other groups ($P < 0.05$). The cell proliferation indexes in the control group were increased when compared to the sham group while they were significantly lower than the EN and CCE groups ($P < 0.05$, Table 1).

Apoptotic index

Apoptosis in the control group was markedly increased compared to other groups. The apoptotic index in subjects fed with CCE was significantly lower than those in the EN and control groups ($P < 0.05$), and it was found to be insignificantly higher than the apoptotic indexes of the sham group ($P < 0.05$, Table 1).

DISCUSSION

Loss of small bowel function caused by extended intestinal resection results in malabsorption and fluid and electrolyte imbalances[1]. In the early postoperative period the first priority of SBS treatment is adequate resuscitation of volume and electrolyte disturbances. When these parameters are stabilized, parenteral nutrition can be started[1-3]. Although parenteral nutrition causes a significant decrease in the mortality rates of SBS, the time required for optimal TPN therapy is too long and has many disadvantages and severe complications[4-7]. Searching for new treatment methods for increasing bowel adaptation mechanisms in order to reduce complications of SBS is an area which many recent studies concentrated on. The hormones; bombesin, growth factors, insulin like growth factors, ghrelin, leptin, and EN products; glutamine, fish oil (omega-3 fatty acids), immune

nutrients and fibers, have been investigated, and found to have beneficial effects on bowel adaptation mechanism in SBS[8-10,18]. We designed our research on the use of species of chlorella algae for healing effects because of its high protein, nucleic acid, antioxidant and fiber content[11,12]. In this study, it was observed that enteral feeding with CCE has beneficial effects on intestinal adaptation in rats with SBS. Lengthening of the intestinal villus, increasing crypt depth, intestinal proliferation, mucosal protein, DNA, serum citrulline, protein and albumin levels, and decrease of apoptosis were found in the study. This is the first study to demonstrate the healing effects of chlorella on SBS.

When we look at the current literature, we can find few trials of different algae species[19,20]. Tokida ameliorated murine chronic colitis through down-regulation of interleukin-6 production on colonic epithelial cells. Sakai *et al*[21] have also described that *Sargassum horneri*, a marine brown algae, increases Cl^- absorption in isolated rat colon by activation of leukotrienes. In another study, it was shown that algae extract can reduce inflammation and ultrastructural changes in rats colitis induced by acetic acid[20]. Gonzalez *et al*[22], demonstrated that intestinal myeloperoxidase enzyme levels remarkably decrease in response to algae extract. Dvir and colleagues[23] suggested that red algae can regulate intestinal physiology and lipid metabolism, it can also be used as a functional nutrient. The same investigators claimed that algae-derived polysaccharides can increase jejunal muscular hypertrophy, and algae fiber can lengthen both colon and small bowel and increase colonic transit time by 44% compared to control group. Significant increases in mucosal villous lengthening and crypt depth due to the effects of micro-algae were observed in the same study. Although it is very rare, we can see the use of algae for SBS in the literature.

To our knowledge, if fluid resuscitation is not subsequently provided following the surgery, mortality can be significantly high in animal models of SBS[1]. For this reason, we inserted an intravenous line in rats, before starting TPN, and we resuscitated them with iv fluid for a short period of time. After the third postoperative day, rats received dextrose in addition to EN and CCE. They were weighed daily until the end of the experiment. All rats subjected to SBS lost significant weight. However, weight lost in rats fed with EN and CCE was markedly less than the control group. Though both EN and CCE resulted in weight gain in rats, the CCE group rats gained more weight than the EN group. Both nutritional solutions have almost the same energy distribution, but CCE has higher nucleotide content, and better absorptive and adaptive capacity than the EN, so rats gained more weight in the CCE group than those in other groups. In our previous study, we observed that CCE increases weight gain in malnourished rats.

Recent articles feature the amino acid citrulline, the best marker of intestinal absorptive capacity in SBS due to massive intestinal resection[3-7]. The clinical study of Rhoads *et al*[24], found that there was a correlation between serum citrulline levels and enteral tolerance, and levels of serum citrulline could be used as a predictive test. In our study, fifteenth day serum citrulline levels were significantly lower than those in other groups. However, the CCE group serum citrulline levels were markedly higher than those in the control and EN groups. Another result of absorptive and adaptive responses was the significant increase in serum protein and albumin levels in CCE fed rats when compared to control rats. High amino acid and nucleotide levels in CCE could be responsible for these effects.

Mucosal DNA and BrdU proliferation index for evaluating intestinal proliferation rate were found significantly reduced in SBS rats when compared to control group rats. Mucosal DNA and intestinal proliferation index were markedly higher in CCE and EN groups than those in control group. These parameters also increased in the CCE group compared to the EN group. Excessive amount of nucleic acid, protein, vitamin and other substances in CCE may be responsible for these outcomes.

In conclusion, enteral administration of CCE increases intestinal adaptation and proliferation in experimental SBS. The current study provides preliminary data for future research. More studies are needed to investigate the use of algae species growing in water as a clinical nutrition product.

COMMENTS

Background

Chlorella is a species of green algae that grows in fresh water. The name chlorella is taken from the Greek word meaning "small, fresh green", it contains the highest level of chlorophyll in the world when compared with the all other nutrients. It has been consumed as a food source for centuries mainly in Japan and other Far East countries, and suggestion of its healing properties has enhanced consumption. However, there is little data about the use of chlorella in disease, which is investigated in this experimental study.

Research frontiers

Short bowel syndrome (SBS) which affects both children and adults is a generally seen disease. The basis of treatment for this disease is enteral and intravenous nutrition. Several enteral nutrition (EN) products have been used for SBS. The aim of this study is to evaluate the effect of chlorella in SBS in rats.

Innovations and breakthroughs

In this study, positive effects of orally given chlorella were seen. This is the first study on this subject. It was seen that chlorella increased intestinal adaptation in SBS.

Applications

This experimental study will guide new experimental and clinical studies. Chlorella is an algae which is widely found in both salt and fresh water. As a usage for EN, it can be used for acute pancreatitis, inflammatory bowel diseases, colitis studies and clinical studies.

Peer review

In this experimental study about SBS, orally given CCE showed a positive effect on parameters of intestinal adaptation. Since it is the first study that shows positive effects of algae in SBS, this is an interesting study.

REFERENCES

1 **Collins JB 3rd**, Georgeson KE, Vicente Y, Hardin WD Jr. Comparison of open and laparoscopic gastrostomy and fundoplication in 120 patients. *J Pediatr Surg* 1995; **30**: 1065-1070; discussion 1070-1071

2 **Vanderhoof JA**, Langnas AN. Short-bowel syndrome in

children and adults. *Gastroenterology* 1997; **113**: 1767-1778
3 **Wilmore DW**, Robinson MK. Short bowel syndrome. *World J Surg* 2000; **24**: 1486-1492
4 **Kvietys PR**. Intestinal physiology relevant to short-bowel syndrome. *Eur J Pediatr Surg* 1999; **9**: 196-199
5 **Weale AR**, Edwards AG, Bailey M, Lear PA. Intestinal adaptation after massive intestinal resection. *Postgrad Med J* 2005; **81**: 178-184
6 **Riecken EO**, Stallmach A, Zeitz M, Schulzke JD, Menge H, Gregor M. Growth and transformation of the small intestinal mucosa--importance of connective tissue, gut associated lymphoid tissue and gastrointestinal regulatory peptides. *Gut* 1989; **30**: 1630-1640
7 **Messing B**, Crenn P, Beau P, Boutron-Ruault MC, Rambaud JC, Matuchansky C. Long-term survival and parenteral nutrition dependence in adult patients with the short bowel syndrome. *Gastroenterology* 1999; **117**: 1043-1050
8 **Jackson CS**, Buchman AL. The nutritional management of short bowel syndrome. *Nutr Clin Care* 2004; **7**: 114-121
9 **Byrne TA**, Wilmore DW, Iyer K, Dibaise J, Clancy K, Robinson MK, Chang P, Gertner JM, Lautz D. Growth hormone, glutamine, and an optimal diet reduces parenteral nutrition in patients with short bowel syndrome: a prospective, randomized, placebo-controlled, double-blind clinical trial. *Ann Surg* 2005; **242**: 655-661
10 **O'Dwyer ST**, Smith RJ, Hwang TL, Wilmore DW. Maintenance of small bowel mucosa with glutamine-enriched parenteral nutrition. *JPEN J Parenter Enteral Nutr* 1989; **13**: 579-585
11 **Lahaye M**. Marine algae as sources of fibers: Determination of soluble and insoluble dietary fiber contents in some sea vegetables. *J Sci Food Agric* 1991; **54**: 587-594
12 **Michel C**, Benard C, Lahaye, M, Formaglio D, Kaeffer B, Quemener B, Berot S, Yvin JC, Blottiere HM, Cherbut C. Algal oligosaccharides as functional foods: in vitro study of their cellular and fermentative effects; Les oligosides algaux comme aliments fonctionnels: etude in vitro de leurs effets cellulaires et fermentaires. *Sciences des aliments* 1999; **19**: 311-332
13 **Salman B**, Kerem M, Bedirli A, Katircioglu H, Ofluoglu E, Akin O, Onbasilar I, Ozsoy S, Haziroglu R. Effects of Cholerella sp. microalgae extract on colonic anastomosis in rats with protein-energy malnutrition. *Colorectal Dis* 2008; **10**: 469-478
14 **Rippka R**. Isolation and purification of cyanobacteria. *Methods Enzymol* 1988; **167**: 3-27
15 **Khan NH**, Rahman M, Nur-e-Kamal MS. Antibacterial activity of Euphorbia thymifolia Linn. *Indian J Med Res* 1988; **87**: 395-397
16 **Vlachos V**, Critchley AT, von Holy A. Establishment of a protocol for testing antimicrobial activity in southern African macroalgae. *Microbios* 1996; **88**: 115-123
17 **Chomczynski P**. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 1993; **15**: 532-534, 536-537
18 **Booth IW**. Enteral nutrition as primary therapy in short bowel syndrome. *Gut* 1994; **35**: S69-S72
19 **Matsumoto S**, Nagaoka M, Hara T, Kimura-Takagi I, Mistuyama K, Ueyama S. Fucoidan derived from Cladosiphon okamuranus Tokida ameliorates murine chronic colitis through the down-regulation of interleukin-6 production on colonic epithelial cells. *Clin Exp Immunol* 2004; **136**: 432-439
20 **Berge JP**, Debiton E, Dumay J, Durand P, Barthomeuf C. In vitro anti-inflammatory and anti-proliferative activity of sulfolipids from the red alga Porphyridium cruentum. *J Agric Food Chem* 2002; **50**: 6227-6232
21 **Sakai H**, Uchiumi T, Lee JB, Ohira Y, Ohkura J, Suzuki T, Hayashi T, Takeguchi N. Leukotrienes-mediated effects of water extracts from Sargassum horneri, a marine brown alga, on Cl- absorption in isolated rat colon. *Jpn J Physiol* 2004; **54**: 71-77
22 **Gonzalez R**, Rodriguez S, Romay C, Ancheta O, Gonzalez A, Armesto J, Remirez D, Merino N. Anti-inflammatory activity of phycocyanin extract in acetic acid-induced colitis in rats. *Pharmacol Res* 1999; **39**: 55-59
23 **Dvir I**, Chayoth R, Sod-Moriah U, Shany S, Nyska A, Stark AH, Madar Z, Arad SM. Soluble polysaccharide and biomass of red microalga Porphyridium sp. alter intestinal morphology and reduce serum cholesterol in rats. *Br J Nutr* 2000; **84**: 469-476
24 **Rhoads JM**, Plunkett E, Galanko J, Lichtman S, Taylor L, Maynor A, Weiner T, Freeman K, Guarisco JL, Wu GY. Serum citrulline levels correlate with enteral tolerance and bowel length in infants with short bowel syndrome. *J Pediatr* 2005; **146**: 542-547

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RAPID COMMUNICATION

Risk factors of thrombosis in abdominal veins

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Abstract

AIM: To estimate the prevalence of inherited and acquired thrombophilic risk factors in patients with abdominal venous thrombosis and to compare the risk factor profiles between Budd-Chiari syndromes (BCS) and splanchnic vein thrombosis (SVT).

METHODS: In this retrospective study, 36 patients with abdominal venous thrombosis were studied. The patients were divided into Budd-Chiari group (hepatic vein, IVC thrombosis) and splanchnic venous thrombosis group (portal, splenic, superior mesenteric veins) based on the veins involved. Hereditary and acquired thrombophilic risk factors were evaluated in all patients.

RESULTS: Twenty patients had SVT, 14 had BCS, and 2 had mixed venous thrombosis. Ten patients (28%) had hereditary and 10 patients (28%) acquired thrombophilic risk factors. The acquired risk factors were significantly more common in the SVT group (SVT *vs* BCS: 45% *vs* 7%, χ^2 = 5.7, P = 0.02) while hereditary risk factors did not show significant differences between the two groups (SVT *vs* BCS: 25% *vs* 36%, χ^2 = 0.46, P = 0.7). Multiple risk factors were present in one (7%) patient with BCS and in 3 patients (15%) with SVT. No risk factors were identified in 57% of patients with BCS and in 45% of patients with SVT.

CONCLUSION: Hereditary and acquired risk factors play an important role in the etiopathogenesis of abdominal venous thrombosis. Acquired risk factors are significantly more common in SVT patients while hereditary factors are similar in both groups.

Key words: Budd Chiari syndrome; Splanchnic vein thrombosis; Risk factors; Hereditary; Risk comparison

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INTRODUCTION

Abdominal venous thrombosis may present as Budd-Chiari Syndrome (BCS) (thrombosis of inferior vena cava and/or hepatic veins) or splanchnic venous thrombosis (SVT) (occlusion of portal, splenic, superior or inferior mesenteric veins). Hereditary and acquired risk factors have been implicated in the etiopathogenesis of abdominal venous thrombosis[1,2]. Hereditary risk factors for thrombophilia include Factor V Leiden gene mutation, prothrombin gene mutation, homozygous methyl tetrahydrofolate reductase (*MTHFR*) gene mutation, and deficiencies of coagulation inhibitor protein C, protein S and antithrombin Ⅲ (AT Ⅲ)[3-7]. Causes of acquired thrombophilia are myeloproliferative disorders, malignancy, surgery, antiphospholipid syndrome, pregnancy, oral contraceptives, and infection[8-11]. Identification of these risk factors may help in evaluation, planning therapy, or screening family members to evaluate an individual risk.

There are few studies from South Asian regions which have comprehensively evaluated prothrombotic risk factors in BCS and portal venous thrombosis (PVT)[12,13]. These studies did not assess risk factors in patients with mesenteric venous thrombosis. Other studies have evaluated individual risk factors or multiple risk factors in single venous thrombosis[14-17]. The aim

of the study was to analyse prothrombotic etiological profiles (hereditary and acquired) in patients with abdominal venous thrombosis and to compare the profiles of the BCS and SVT groups.

MATERIALS AND METHODS

Patients admitted with abdominal venous thrombosis that had complete etiological work up during the period July 1997 to June 2006 were included in the study. Patients with incomplete evaluation (acute thrombosis or on anticoagulants) were excluded. Diagnosis of thrombosis was based on Doppler sonography, abdominal computed tomography (CT), or venography. For all selected patients, clinical information and laboratory data were collected by a standardized review of medical charts using uniform structured data forms. Details of acquired prothrombotic risk factors like abdominal surgery, oral contraceptives, pregnancy, liver cirrhosis, antiphospholipid syndrome, infection, or others were also obtained.

Genetic tests for mutation in Factor V Leiden gene (1691, G-A), *MTHFR* gene (677 C-T), and prothrombin gene (20210, G-A) were done in all the patients by PCR amplification of the respective gene segments[18-20]. The amplified products were subjected to restriction digestion fragment length polymorphism (RFLP) analysis. Protein C and AT Ⅲ were assessed using chromogenic assays, done on the coagulation analyzer (Dade Behring's Sysmex CA 1500). Free protein S was estimated by an immunoassay (Chromogenix Coamatic Protein S Free, IL) done on the ACL Advance (Instrumentation Laboratory). The assays for protein C, protein S, and AT Ⅲ were run concurrently with normal control and abnormal control (substrate present in low level simulating deficiency states) samples for validation as well as comparison with normal. The normal reference ranges of various tests were protein C: 50%-150% of normal; protein S: 50%-150% of normal; AT Ⅲ: 80%-120% of normal. Patients were considered to have protein C, protein S, or AT Ⅲ deficiency only if liver dysfunction was ruled out.

Statistical analysis

Comparison between the BCS and the SVT group was done by Fischer's exact test for categorical variables and Mann Whitney *U* test for continuous variables. A *P* value of < 0.05 was considered significant. All analysis was performed in SPSS for Windows Version 11.

RESULTS

Thirty-six patients with thrombosis of abdominal veins were studied. The mean age of the patients was 36.7 years (range: 3-69 years). There were 24 males (67%) and 12 females (33%). Abdominal pain, the commonest symptom, was seen in 16 (44%), hepatomegaly in 4 (11%), splenomegaly in 10 (28%), and ascites in 13 (36%) patients. Acute presentation was more common in SVT (40%) than in BCS (21%). Diagnosis of abdominal venous thrombosis was made by Doppler sonography in 21 patients (58%), CECT abdomen in 10 (28%), and venography in 5 (14%) patients. Twenty patients had thrombosis of splanchnic veins (SVT), 14 had thrombosis of inferior vena cava and/or hepatic vein (BCS) and 2 had thrombosis in both splanchnic and IVC/hepatic veins.

The site of thrombosis along with details of hereditary and acquired risk factors in all patients studied is shown in Table 1. Hereditary risk factors were present in 10 (28%) patients and acquired risk factors in 10 (28%) patients. The most common hereditary risk factors were Factor V Leiden gene mutation (11%) and AT Ⅲ deficiency (11%) followed by protein C deficiency (8%). None of the patients had a prothrombin gene mutation, protein S deficiency, or was homozygous for *MTHFR* gene mutation. *MTHFR* mutation (heterozygous) was seen in 22% patients, which is not considered a risk factor for thrombosis. In the BCS group (14 patients): IVC obstruction alone was present in 5 patients, hepatic vein (HV) obstruction alone in 6 patients, and IVC + HV obstruction in 3 patients. Hereditary risk factors were present in 5 (36%) patients and acquired risk factor in one (7%) patient. In SVT group (20 patients): portal vein (PV) obstruction alone was present in 4 patients, splenic vein (SV) obstruction alone in 2 patients, superior mesenteric vein (SMV) obstruction alone in 1 patient, PV + SMV obstruction in 3 patients, and PV + SV + SMV obstruction in 10 patients. Hereditary risk factors were present in 5 (25%) patients and acquired risk factors in 9 (45%) patients.

Comparison of risk factor profiles between the BCS and the SVT group is shown in Table 2. Hereditary risk factors were higher in the BCS group (BCS *vs* SVT: 36% *vs* 25%, $P = 0.7$), but this difference did not reach statistical significance. Acquired risk factors were significantly higher in the SVT group (SVT *vs* BCS: 45% *vs* 7%, $P = 0.02$). The prevalence of multiple risk factors in the BCS and the SVT group are shown in Table 3. More than one risk factor was seen in 1 (7%) patient in the BCS group and in 4 (20%) patients in the SVT group. No risk factor was identified in 57% of patients in the BCS group and in 45% of patients in the SVT group.

DISCUSSION

This study evaluated hereditary and acquired risk factors in 36 patients with abdominal venous thrombosis. Hereditary risk factors were identified in 36% of patients with BCS and in 25% of patients with SVT. Acquired risk factors were detected in 7% of patients with BCS and in 45% of patients with SVT.

Prevalence of Factor V Leiden mutation (FVLM), the most common cause of inherited thrombophilia, is variable in different populations[21]. Risk of venous thrombosis is 5- to 8-fold in heterozygotes and 50- to 80-fold in mutation homozygotes[3]. Janssen *et al* showed that prevalence of FVLM in BCS (26%) and PVT (8%) was higher than in controls (3%) suggesting that FVLM is an important risk factor for BCS (OR 11.3) and PVT (OR 2.7)[22]. Mohanty *et al* also found FVLM to be an important risk factor in BCS (26%; OR 14.5) and in PVT (6%; OR 2.3)[12]. Bhattacharyya *et al* demonstrated FVLM mutation in 17% of BCS and in 3% of patients with

Table 1 Site of thrombosis and presence of risk factors in individual patients

Patient No.	Group	Site	Age(yr)	Sex	Hereditary risk factors						Acquired risk factors
					FVL	PT	MTHFR[1]	PrC	PrS	AT Ⅲ	
1	IVC and/or	Ⅳ	12	M	-,-	-,-	-,-	N	N	N	
2	Hepatic vein	Ⅳ	43	M	-,-	-,-	-,-	N	N	N	Past peripheral DVT
3	thrombosis	Ⅳ	45	M	-,-	-,-	-,-	N	N	N	
4	(BCS)	H	20	F	+,-	-,-	-,-	N	N	N	
5		Ⅳ + H	39	M	-,-	-,-	-,-	N	N	N	
6		H	42	F	-,-	-,-	-,-	N	N	N	
7		H	20	M	-,-	-,-	+,-	N	N	N	
8		Ⅳ	49	M	-,-	-,-	-,-	N	N	N	
9		H	4	M	-,-	-,-	-,-	N	N	N	
10		Ⅳ	46	M	-,-	-,-	+,-	N	N	Y	
11		Ⅳ + H	54	M	+,-	-,-	+,-	N	N	N	
12		Ⅳ + H	5	M	-,-	-,-	+,-	Y	N	Y	
13		H	40	F	-,-	-,-	-,-	N	N	N	
14		H	28	M	-,-	-,-	+,-	N	N	Y	
15	Splanchnic	P + SP + SM	55	F	-,-	-,-	-,-	Y	N	N	
16	vein	P + SP + SM	49	F	-,-	-,-	-,-	N	N	N	
17	thrombosis	P + SP + SM	44	F	-,-	-,-	-,-	N	N	N	Abdominal surgery
18	(SVT)	SP	35	M	-,-	-,-	-,-	N	N	N	
19		P	3	M	-,-	-,-	+,-	Y	N	N	Abdominal surgery
20		P	22	F	-,-	-,-	-,-	N	N	N	Cirrhosis
21		P + SP + SM	51	F	-,-	-,-	-,-	N	N	N	Past peripheral DVT
22		P + SM + IM	47	F	-,-	-,-	-,-	N	N	N	
23		P	30	M	-,-	-,-	-,-	N	N	N	
24		P + SM	37	M	-,-	-,-	-,-	N	N	N	Cirrhosis
25		P + SM	43	M	-,-	-,-	-,-	N	N	N	
26		SM	31	M	+,-	-,-	-,-	N	N	N	APLA
27		P	30	M	-,-	-,-	-,-	N	N	Y	
28		P + SP + SM	28	M	-,-	-,-	-,-	N	N	N	
29		SP	20	M	-,-	-,-	-,-	N	N	N	Acute pancreatitis
30		P + SP + SM	62	M	-,-	-,-	-,-	N	N	N	
31		P + SP + SM	69	M	-,-	-,-	-,-	N	N	N	Cirrhosis, HCC
32		P + SM	49	F	-,-	-,-	-,-	N	N	N	
33		P + SP + SM	53	M	+,-	-,-	-,-	N	N	N	Polycythemia, APLA
34		P + SP + SM	42	F	-,-	-,-	+,-	N	N	N	
35	BCS + SVT	Ⅳ + H + P	50	F	-,-	-,-	+,-	N	N	N	
36		H + P	25	M	-,-	-,-	-,-	N	N	N	

Ⅳ: Inferior vena cava; H: Hepatic vein; P: Portal vein; SP: Splenic vein; SM: Superior mesenteric vein; IM: Inferior mesenteric vein; FVL: Factor Ⅴ Leiden gene; PT: Prothrombin gene; MTHFR: Methyl tetrahydrate folate reductase gene; PrC: Protein C deficiency; PrS: Protein S deficiency; AT Ⅲ: Antithrombin Ⅲ deficiency; Y: Yes; N: No; APLA: Antiphospholipid antibody. -,-: Wild type; -,+: Heterozygous mutation. [1]Heterozygous *MTHFR* gene mutation is not considered a risk factor of thrombosis.

Table 2 Characteristics and risk factors of patients with BCS and SVT

	BCS (n = 14)	SVT (n = 20)	P[1]
Age: Median (IQR)	39.5 (27.25) yr	42.5 (20.5) yr	0.18
Female	21.4%	40%	0.30
Acute presentation	21.4%	40%	0.30
Hereditary risk factors	35.7%	25%	0.70
Factor Ⅴ Leyden mutation	14.3%	10%	0.55
Prothrombin gene mutation	0%	0%	-
Homozygous *MTHFR* gene mutation	0%	0%	-
Protein C deficiency	7.1%	10%	1.0
Protein S deficiency	0%	0%	-
AT Ⅲ deficiency	21.4%	5%	0.28
Acquired risk factors	7.1%	45%	0.02
No risk factor	57.1%	45%	0.70

[1]Fisher's exact test for categorical variables and Mann Whitney's U test for continuous variable.

Table 3 Prevalence of multiple risk factors (inherited and acquired) among patients with BCS and SVT n (%)

Number of risk factors	BCS (n = 14)	SVT (n = 20)	Total (n = 34)
0	8 (57)	9 (45)	17 (50)
1	5 (36)	7 (35)	12 (35)
2	1 (7)	3 (15)	4 (12)
3	-	1 (5)	1 (3)

PVT[13]. Koshy *et al* showed that the prevalence of FVLM was similar in patients with PVT (3%) and controls (1%) and suggested that FVLM is not associated with PVT in India[14]. Similar observations were made by Sharma *et al* who demonstrated FVLM in 1.6% of patients with PVT and in 4% of controls[15]. In the present study, 14% of patients with BCS and 10% with SVT were heterozygotes, both higher than control data (1%-4%) reported earlier from India[12,14,15]. Though the numbers of patients in the study are small, results suggest that FVLM may be a risk factor in BCS and SVT.

Prothrombin gene mutation, a risk factor for venous thrombosis (homozygote: 10-fold; heterozygote: 2- to 4-fold) is rare in African and Asian populations compared to Caucasians[23]. None of the five Indian

studies have shown this gene mutation in cases or controls[12,13,15-17]. We also did not detect this mutation in any of our patients. Prevalence of heterozygote *MTHFR* gene mutation in patients with venous thrombosis is similar to healthy controls suggesting that this mutation is not an important prothrombotic factor[24]. It has been shown that homozygote *MTHFR* mutation, one of the causes of hyperhomocystinaemia (risk factor for vascular disease), is a risk factor for venous thrombosis[19,24]. None of the patients in the present study were homozygous for the *MTHFR* mutation. Three patients had heterozygote *MTHFR* mutation as the only abnormality. They were presumed to have idiopathic abdominal venous thrombosis as heterozygote *MTHFR* mutation alone is not considered a significant prothrombotic risk factor. Five heterozygous patients had additional hereditary or acquired risk factors. In a study from Northern India, Bhattacharyya *et al* investigated 57 BCS and 48 PVT patients, and reported none were homozygous for *MTHFR* gene mutation. Heterozygous mutations were seen in 24% of BCS and 21% of PVT patients[13].

Indian and Western studies have shown that protein C deficiency is the second most common cause of inherited thrombophilia in patients with BCS and PVT[12,13,22]. Amarapurkar *et al* showed that protein C deficiency was the commonest hereditary risk factor (26%) in a study on 28 patients with mesenteric venous thrombosis[25]. Protein C was also the commonest risk factor (38% patients) in a series of 16 patients with mesenteric venous thrombosis reported by Harward *et al*[26]. In the present study, protein C deficiency was demonstrated in 7% of patients with BCS and in 10% of patients with SVT. Prevalence of protein S, and AT Ⅲ deficiency as risk factors for inherited thrombophilia in patients with BCS and PVT were low in Indian and Western studies[12,13,22]. Protein S deficiency was not detected in any of our patients. AT Ⅲ deficiency was higher in patients with BCS (21%) as compared to those with SVT (5%). Diagnosis of inherited deficiencies of protein C, protein S, and AT Ⅲ, as a cause of abdominal venous thrombosis is difficult, because acquired deficiencies develop in liver failure, acute thrombosis, and during anticoagulant therapy[27]. None of the patients in the present study with protein C and AT Ⅲ deficiency had liver failure or were on anticoagulant therapy.

Comparison of prothrombotic risk factor profiles between BCS and SVT showed a trend for hereditary risk factors to be more frequent in BCS (BCS *vs* SVT: 35.7% *vs* 25%; $P = 0.7$); two other Indian studies have made similar observations of hereditary factors being more frequent in BCS group compared to PVT group[12,13]. Studies on prevalence of acquired risk factors in abdominal venous thrombosis have shown variable results. Denninger *et al* and Janssen *et al* have shown that acquired risk factors are more frequent in PVT than in BCS[22,28]. Mohanty *et al* found the frequency of acquired risk factors to be similar in BCS and PVT[12]. In our study, acquired risk factors were significantly more common in the SVT group (BCS *vs* SVT: 7% *vs* 45%; $P = 0.02$) suggesting that SVT is a heterogeneous disease where hereditary and local risk factors play important roles.

No risk factor was identified in 57% of BCS and 45% of patients with SVT. One possible reason may be the low prevalence of myeloproliferative disorders in our series (one patient). Myeloproliferative disorders (overt or latent) have been shown as an important risk factor in previous studies on abdominal vein thrombosis[28-31]. Tests for detecting latent myeloproliferative disorders (formation of "spontaneous" erythroid colonies in cultures of bone marrow progenitor cells in erythropoietin-poor medium[32,33]) were not performed on our patients. In a study from Western India, an etiological factor could be found in 59% of the BCS and 30% of the PVT patients[12]. Interestingly, in this study also, none had a myeloproliferative disorder.

Previous studies have suggested that venous thrombosis results from coexistence of several risk factors[28]. In the present study, ⩾ 2 risk factors were detected in 7% of BCS and in 20% of patients with SVT.

Hereditary and acquired risk factors play an important role in etiopathogenesis of abdominal venous thrombosis. Acquired risk factors are significantly more common in patients with SVT while hereditary risk factors are similar in patients with BCS and SVT. Recognition and evaluation of these risk factors may help in therapy and prevention of disease progression. As a significant number of patients lack obvious etiology further research is required to identify as yet unrecognized risk factors.

COMMENTS

Background

Abdominal venous thrombosis may present as Budd-Chiari Syndrome (BCS) or splanchnic venous thrombosis (SVT). Hereditary and acquired risk factors are implicated in the etiopathogenesis of abdominal venous thrombosis. There are few systematic studies that have comprehensively evaluated both hereditary and acquired factors in BCS and SVT. Most studies have evaluated either a single prothrombotic risk factor or multiple risk factors in a single vein.

Research frontiers

Concept of multifactorial theory of thrombogenesis suggests that thrombosis occurs by activation of a trigger factor (acquired) in a thrombophilic milieu (hereditary). The prevalence of inherited risk factors is variable between populations throughout the world. Possible reasons are small numbers of patients studied, non-standardized evaluation of parameters tested, and genetic differences between patient populations. Data need to be generated by good studies from different geographical areas in the world. Etiological factors for abdominal venous thrombosis were identified in 70%-80% of patients in Western studies. In Indian studies, no risk factor was identified in half the patients suggesting that other unknown hereditary/risk factors may be operating in these patients.

Innovations and breakthroughs

The present study suggests that acquired risk factors which are preventable are important in etiopathagenesis of SVT. As no risk factors were identified in about half the patients, research needs to be ongoing to identify unknown hereditary/ acquired risk factors operating in these patients.

Applications

Abdominal venous thrombosis is a life threatening condition caused by single or multiple, hereditary or acquired prothrombotic risk factors. Prevention and therapy with non-invasive techniques and new anticoagulant drugs are now possible. Complete thrombophilia screening is, therefore, important for risk assessment, and therapy in patients with abdominal venous thrombosis. With continuing search for hereditary risk factors (genetic molecular defects), true

idiopathic thrombotic disease will become uncommon.

Peer review

This paper investigates genetic and acquired risk factors in patients with thromboembolism in abdominal veins. The authors make a difference between hereditary and acquired risk. It's a nice study.

REFERENCES

1 **Egesel T**, Buyukasik Y, Dundar SV, Gurgey A, Kirazli S, Bayraktar Y. The role of natural anticoagulant deficiencies and factor V Leiden in the development of idiopathic portal vein thrombosis. *J Clin Gastroenterol* 2000; **30**: 66-71

2 **Dilawari JB**, Bambery P, Chawla Y, Kaur U, Bhusnurmath SR, Malhotra HS, Sood GK, Mitra SK, Khanna SK, Walia BS. Hepatic outflow obstruction (Budd-Chiari syndrome). Experience with 177 patients and a review of the literature. *Medicine* (Baltimore) 1994; **73**: 21-36

3 **Bayraktar Y**, Harmanci O. Etiology and consequences of thrombosis in abdominal vessels. *World J Gastroenterol* 2006; **12**: 1165-1174

4 **Deltenre P**, Denninger MH, Hillaire S, Guillin MC, Casadevall N, Briere J, Erlinger S, Valla DC. Factor V Leiden related Budd-Chiari syndrome. *Gut* 2001; **48**: 264-268

5 **Margaglione M**, Brancaccio V, Giuliani N, D'Andrea G, Cappucci G, Iannaccone L, Vecchione G, Grandone E, Di Minno G. Increased risk for venous thrombosis in carriers of the prothrombin G-->A20210 gene variant. *Ann Intern Med* 1998; **129**: 89-93

6 **Pabinger I**, Schneider B. Thrombotic risk in hereditary antithrombin III, protein C, or protein S deficiency. A cooperative, retrospective study. Gesellschaft fur Thrombose- und Hamostaseforschung (GTH) Study Group on Natural Inhibitors. *Arterioscler Thromb Vasc Biol* 1996; **16**: 742-748

7 **Franco RF**, Reitsma PH. Genetic risk factors of venous thrombosis. *Hum Genet* 2001; **109**: 369-384

8 **Espinosa G**, Font J, Garcia-Pagan JC, Tassies D, Reverter JC, Gaig C, Cervantes F, Cervera R, Bosch J, Ingelmo M. Budd-Chiari syndrome secondary to antiphospholipid syndrome: clinical and immunologic characteristics of 43 patients. *Medicine* (Baltimore) 2001; **80**: 345-354

9 **Valla D**, Le MG, Poynard T, Zucman N, Rueff B, Benhamou JP. Risk of hepatic vein thrombosis in relation to recent use of oral contraceptives. A case-control study. *Gastroenterology* 1986; **90**: 807-811

10 **Walker ID**. Thrombophilia in pregnancy. *J Clin Pathol* 2000; **53**: 573-580

11 **Matei D**, Brenner B, Marder VJ. Acquired thrombophilic syndromes. *Blood Rev* 2001; **15**: 31-48

12 **Mohanty D**, Shetty S, Ghosh K, Pawar A, Abraham P. Hereditary thrombophilia as a cause of Budd-Chiari syndrome: a study from Western India. *Hepatology* 2001; **34**: 666-670

13 **Bhattacharyya M**, Makharia G, Kannan M, Ahmed RP, Gupta PK, Saxena R. Inherited prothrombotic defects in Budd-Chiari syndrome and portal vein thrombosis: a study from North India. *Am J Clin Pathol* 2004; **121**: 844-847

14 **Koshy A**, Jeyakumari M. Factor V Leiden is not commonly associated with idiopathic portal vein thrombosis in southern India. *Indian J Gastroenterol* 2006; **25**: 140-142

15 **Sharma S**, Kumar SI, Poddar U, Yachha SK, Aggarwal R. Factor V Leiden and prothrombin gene G20210A mutations are uncommon in portal vein thrombosis in India. *Indian J Gastroenterol* 2006; **25**: 236-239

16 **Koshy A**, Jeyakumari M. Prothrombin G20210A gene variant is not associated with idiopathic portal vein thrombosis in an area endemic for portal vein thrombosis. *Ann Hematol* 2006; **85**: 126-128

17 **Kumar SI**, Kumar A, Srivastava S, Saraswat VA, Aggarwal R. Low frequency of factor V Leiden and prothrombin G20210A mutations in patients with hepatic venous outflow tract obstruction in northern India: a case-control study. *Indian J Gastroenterol* 2005; **24**: 211-215

18 **Bertina RM**, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; **369**: 64-67

19 **Frosst P**, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995; **10**: 111-113

20 **Poort SR**, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996; **88**: 3698-3703

21 **Lucotte G**, Mercier G. Population genetics of factor V Leiden in Europe. *Blood Cells Mol Dis* 2001; **27**: 362-367

22 **Janssen HL**, Meinardi JR, Vleggaar FP, van Uum SH, Haagsma EB, van Der Meer FJ, van Hattum J, Chamuleau RA, Adang RP, Vandenbroucke JP, van Hoek B, Rosendaal FR. Factor V Leiden mutation, prothrombin gene mutation, and deficiencies in coagulation inhibitors associated with Budd-Chiari syndrome and portal vein thrombosis: results of a case-control study. *Blood* 2000; **96**: 2364-2368

23 **Rosendaal FR**, Doggen CJ, Zivelin A, Arruda VR, Aiach M, Siscovick DS, Hillarp A, Watzke HH, Bernardi F, Cumming AM, Preston FE, Reitsma PH. Geographic distribution of the 20210 G to A prothrombin variant. *Thromb Haemost* 1998; **79**: 706-708

24 **Li XM**, Wei YF, Hao HL, Hao YB, He LS, Li JD, Mei B, Wang SY, Wang C, Wang JX, Zhu JZ, Liang JQ. Hyperhomocysteinemia and the MTHFR C677T mutation in Budd-Chiari syndrome. *Am J Hematol* 2002; **71**: 11-14

25 **Amarapurkar DN**, Patel ND, Jatania J. Primary mesenteric venous thrombosis: a study from western India. *Indian J Gastroenterol* 2007; **26**: 113-117

26 **Harward TR**, Green D, Bergan JJ, Rizzo RJ, Yao JS. Mesenteric venous thrombosis. *J Vasc Surg* 1989; **9**: 328-333

27 **Tripodi A**, Mannucci PM. Abnormalities of hemostasis in chronic liver disease: reappraisal of their clinical significance and need for clinical and laboratory research. *J Hepatol* 2007; **46**: 727-733

28 **Denninger MH**, Chait Y, Casadevall N, Hillaire S, Guillin MC, Bezeaud A, Erlinger S, Briere J, Valla D. Cause of portal or hepatic venous thrombosis in adults: the role of multiple concurrent factors. *Hepatology* 2000; **31**: 587-591

29 **Valla D**, Casadevall N, Lacombe C, Varet B, Goldwasser E, Franco D, Maillard JN, Pariente EA, Leporrier M, Rueff B. Primary myeloproliferative disorder and hepatic vein thrombosis. A prospective study of erythroid colony formation in vitro in 20 patients with Budd-Chiari syndrome. *Ann Intern Med* 1985; **103**: 329-334

30 **De Stefano V**, Teofili L, Leone G, Michiels JJ. Spontaneous erythroid colony formation as the clue to an underlying myeloproliferative disorder in patients with Budd-Chiari syndrome or portal vein thrombosis. *Semin Thromb Hemost* 1997; **23**: 411-418

31 **Valla D**, Casadevall N, Huisse MG, Tulliez M, Grange JD, Muller O, Binda T, Varet B, Rueff B, Benhamou JP. Etiology of portal vein thrombosis in adults. A prospective evaluation of primary myeloproliferative disorders. *Gastroenterology* 1988; **94**: 1063-1069

32 **Pagliuca A**, Mufti GJ, Janossa-Tahernia M, Eridani S, Westwood NB, Thumpston J, Sawyer B, Sturgess R, Williams R. In vitro colony culture and chromosomal studies in hepatic and portal vein thrombosis--possible evidence of an occult myeloproliferative state. *Q J Med* 1990; **76**: 981-989

33 **Hirshberg B**, Shouval D, Fibach E, Friedman G, Ben-Yehuda D. Flow cytometric analysis of autonomous growth of erythroid precursors in liquid culture detects occult polycythemia vera in the Budd-Chiari syndrome. *J Hepatol* 2000; **32**: 574-578

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RAPID COMMUNICATION

Laryngopharyngeal reflux in patients with reflux esophagitis

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Abstract

AIM: To assess the prevalence of laryngopharyngeal reflux (LPR) in patients with reflux esophagitis and disclose factors contributing to the development of LPR.

METHODS: A total of 167 patients who proved to have reflux esophagitis by endoscopy were enrolled. They received laryngoscopy to grade the reflux findings for the diagnosis of LPR. We used validated questionnaires to identify the presence of laryngopharyngeal symptoms, and stringent criteria of inclusion to increase the specificity of laryngoscopic findings. The data of patients were analyzed statistically to find out factors related to LPR.

RESULTS: The prevalence rate of LPR in studied subjects with reflux esophagitis was 23.9%. Age, hoarseness and hiatus hernia were factors significantly associated with LPR. In 23 patients with a hiatus hernia, the group with LPR was found to have a lower trend of esophagitis grading.

CONCLUSION: Laryngopharyngeal reflux is present in patients with reflux esophagitis, and three predicting factors were identified. However, the development of LPR might be different from that of reflux esophagitis. The importance of hiatus hernia deserves further study.

Key words: Laryngopharyngeal reflux; Gastroesophageal reflux disease; Reflux esophagitis

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INTRODUCTION

Gastroesophageal reflux disease (GERD) has been known to be a common medical condition affecting approximately 35%-40% of the adult population in the Western world[1,2]. Forty-four of all Americans suffer from heartburn at least once per month, 20% at least once per week[3]. The role of GERD in causing extra-esophageal symptoms is also increasingly being recognized[4]. Chronic laryngeal signs and symptoms associated with GERD are often referred to as reflux laryngitis or laryngopharyngeal reflux (LPR)[5]. But, not all episodes of GERD are associated with LPR and not all patients with LPR have typical features of GERD[6]. Classic reflux symptoms (heartburn and regurgitation), which are referred to as "typical GERD," may be absent in more than half the patients presenting with extra-esophageal manifestations[7,8]. The "silent reflux" contributes to the difficulty in making a definite and correct diagnosis. The extra-esophageal manifestations of GERD provide the most challenging areas to perform good research[9].

Furthermore, the prevalence of LPR in the patients with GERD has not been studied well in the past. The kind of patients with GERD who are more associated with LPR is still unknown. The cause-and-effect relationship between GERD and LPR remains elusive.

In patients with esophageal syndromes of GERD, reflux esophagitis (RE) is more easily diagnosed definitely by endoscopy than others. The objectives of our current study were to determine the prevalence of LPR in the patients with RE and to find out the factors that contribute to the development of LPR.

MATERIALS AND METHODS

Recruitment of patients

Consecutive patients who were diagnosed to have RE by gastroendoscopic examination due to various symptoms, such as epigastric pain, acid regurgitation, heartburn, nausea, abdominal fullness sensation, and so on, at the gastrointestinal clinic of Cathay General Hospital from September 2006 to October 2007 were enrolled. These qualified patients then were referred to the otorhinolaryngologic clinic for the further work-up of a laryngoscopic examination. Written, informed consent had to be provided by the participants before the endoscopic examination.

To improve the specificity of our study, the inclusion criteria of the patients were very strict. Patients were excluded from the study if they had a history of respiratory or gastrointestinal malignancy; radiation therapy to the head and neck, lung, or gastrointestinal tract; gastroesophageal surgery; use of H_2-receptor antagonists or proton pump inhibitors in previous 1 mo; past or present smoker; excessive alcohol consumption; chronic cough attributable to known chronic pulmonary or tracheobronchial disease; professional voice users (e.g. singer, teacher); excessive voice use; exposure to occupational or environmental pollutants; history of seasonal allergic rhinitis; pharyngolaryngeal infection in the previous 3 mo; tracheal intubation in the previous 12 mo and use of inhaled corticosteroids[10,11].

Gastroendoscopic examination

All subjects received conventional endoscopic imaging, as well as imaging with the narrow-band imaging (NBI) system by using a video endoscope (GIF-H 260; Olympus Optical Co, Ltd, Tokyo, Japan). A group of experienced endoscopists performed the endoscopic examination. NBI is a novel, non-invasive optical technique that adjusts reflected light to enhance the contrast between the esophageal mucosa and the gastric mucosa[12]. The Los Angeles classification was used to grade esophagitis. A hiatus hernia was diagnosed if the hernia sac was more than 2 cm in length. We did not include patients suspected to have Barrett's esophagus due to diagnostic complexity.

Questionnaire

The qualified participants needed to complete a questionnaire by answering "yes" or "no" with the aid of research nurses, right after the gastroendoscopic examination. It was used to identify the presence of any throat or reflux symptoms (cough, hoarseness, throat clearing, sore throat, thick drainage, globus sensation, bad taste in the mouth, swallowing problems, chest pain). Subjects were also asked to score the severity of each symptom based on a graded scale of 1 to 4 [1 = rare (once a month or less), 2 = occasional (2-3 times a month), 3 = frequent (several times a week); 4 = all the time (several times daily)]. The graded scales of more than 2 were considered significant, and the symptoms with such a scale could be included into the study[13].

Laryngoscopic examination

Each patient at the otorhinolaryngologic clinic underwent an endoscopic examination (Hopkins 70℃ Telescope, Model 8706 CA, Karl Storz, Germany) of the larynx by two well-trained otolaryngologists, both with experience of over 10 years and good consensus, to grade the laryngoscopic findings. The otolaryngologists were not aware of the results of the questionnaire before the laryngoscopic examination. A reflux finding score (RFS) was obtained based on the laryngeal examination scoring system by Belafsky *et al*[14]. A RFS of > 7 was considered abnormal and to have LPR. Laryngeal signs suspected to be reflux-related were determined based on an agreement of the two experts.

Statistical analysis

Statistical analyses were performed using the Stata 8.0 for Window (STATA Corp, College Station, TX). Patient characteristics were compared using the Student's *t* test and Pearson's λ^2 test for proportions. A logistic regression model was used to adjust for confounding covariates including, age, sex, BMI, disease (presence or absence), a hiatus hernia (presence or absence), and the grading of LA classification (A, B, C) *etc.* A two-tailed *P* value of less than 0.05 was taken to indicate statistical significance.

RESULTS

Two hundred twenty-two patients with endoscopically proven RE initially were included in the study. However, 13 patients did not visit otorhinolayngeal clinic due to personal reasons and 42 patients were excluded because they did not meet the strict inclusion criteria. Therefore, a total of 167 patients (80 males and 87 females) were enrolled in this study. The demographic characteristics of the studied subjects are listed in Table 1. 96.4% of the patients belonged to the groups of esophagitis grade A and B; only 3.6% were of grade C. A hiatus hernia was found in 13.8% of the patients.

Table 2 shows comparisons of demographic characteristics of patients with and without LPR. Among the 167 patients, 23.9% (40 cases) were diagnosed to have LPR. The difference in age between the patients with and without LPR was significant (45.2 *vs* 49.9 years, $P = 0.04$). The patients with LPR were younger than the ones without LPR. The presence of hoarseness symptom was significantly higher in the group with LPR (55.0% *vs* 26.8%, $P = 0.001$). In addition, a hiatus hernia was found more frequently in the group with LPR (27.5% *vs* 9.5%, $P = 0.004$).

If we combined the symptom of hoarseness and presence of a hiatus hernia together, the prediction of LPR was much higher (odds ratio increased up to 12.3, Table 3).

We also made a detailed analysis in the patients with a hiatus hernia. The distribution of esophagitis grading between the groups with and without LPR were compared. Of interest, in 23 patients with a hiatus hernia, the group with LPR (11 patients) had a relatively lower trend of esophagitis grading (LA grade A/B/C:

Table 1 Demographic characteristics of 167 patients

Demographic characteristics	*n*
Gender (male/female)	80/87
Age (yr)	
mean ± SD	48.8 ± 12.8
Range	21- 81
BMI (kg/m^2)	
mean ± SD	23.4 ± 3.2
Range	16.1-36.3
LPR symptoms (%)	
Hoarseness	56 (33.5)
Globus	56 (33.5)
Cough	46 (27.5)
Throat clearing	59 (35.3)
LA grade (%)	
A	118 (70.7)
B	43 (25.7)
C	6 (3.6)
Hiatus hernia (%)	23 (13.8)

BMI: Body mass index; LPR: Laryngopharyngeal reflux; LA grade: The grade of Los Angeles classification of esophagitis.

Table 2 Comparisons of demographic characteristics of patients with and without LPR

	LPR (*n* = 40)	Non-LPR (*n* = 127)	*P*
Gender (%)			0.67
Male	18 (45.0)	62 (48.8)	
Female	22 (55.0)	65 (51.2)	
Age (yr)	45.2 ± 11.9	49.9 ± 12.9	0.04
BMI (kg/m^2)	23.3 ± 3.2	23.4 ± 3.2	0.88
LPR symptoms (%)			
Hoarseness	22 (55.0)	34 (26.8)	0.001
Globus	14 (35.0)	42 (33.1)	0.82
Cough	12 (30.0)	34 (26.8)	0.69
Throat clearing	16 (40.0)	43 (33.9)	0.47
LA grade (%)			0.68
A	29 (72.5)	89 (70.1)	
B	9 (22.5)	34 (26.8)	
C	2 (5.0)	4 (3.1)	
Hiatus hernia (*n*, %)	11 (27.5)	12 (9.5)	0.004

BMI: Body mass index; LPR: Laryngopharyngeal reflux; LA grade: The grade of Los Angeles classification of esophagitis.

9, 81.8%/2, 18.2%/0, 0%), whereas the group without LPR (12 patients) had a higher trend of grading (LA grade A/B/C: 4, 33.3%/6, 50.0%/2, 16.7%). The difference was statistically significant ($P = 0.04$).

DISCUSSION

The association between LPR and GERD has not been firmly established yet[6]. Not all patients with GERD will develop LPR. On the other hand, it is estimated that 50%-60% of chronic laryngitis and difficult-to-treat sore throat may be related to GERD[8]. The causal association between acid reflux and laryngitis is highly plausible considering the close anatomical relationship. The vagally mediated reflexes (bronchospasm, laryngospasm and cough) stimulated by esophageal acid is also implicated in the pathogenesis of GERD-related extra-esophageal disorder[11,15].

Table 3 Logistic regression analyses on predictors of LPR

	Odds ratio	
	Model 1	Model 2
Gender		
Female	-	-
Male	0.72	0.82
Age	0.96[1]	0.96[1]
BMI	0.98	0.99
LPR symptoms		
Hoarseness	4.12[1]	-
Globus	1.77	1.21
Cough	0.91	1.23
Throat clearing	0.82	1.24
LA grade		
A	-	-
B	0.81	0.76
C	2.35	2.21
Hiatus hernia	4.78[1]	-
Hiatus hernia and Hoarseness	-	12.3[1]

BMI: Body mass index; LPR: Laryngopharyngeal reflux; LA grade: The grade of Los Angeles classification of esophagitis. [1]*P* value is significant at the 0.05 level.

Currently, there is no "gold-standard" for the diagnosis of LPR. Ambulatory 24-h dual or triple probe pH-metry was once considered the best method for reflux testing[16] but the position of the probes makes the measurement not easy to interpret, and there is no consensus about the pathological reflux at the level of laryngopharynx[6]. Moreover, extra-esophageal reflux is also intermittent. A negative pH study does not rule out extra-esophageal reflux[17]. The empiric therapy with aggressive acid suppression, usually BID dosing of proton-pump inhibitors (PPIs), is currently recommended as the most practical and cost effective approach for the patients suspected with extra-esophageal presentations of GERD[16]. Nevertheless, this therapeutic trial for the diagnosis of LPR could not provide direct evidence of pathologic imaging changes of patients, about which most clinicians want to learn.

Laryngeal examination with special emphasis on the posterior location of tissue injury can be helpful for the diagnosis of LPR[18]. The severity of mucosal injury may be graded according to the RFS by Belafsky 2001[14]. The RFS is an 8-item clinical severity scale based on findings during fiberoptic laryngoscopy. However, this RFS system has been criticized to have high inter- or intra-observer variability and low specificity for reflux laryngitis[6,10,19]. Therefore, it is very important to exclude meticulously other potential etiologies that can lead to laryngeal irritation. In our study, we did a very stringent selection of the patients to avoid the secondary causes of chronic laryngitis, such as smoking, alcohol, excessive voice use, allergies, or asthma.

The NBI system we used on gastroendoscopic examinations could offer a better image of capillary patterns and, thus, enhance the contrast between the esophageal and gastric mucosa and facilitate the endoscopic evaluation of esophagitis[20,21]. The better depictions of small erosive foci improves the intra- and inter-observer reproducibility in the grading of esophagitis, especially

in the grading of class A or B esophagitis[12], which was very helpful in our study.

In our study, LPR was present in 23.9% of the studied subjects with RE. In the past, Koufman described posterior laryngitis in 74% and laryngeal edema with erythema in 60% of all patients with GERD[16]. Tauber *et al* also reported 85% of GERD-positive patients had posterior laryngitis and 69% had laryngitis with an interarytenoid erythema and edema[22]. Our prevalence rate of LPR is much lower than theirs; the different sample size of patients and method of enrollment in our research must have influenced the results. Because we used very stringent criteria to enroll the patients, it was possible that we missed some cases and underestimated the prevalence rate of LPR. In fact, this kind of report is quite rare in the literature. Most papers dealt only with the prevalence rate of GERD (ranging 20%-50%) in patients with LPR[4,21,23,24].

Our results indicated that age, hoarseness and a hiatus hernia could be the predicting factors of LPR in the patients with RE. However, gender, body mass index, and the severity of esophagitis were not associated. A large cohort study performed by Jaspersen *et al* reported female gender, higher age, severe esophagitis, longer duration of GERD and smoking were significantly related to the extra-esophageal disorder[25]. Their risk factors were not the same as ours, which might be caused by the recruitment method they used. Though the case number of their study was large, they did not exclude the patients strictly and included patients who smoked. The patients they studied did not receive a laryngoscopic examination, and solely relied on the "symptom questionnaire" for the diagnosis of extra-esophageal disorders, which could be another factor that would induce diverse outcomes.

Increased GERD severity due to degradation of the gastroesophageal junction and impaired esophageal clearance was found in the elderly[26]. Yet, age as a factor contributing to LPR seldom has been mentioned before. In the present study, the RE patients with LPR were of a younger age than the patients without LPR. This finding is contradictory to the result of Jaspersen's study, in which they noted higher age was a risk factor for the occurrence of extra-esophageal disorder[25]. The opposite results again might be attributed to the different recruitment and research methods. However, the drawback of our study was that we had fewer patients. According to our findings, higher age, which implies the probable longer duration of GERD, is not essential for the development of LPR. In addition, our study also indicated the severity of RE had nothing to do with the occurrence of LPR. Therefore, the existence of LPR seems to be not associated with the duration or severity of RE.

LPR may have several clinical symptoms. Among them, throat-clearing, persistent cough, globus and hoarseness are the most common complaints[24]. In our study, the prevalence of hoarseness in all the patients was 33.5%. When we made comparison between the patients with and without LPR, the rate of hoarseness became 55.0% *vs* 26.8%, which was statistically significant. Our result indicated that more than 50% of the RE patients with LPR had the symptom of hoarseness. As for the other symptoms (globus, throat discomfort and persistent cough), we did not find significant differences between the two groups.

Hoarseness is a common complaint of the patients at the otorhinolaryngologic clinic. Underlying causes include malignancy, vocal cord palsy, polyps and nodules of the vocal cords, laryngitis and functional disorders. Acute laryngitis is usually infective, whereas chronic laryngitis may result from a spectrum of insults including cigarette smoking, dehydration, acid reflux and muscular imbalance[6]. Hoarseness is not specific for LPR. Therefore, we must exclude several other possible causes before we can make sure the laryngitis-related hoarseness is induced solely by acid reflux. In our patients with RE, an additional symptom of hoarseness might reflect that the acid reflux has gone beyond the upper esophageal sphincter and injured the vocal cord. Extra-esophageal manifestation of GERD, thus, might be incurred.

Hiatus hernias have a higher detection rate in Western populations, ranging between 14.5% and 22%[27]. In the Far East, the prevalence rate is much lower, 7% of 464 subjects in Taiwan[28], 2.9% of 11943 subjects in Singapore[29], and 17.5% of 6010 individuals in Japan were reported[30]. In a recent series in Taiwan, hiatus hernia was found in 18.8% of patients with erosive esophagitis[31]. In our research, it was 13.8% of the studied subjects, which was also higher than that of the normal population here.

A hiatus hernia can disrupt both the anatomy and physiology of the normal anti-reflux mechanism. It is associated with decreased esophageal peristalsis; it also increases the cross-sectional area of the esophago-gastric junction and acts as a reservoir allowing reflux from the hernia sac into the esophagus during swallowing. The presence of a hiatus hernia is associated with symptoms of gastroesophageal reflux, and increased prevalence and severity of RE[27]. Because the presence of a hiatus hernia would increase esophageal acid exposure, it is emerging as an important factor in the pathogenesis of GERD[27].

In our study, a confirmed hiatus hernia was found to be a risk factor contributing to LPR in the patients with RE. Considering the possible mechanism of reflux-related extra-esophageal disorders, it will not be surprising to disclose the importance of a hiatus hernia in these patients. With the existence of a hiatus hernia, the acid reflux could be potentiated and would result in more mucosal injury up to larynx. Animal studies have shown that even minute amounts of gastric acid and pepsin on laryngeal mucosa can induce significant inflammation and edema[32,33]. Further work is still needed to understand how a hiatus hernia influences the progression of GERD and its complications. At present, a hiatus hernia is known to be a marker of severe GERD[27] and must have a contributing effect in the pathogenesis of LPR.

In our patients with a hiatus hernia, we also analyzed their grade of esophagitis. Between the groups with and without LPR, the result was quite interesting and surprising. In this category, the patients with LPR had

a milder form of esophagitis than the ones without LPR. This finding again supports the concept that the development of LPR is not related to the severity of RE. To the contrary, LPR can be seen more frequently in the patients with mild RE when a confirmed hiatus hernia is present. Of interest, Li *et al* just reported that a hiatus hernia was found to be associated with more severe esophagitis in patients with RE[34]. Therefore, the development of LPR must be different from that of simple RE without LPR. Moreover, our patients who coexisted with a hiatus hernia and hoarseness had a very high odds ratio for LPR. Combining these two factors clinically, we could predict the presence of LPR more accurately in the patients with RE.

Regretfully, we had only 6 patients (3.6%) with LA grade C esophagitis and no patient with grade D. Thus, in our research, several factors could not be viewed and studied with the entire esophagitis spectrum from grade A to D. Another drawback of our study is that we did not include patients suspected to have Barrett's esophagus or endoscopically suspected esophageal metaplasia, which could be another intriguing field to see the relationship between GERD and extra-esophageal syndromes.

In conclusion, our study revealed that age, hoarseness and a confirmed hiatus hernia were the factors related to LPR in the patients with RE. LPR could be associated with RE, but the definite cause-and-effect relationship is still unknown. Our research was only a hospital-based study; more case numbers and convincing data are necessary in the future. Based on the aforementioned findings, the development of LPR seems to be different from that of RE. The importance of a confirmed hiatus hernia in LPR deserves further study.

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COMMENTS

Background

The role of gastroesophageal reflux disease (GERD) in causing laryngopharyngeal reflux (LPR) is being increasingly recognized, but the cause-and-effect relationship between them remains elusive.

Research frontiers

This research is to assess the prevalence of LPR in the patients with reflux esophagitis (RE), and also to identify the factors contributing to the development of LPR.

Innovations and breakthroughs

The prevalence rate of LPR in our studied subjects with RE was 23.9%. Age, hoarseness, and hiatus hernia were the factors significantly associated with LPR. In addition, the patients who coexisted with a hiatus hernia and hoarseness had a very high odds ratio (12.3) for LPR. Another interesting finding was that in 23 patients with a hiatus hernia, the group with LPR was incidentally revealed to have lower trend of esophagitis grading.

Applications

LPR is present in patients with RE and three predicting factors could be identified. Combining the two factors of hoarseness and hiatus hernia together, we could predict the presence of LPR more accurately in the patients with RE. However, the development of LPR seems to be different from that of RE, based on the findings of this research. The importance of hiatus hernia in LPR deserves further study.

Peer review

In this study, the authors ascertained the association of LPR with GERD and analyzed the factors related to the development of LPR. The results could be very important because the readers could learn the newest knowledge and understand the future perspectives in this field.

REFERENCES

1 **Spechler SJ**. Epidemiology and natural history of gastro-oesophageal reflux disease. *Digestion* 1992; **51** Suppl 1: 24-29
2 **Vaezi MF**. Therapy Insight: gastroesophageal reflux disease and laryngopharyngeal reflux. *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 595-603
3 **Locke GR 3rd**, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ 3rd. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. *Gastroenterology* 1997; **112**: 1448-1456
4 **Jonaitis L**, Pribuisiene R, Kupcinskas L, Uloza V. Laryngeal examination is superior to endoscopy in the diagnosis of the laryngopharyngeal form of gastroesophageal reflux disease. *Scand J Gastroenterol* 2006; **41**: 131-137
5 **Richardson BE**, Heywood BM, Sims HS, Stoner J, Leopold DA. Laryngopharyngeal reflux: trends in diagnostic interpretation criteria. *Dysphagia* 2004; **19**: 248-255
6 **Hopkins C**, Yousaf U, Pedersen M. Acid reflux treatment for hoarseness. *Cochrane Database Syst Rev* 2006; CD005054
7 **Koufman JA**. Laryngopharyngeal reflux is different from classic gastroesophageal reflux disease. *Ear Nose Throat J* 2002; **81**: 7-9
8 **Vaezi MF**. Extraesophageal manifestations of gastroesophageal reflux disease. *Clin Cornerstone* 2003; **5**: 32-38; discussion 39-40
9 **Richter JE**. Extraesophageal presentations of gastroesophageal reflux disease: an overview. *Am J Gastroenterol* 2000; **95**: S1-S3
10 **Vaezi MF**, Richter JE, Stasney CR, Spiegel JR, Iannuzzi RA, Crawley JA, Hwang C, Sostek MB, Shaker R. Treatment of chronic posterior laryngitis with esomeprazole. *Laryngoscope* 2006; **116**: 254-260
11 **Qua CS**, Wong CH, Gopala K, Goh KL. Gastro-oesophageal reflux disease in chronic laryngitis: prevalence and response to acid-suppressive therapy. *Aliment Pharmacol Ther* 2007; **25**: 287-295
12 **Lee YC**, Lin JT, Chiu HM, Liao WC, Chen CC, Tu CH, Tai CM, Chiang TH, Chiu YH, Wu MS, Wang HP. Intraobserver and interobserver consistency for grading esophagitis with narrow-band imaging. *Gastrointest Endosc* 2007; **66**: 230-236
13 **Milstein CF**, Charbel S, Hicks DM, Abelson TI, Richter JE, Vaezi MF. Prevalence of laryngeal irritation signs associated with reflux in asymptomatic volunteers: impact of endoscopic technique (rigid vs. flexible laryngoscope). *Laryngoscope* 2005; **115**: 2256-2261
14 **Belafsky PC**, Postma GN, Koufman JA. The validity and reliability of the reflux finding score (RFS). *Laryngoscope* 2001; **111**: 1313-1317
15 **Close LG**. Laryngopharyngeal manifestations of reflux: diagnosis and therapy. *Eur J Gastroenterol Hepatol* 2002; **14** Suppl 1: S23-S27
16 **Farrokhi F**, Vaezi MF. Extra-esophageal manifestations of gastroesophageal reflux. *Oral Dis* 2007; **13**: 349-359
17 **Richter JE**. Ambulatory esophageal pH monitoring. *Am J Med* 1997; **103**: 130S-134S
18 **Vaezi MF**. Sensitivity and specificity of reflux-attributed laryngeal lesions: experimental and clinical evidence. *Am J Med* 2003; **115** Suppl 3A: 97S-104S
19 **Vaezi MF**, Hicks DM, Abelson TI, Richter JE. Laryngeal signs and symptoms and gastroesophageal reflux disease (GERD): a critical assessment of cause and effect association. *Clin Gastroenterol Hepatol* 2003; **1**: 333-344

20 **Gono K**, Obi T, Yamaguchi M, Ohyama N, Machida H, Sano Y, Yoshida S, Hamamoto Y, Endo T. Appearance of enhanced tissue features in narrow-band endoscopic imaging. *J Biomed Opt* 2004; **9**: 568-577
21 **Hamamoto Y**, Endo T, Nosho K, Arimura Y, Sato M, Imai K. Usefulness of narrow-band imaging endoscopy for diagnosis of Barrett's esophagus. *J Gastroenterol* 2004; **39**: 14-20
22 **Tauber S**, Gross M, Issing WJ. Association of laryngo-pharyngeal symptoms with gastroesophageal reflux disease. *Laryngoscope* 2002; **112**: 879-886
23 **Cammarota G**, Galli J, Agostino S, De Corso E, Rigante M, Cianci R, Cesaro P, Nista EC, Candelli M, Gasbarrini A, Gasbarrini G. Accuracy of laryngeal examination during upper gastrointestinal endoscopy for premalignancy screening: prospective study in patients with and without reflux symptoms. *Endoscopy* 2006; **38**: 376-381
24 **Ahmed TF**, Khandwala F, Abelson TI, Hicks DM, Richter JE, Milstein C, Vaezi MF. Chronic laryngitis associated with gastroesophageal reflux: prospective assessment of differences in practice patterns between gastroenterologists and ENT physicians. *Am J Gastroenterol* 2006; **101**: 470-478
25 **Jaspersen D**, Kulig M, Labenz J, Leodolter A, Lind T, Meyer-Sabellek W, Vieth M, Willich SN, Lindner D, Stolte M, Malfertheiner P. Prevalence of extra-oesophageal manifestations in gastro-oesophageal reflux disease: an analysis based on the ProGERD Study. *Aliment Pharmacol Ther* 2003; **17**: 1515-1520
26 **Lee J**, Anggiansah A, Anggiansah R, Young A, Wong T, Fox M. Effects of age on the gastroesophageal junction, esophageal motility, and reflux disease. *Clin Gastroenterol Hepatol* 2007; **5**: 1392-1398
27 **Gordon C**, Kang JY, Neild PJ, Maxwell JD. The role of the hiatus hernia in gastro-oesophageal reflux disease. *Aliment Pharmacol Ther* 2004; **20**: 719-732
28 **Yeh C**, Hsu CT, Ho AS, Sampliner RE, Fass R. Erosive esophagitis and Barrett's esophagus in Taiwan: a higher frequency than expected. *Dig Dis Sci* 1997; **42**: 702-706
29 **Kang JY**, Tay HH, Yap I, Guan R, Lim KP, Math MV. Low frequency of endoscopic esophagitis in Asian patients. *J Clin Gastroenterol* 1993; **16**: 70-73
30 **Furukawa N**, Iwakiri R, Koyama T, Okamoto K, Yoshida T, Kashiwagi Y, Ohyama T, Noda T, Sakata H, Fujimoto K. Proportion of reflux esophagitis in 6010 Japanese adults: prospective evaluation by endoscopy. *J Gastroenterol* 1999; **34**: 441-444
31 **Lin BR**, Wong JM, Yang JC, Wang JT, Lin JT, Wang TH. Limited value of typical gastroesophageal reflux disease symptoms to screen for erosive esophagitis in Taiwanese. *J Formos Med Assoc* 2003; **102**: 299-304
32 **Little FB**, Koufman JA, Kohut RI, Marshall RB. Effect of gastric acid on the pathogenesis of subglottic stenosis. *Ann Otol Rhinol Laryngol* 1985; **94**: 516-519
33 **Ludemann JP**, Manoukian J, Shaw K, Bernard C, Davis M, al-Jubab A. Effects of simulated gastroesophageal reflux on the untraumatized rabbit larynx. *J Otolaryngol* 1998; **27**: 127-131
34 **Li W**, Zhang ST, Yu ZL. Clinical and endoscopic features of Chinese reflux esophagitis patients. *World J Gastroenterol* 2008; **14**: 1866-1871

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RAPID COMMUNICATION

Macro-regenerative nodules in biliary atresia: CT/MRI findings and their pathological relations

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Abstract

AIM: To describe the radiological findings of a macro-regenerative nodule (MRN) in the liver of pre-transplantation biliary atresia (BA) patients and to correlate it with histological findings.

METHODS: Between August 1990 and November 2007, 144 BA patients underwent liver transplantation (LT) at our institution. The pre-transplantation computer tomography (CT) and magnetic resonance imaging (MRI) findings were reviewed and correlated with the post-transplantation pathological findings.

RESULTS: Nine tumor lesions in 7 patients were diagnosed in explanted livers. The post-transplantation pathological findings showed that all the lesions were MRNs without malignant features. No small nodule was detected by either MRI or CT. Of the 8 detectable lesions, 6 (75%) were in the central part of the liver, 5 (63%) were larger than 5 cm, 5 (63%) had intra-tumor tubular structures, 3 (38%) showed enhancing fibrous septa, 3 (38%) had arterial enhancement in CT, one (13%) showed enhancement in MRI, and one (13%) had internal calcifications.

CONCLUSION: Although varied in radiological appearance, MRN can be differentiated from hepatocellular carcinoma (HCC) in most of BA patients awaiting LT. The presence of an arterial-enhancing nodule does not imply that LT is withheld solely on the basis of presumed malignancy by imaging studies. Liver biopsy may be required in aid of diagnostic imaging to exclude malignancy.

Key words: Biliary atresia; Macro-regenerative nodule; Liver neoplasm; Liver transplantation; Computed tomography; Magnetic resonance imaging

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INTRODUCTION

Biliary atresia (BA), the congenital absence or destruction of the intra- or extra-hepatic biliary system[1], affects about 5-10/100 000 live births[2]. Porto-enterostomy is typically performed as soon as possible in these children[3,4]. However, liver cirrhosis and its complications may develop in some patients even after a successful porto-enterostomy. Liver transplantation (LT) is, thus, beneficial to BA, and is the leading reason for LT in children[5].

Macro-regenerative nodule (MRN), defined as a regenerating liver nodule > 0.5 cm in size, is occasionally encountered in cirrhotic livers[6] and may mimic a hepatocellular carcinoma (HCC)[7,8]. Differentiating this benign entity from HCC may be challenging, but is very important when considering patients for LT. Although the clinical importance of MRN in BA patients have been discussed[9], further details of its computer tomography (CT) and magnetic resonance imaging (MRI) radiological appearances remain to be elucidated. The objective of this study was to describe the CT and MRI appearances of MRN in BA patients, and their radiological importance.

MATERIALS AND METHODS

Patients

We reviewed the images, medical records, and pathological reports of 144 BA patients who underwent LT from August 1990 to November 2007 in Chang Gung Memorial Hospital-Kaohsiung Medical Center, Taiwan, China. The diagnosis of BA was proven by surgical and pathological findings after porto-enterostomy in all patients. Of the 144 patients, routine liver CT angiography and MRI were not performed in 33 patients before September 2000 because no standard procedure was available. However, there were no liver masses described in the pathological reports in these 33 patients. A total of 111 patients were included in this study.

CT

Preoperative imaging evaluation was performed in the 111 patients using Somaton plus 4 spiral CT scanner (Siemens, Erlangen, Germany). Sedation using intravenous propofol (0.5-1 mg/kg body weight) without tracheal intubation was given in uncooperative patients. The scanning protocol was 5-mm collimation and a 1:1.5 pitch. The images were subsequently reconstructed at a 4- or 5-mm interval with scanning range from lung base to liver edge. Non-contrast enhanced scanning was performed followed by contrast enhanced scans utilizing an intravenous contrast medium (1.5-2 mL/kg body weight) injected at 1.5 mL/s with an automated power injector *via* a 22- or 24-gauge intravenous catheter. The arterial phase acquisition started at 20-25 s, porto-venous phase at 60-70 s and equilibrium phase at 3-5 min after intravenous administration of a contrast medium.

MRI

Seventy-nine of the 111 patients underwent MRI examination. We used a 1.5-T superconducting imager (Gyroscan Intera, Philips Medical system, Netherland B.V.) equipped with a phase-array body-coil. The liver was imaged in the axial planes with the following sequences: T1WI (GRE), T2WI (SENSE) and contrast-enhanced T1WI (GR). T1WI (GRE) was conducted using the following parameter: a repetition time/ echo time of 10/4.6 milliseconds. The T2WI (SENSE) was imaged using the following parameter: a repetition time/ echo time of 600/80 milliseconds.

For all the pulse sequences, a 5-8 mm thick slice was used with a 2 mm gap, 256 × 256 matrix size, echo train length of 1, number of average of 1 and a 35-40 field of view, depending on the size of the individual patient's liver.

A contrast medium was administrated using gadolinium-DTPA (0.1 mmole/kg body weight, Magnevist, Schering, Berlin, Germany) followed by a 20-mL saline flush. The delay for image acquisition timing was determined with a bolus tracking technique. Image reconstruction with 5-8 mm thickness was performed with source images at a MRI workstation.

Image interpretation

Two radiologists experienced in reading abdominal radiography retrospectively reviewed the images from the picture archiving and communicating system (PACS, GE medics) or from the patient's file storage (before 2002). Arterial, porto-venous and equilibrium phase images were interpreted conjointly. Preoperative assessment of the liver nodules included the number, location, size (the largest diameter in 3D orthogonal view), morphology, enhancing pattern, and signal intensity in MRI.

Histopathologic review

All explanted livers were serially sliced at 0.5 cm intervals and carefully inspected to detect the focal lesions seen during preoperative imaging. The size and location of all visible nodules were recorded at gross inspection. All macroscopic nodules were examined microscopically for histological identification and differentiation. Representative sections of the liver were also examined.

Serum alpha-fetoprotein (AFP)

Serum AFP values were determined in all patients at the time of imaging or before transplantation.

RESULTS

Nine MRN were detected in the explanted liver of 7 (4.8%) out of the 111 patients. The mean nodule diameter was 5.9 cm (range 1.6-9 cm). The MRN were located in the medial aspect (hepatic segments 4, 5, and 8) of 6 (67%) patients and in the lateral part of the liver (hepatic segments 2, and 6) of 3 (33%). The margin of the nodules was well-defined in all specimens. Of these 9 MRN, 7 were detected by CT and 7 by MRI. One was not detected by either MRI or CT, and one was found by MRI only. One patient with MRN did not undergo MRI. The CT and MRI findings of the 8 detectable MRN are listed in Table 1. At CT, the MRN were hyperdense compared with the surrounding liver parenchyma before contrast in 6 (75%) nodules (Figure 1A), the other 3 (38%) nodules were isodense (Figure 2A). After contrast medium enhancement, one nodule (13%) showed prominent enhancement both in arterial phase and in porto-venous phase, two nodules (25%) showed early enhancement and early wash-out pattern (Figure 3A), and four (50%) nodules showed no enhancement.

At MRI, the nodule was isointense to hyperintense on T1WI sequences and hypointense in T2WI in 5 (63%) nodules (Figure 1B) with one nodule showing T2WI central hyperintensity. Two nodules (25%) were hypointense on T1WI and hyperintense in T2WI (Figure 2B). After contrast enhancement, only one nodule showed enhancement. The other characteristic radiological findings in CT and MRI included stretching of intratumor tubular structures (5 lesions) (Figures 1C and 3B), fibrous septa in the periphery of the nodules (2 lesions), and internal calcifications (1 lesion). The septa were hypointense both before and after enhancement in T1WI.

Histopathology displayed that all lesions were MRN (Figures 1D and 3C), which were described as well-circumscribed liver cell nodules showing proliferating uniform liver cells bearing uniform round nuclei and

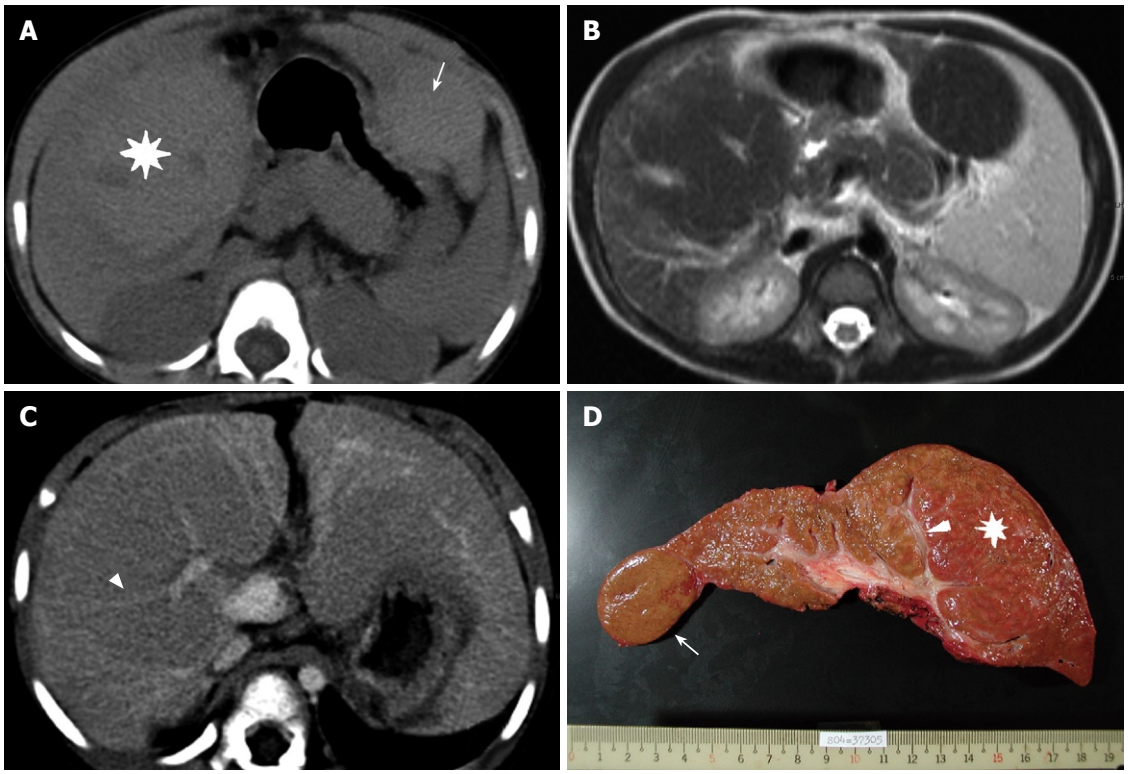

Figure 1 A 3-year-old BA girl with segments 5-8 (asterisk) and segment 2 (arrow) MRN. **A**: The density of the MRN is slightly higher than that in the surrounding liver parenchyma during pre-enhanced phase of the CT; **B**: FSE/T2WI MRI shows a lower signal intensity in the MRN than in the surrounding liver; **C**: During portovenous phase of the CT, the tubular structure and splaying portal veins can be seen in the MRN (arrowhead); **D**: The explanted liver and intra-tumoral portal tract can be seen (arrowhead).

Table 1 Summary of CT and MRI characteristics

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Size (cm)	9; 5; 1.6[1]	4.5	2.1	3.5	7	7.5	13
Location (segment)	S5-8/S2	S6	S8	S5	S4-5-8	S4-5-8	S5-6-7-8
Density in CT (-) relative to liver parenchyma	Hyperdense; Hyperdense	Hyperdense	Isodense	Isodense	Hyperdense	Hyperdense	Hyperdense
Enhancement in CT	No	Early enhancement and early wash-out	Delayed portovenous enhancement	No	Arterial and portovenous enhancement	No	Early patchy enhancement early wash-out
MRI T1WI/T2WI/C+	Hyperintense/ Hypoinstense	Hyperintense/ Hypoinstense	Hypoinstense/ Hyperintense	Hypoinstense/ Hyperintense	Not done	Hyperintense/ Hypoinstense	Isointense/ Hypoinstense
Septa T1WI/T2WI/C+	Not discernible	Hypoinstense/ Hypointense/ Enhanced	Not discernible	Not discernible	Not done	Hypoinstense/ Hypointense/ Enhanced	Not discernible
Presence of internal tubular structure (portal tract)	Yes; yes	No	No	No	Yes	Yes	Yes
Calcifications	No	No	No	Yes	No	No	No

[1]This small nodule is not detected by either CT or MRI.

eosinophilic cytoplasm. No cellular atypia was found. The liver cells were arranged in one- to two-cell thick plates with intervening sinusoids (Figure 3D). No apparent sinusoidal capillarization was seen in the tumor lesion and no malignant foci were identified. Abortive portal tract formation was also noted. In all the seven MRN patients, the AFP level was < 3 ng/mL before LT.

DISCUSSION

Multi-acinar MRN, first described by Edmondson in 1976[10], are sometimes seen in the cirrhotic liver. In 1996, an International Working Party defined MRN as "at least 5 mm regenerative nodules containing more than one portal tract"[10]. The reported prevalence in autopsy and explanted series varies from 14.2% in nodules > 1 cm in diameter to 37% in nodules > 0.5 cm in diameter[6,11,12]. It has been proposed that nodules > 2 cm in diameter in a background of cirrhosis are almost always dysplastic. However, these data were derived mainly from viral- or alcoholic-related cirrhotic livers. The smallest lesion found at gross pathology in our series was 1.6 cm in

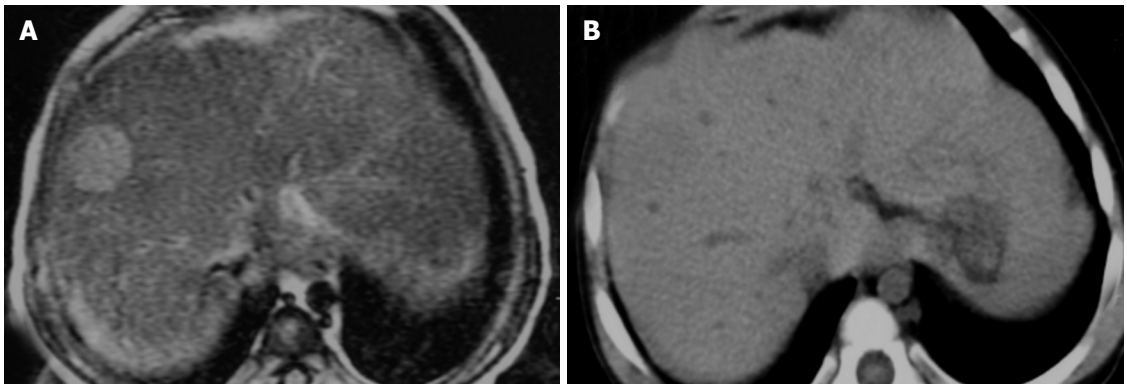

Figure 2 A 16-month-old BA girl with a 2.1 cm MRN in segment 8. **A**: During pre-enhanced phase of the CT, no nodule can be seen; **B**: The signal intensity is hyperinstense on T2WI MRI.

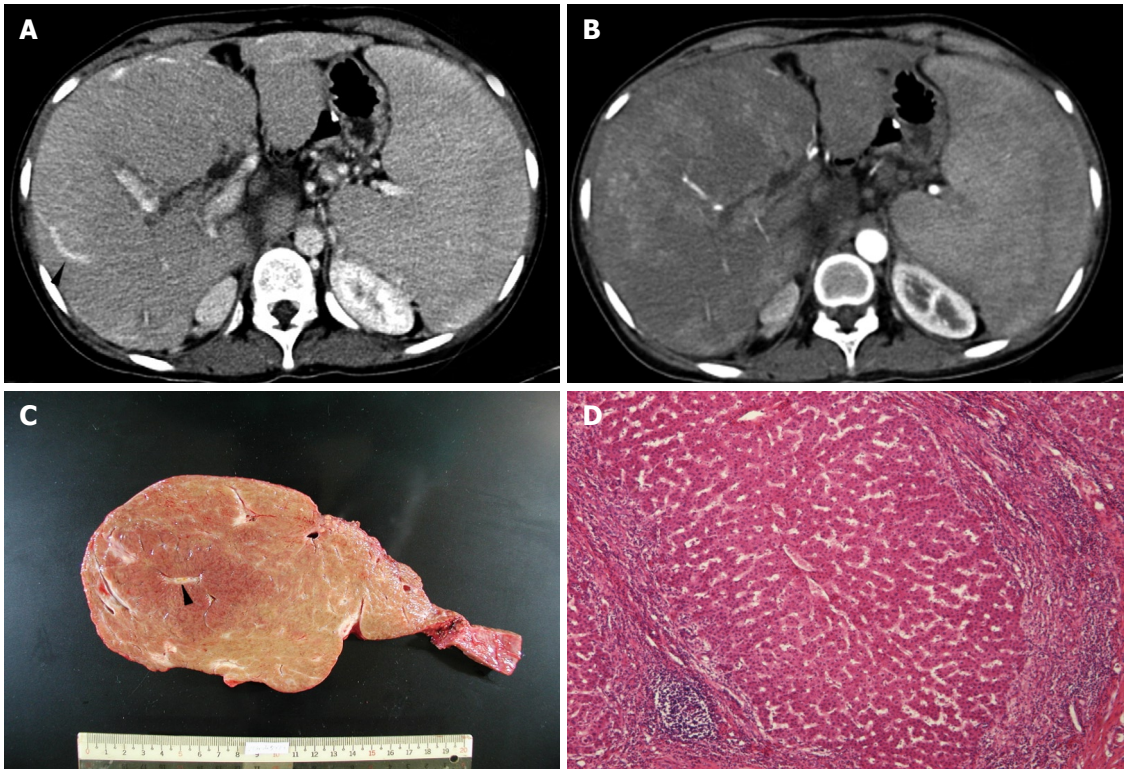

Figure 3 Patchy arterial enhancement within the mass in arterial phase of the CT (**A**), tubular structure (arrowhead) within the mass during portovenous phase of the CT (**B**), the explanted liver and intra-tumoral portal tract (arrowhead) (**C**), and microscopic examination (HE) showing uniform benign-looking liver cells arranged in one- to two-cell thick plates with intervening sinusoids and surrounding fibrous septa infiltrated with lymphocyte (**D**) in a 3-year-old BA girl with 13 cm MRN in right liver.

diameter and 56% (5/9) of the nodules were > 5 cm in diameter (Figure 1A). In the literature, lesions > 10 cm in diameter have been reported[13,14].

MRN can be divided into siderotic and non-siderotic types[15] based on the presence of iron deposition within the mass. The presence of iron results in hypointense signal especially with longer TE MRI pulse sequences[16]. In this study, 63% (5/8) of the regenerating nodules fit this pattern (Figure 1B)[17,18]. In non-siderotic regenerating nodules (2/8), the patterns included T1 hypointensity and T2 hyperintensity (Figure 2B). These non-siderotic MRN are difficult to see on pre-enhanced CT due to their isodensity with the surrounding liver parenchyma (Figure 2A). Because of the retrospective design of the current study, our methodology did not include histopathologic proof that the decreased signal intensity seen was caused by hepatic iron deposition. However, the same imaging techniques have been used to detect hepatic iron overload and siderotic regenerative nodules, and the results were confirmed with quantitative histopathologic measurements[19-21].

In CT hepatic angiography, MRN have been characterized as non-enhancing nodules surrounded by enhancing fibrous septa[22]. However, in our study, this structural pattern was not easily seen. Only 25% (2/8) in our cases had broad vascular septa partially discernable by MRI. These septa were hypointense on T1W images, perhaps due to the fibrous component of the septum

itself. Enhancing septa were seen only in two nodules in this series after gadolinium-DTPA administration.

Fifty-three percent (5/8) of the MRN in our series had characteristic internal enhancing tubular structures during porto-venous phase. This was particularly true in the larger masses (Figures 1C and 3B). The enhancing tubular structures correlated with a distorted portal vein accompanying a bile duct on histopathology (Figures 1D and 3C). MRN virtually always contain some normal-appearing abortive portal tracts with complete portal veins, hepatic arteries and bile ducts[10]. Importantly, as a regenerative nodule progresses to become a dysplastic nodule or early HCC, one may notice the loss of visualization of these portal tracts and development of new arterial vessels, termed non-triadal arteries. These features are often used to differentiate MRN from adenoma or carcinoma pathologically. Therefore, the imaging appearance of these characteristic findings may be useful in diagnosing MRN during the pretransplant survey among BA patients awaiting LT.

Other useful imaging features used to differentiate MRN from HCC have been described elsewhere. First, a MRN is usually hyperdense to liver parenchyma in pre-enhanced study and often (but not always) does not enhance post contrast. A typical HCC is hypodense or isodense to liver parenchyma in pre-enhanced study and shows early arterial enhancement and early washout in porto-venous phase in dynamic-enhanced CT[23]. Second, the signal intensity of MRN in T2WI is often hypointense while malignant tumors are often hyperintense[23,24]. Third, MRN, especially larger ones, show splaying of intratumor portal veins while malignant tumors usually demonstrate displaced or obliterated portal veins. However, there are some overlapping features between MRN and HCC[25]. In a cirrhotic liver, early reports suggested that virtually all arterial-enhancing lesions are HCC. However, arterial enhancement may be seen in a regenerating nodule, non-tumor arterio-portal shunt, and aberrant venous drainage in a cirrhotic liver[8,26,27]. Our series demonstrated that arterial enhancement also occurred in MRN of BA patients.

A MRN has been presumed to be a precancerous lesion in virus-related or alcoholic-related liver cirrhosis because malignant foci are occasionally found in MRN[28,29]. Although rare, one case report has described a small HCC in a BA-related cirrhotic liver[30]. The possibility of early HCC could not be excluded in some of our pre-transplant imaging surveys of BA patients. According to the non-invasive diagnostic criteria for HCC proposed by the European Association for the Study of the Liver[31,32], a diagnosis of HCC is established by the concomitant positive findings in two imaging techniques, or by a positive findings in one imaging technique with an AFP > 400 μg/L. The radiological interpretations for three arterial-enhancing tumor lesions (patient 2, 5 and 7) in this series were HCC initially (Figure 3A). Using the Barcelona Clinic Liver Cancer staging classification and treatment schedule, LT is considered for patients with three nodules < 3 cm in diameter or with one tumor < 5 cm in diameter and liver function impairment[33]. LT may be precluded in patients 5 and 7 if the HCC diagnosis was made based solely on imaging. Furthermore, the AFP values for all cases were < 3 ng/mL. In such a situation, liver biopsy is necessary to confirm the nature of an arterial enhancing mass in order to exclude hepatic malignancy.

In conclusion, MRN are seen in about 5% of patients with BA awaiting LT. CT and MRI imaging features of MRN as described in this review can be useful in differentiating MRN from HCC in most of BA patients awaiting LT. The presence of an arterial-enhancing nodule should not imply that LT should be withheld solely on the basis of presumed malignancy by imaging studies, especially if the AFP value is incongruent with radiographic findings. Liver biopsy may be required in aid of diagnostic imaging to exclude malignancy in these cases.

COMMENTS

Background

End stage liver cirrhosis develops in some biliary atresia (BA) patients later in life. Liver transplantation (LT) is beneficial to such patients. Macro-regenerative nodules (MRNs) in cirrhotic liver are occasionally encountered in computer tomography (CT) / magnetic resonance imaging (MRI) and may be confused with hepatocellular carcinoma (HCC).

Research frontiers

The authors described typical and atypical CT/MRI appearance of the MRN in BA patients, and the criteria for differentiation of MRNs from MRN. The strategy of managing atypical MRNs is also discussed.

Innovations and breakthroughs

This study showed that the majority of MRNs can be easily differentiated from hepatocellullar carcinoma by CT/MRI and unnecessary liver biopsy can be avoided.

Applications

Using the radiographic features presented in this study will help manage BA patients awaiting LT properly.

Terminology

MRN are defined as regenerative nodules larger than 0.5 cm in diameter in a cirrhotic liver. BA is a congenital absence or destruction of the intra- or extra-hepatic biliary system. It affects about 5-10/100 000 live births and is the leading cause for liver transplant in children especially in the oriental population.

Peer review

The authors described the radiological findings of MRN in the liver of pre-transplantation BA patients and correlated it with histological findings. This is an interesting paper, which is informative to readers.

REFERENCES

1 **Sokol RJ**, Mack C. Etiopathogenesis of biliary atresia. *Semin Liver Dis* 2001; **21**: 517-524

2 **Yoon PW**, Bresee JS, Olney RS, James LM, Khoury MJ. Epidemiology of biliary atresia: a population-based study. *Pediatrics* 1997; **99**: 376-382

3 **Hung PY**, Chen CC, Chen WJ, Lai HS, Hsu WM, Lee PH, Ho MC, Chen TH, Ni YH, Chen HL, Hsu HY, Chang MH. Long-term prognosis of patients with biliary atresia: a 25 year summary. *J Pediatr Gastroenterol Nutr* 2006; **42**: 190-195

4 **Sokol RJ**, Mack C, Narkewicz MR, Karrer FM. Pathogenesis and outcome of biliary atresia: current concepts. *J Pediatr Gastroenterol Nutr* 2003; **37**: 4-21

5 **Goss JA**, Shackleton CR, McDiarmid SV, Maggard M, Swenson K, Seu P, Vargas J, Martin M, Ament M, Brill J, Harrison R, Busuttil RW. Long-term results of pediatric liver transplantation: an analysis of 569 transplants. *Ann*

Surg 1998; **228**: 411-420
6 **Furuya K**, Nakamura M, Yamamoto Y, Togei K, Otsuka H. Macroregenerative nodule of the liver. A clinicopathologic study of 345 autopsy cases of chronic liver disease. *Cancer* 1988; **61**: 99-105
7 **Farrell R**, Steinman A, Green WH. Arteriovenous shunting in a regenerating liver simulating hepatoma. Report of a case. *Radiology* 1972; **102**: 279-280
8 **Brancatelli G**, Baron RL, Peterson MS, Marsh W. Helical CT screening for hepatocellular carcinoma in patients with cirrhosis: frequency and causes of false-positive interpretation. *AJR Am J Roentgenol* 2003; **180**: 1007-1014
9 **Liu YW**, Concejero AM, Chen CL, Cheng YF, Eng HL, Huang TL, Chen TY, Wang CC, Wang SH, Lin CC, Yong CC, Yang CH, Jordan AP, Jawan B. Hepatic pseudotumor in long-standing biliary atresia patients undergoing liver transplantation. *Liver Transpl* 2007; **13**: 1545-1551
10 **Terminology of nodular hepatocellular lesions.** International Working Party. *Hepatology* 1995; **22**: 983-993
11 **Le Bail B**, Belleannee G, Bernard PH, Saric J, Balabaud C, Bioulac-Sage P. Adenomatous hyperplasia in cirrhotic livers: histological evaluation, cellular density, and proliferative activity of 35 macronodular lesions in the cirrhotic explants of 10 adult French patients. *Hum Pathol* 1995; **26**: 897-906
12 **Terada T**, Terasaki S, Nakanuma Y. A clinicopathologic study of adenomatous hyperplasia of the liver in 209 consecutive cirrhotic livers examined by autopsy. *Cancer* 1993; **72**: 1551-1556
13 **Wang ZJ**, Coakley FV, Ferrell LD, Rosenthal P. CT and MRI of hepatic pseudotumor in long-standing biliary atresia. *J Comput Assist Tomogr* 2002; **26**: 444-447
14 **Gurkan A**, Sherlock MF, Chui AK, Thomson R, Verran D, Sheil AG, Painter D. A giant multiacinar macroregenerative nodule in an explanted liver. *Transplantation* 2001; **72**: 538-539
15 **Terada T**, Nakanuma Y. Survey of iron-accumulative macroregenerative nodules in cirrhotic livers. *Hepatology* 1989; **10**: 851-854
16 **Krinsky GA**, Lee VS, Theise ND, Weinreb JC, Rofsky NM, Diflo T, Teperman LW. Hepatocellular carcinoma and dysplastic nodules in patients with cirrhosis: prospective diagnosis with MR imaging and explantation correlation. *Radiology* 2001; **219**: 445-454
17 **Ohtomo K**, Itai Y, Ohtomo Y, Shiga J, Iio M. Regenerating nodules of liver cirrhosis: MR imaging with pathologic correlation. *AJR Am J Roentgenol* 1990; **154**: 505-507
18 **Murakami T**, Nakamura H, Hori S, Nakanishi K, Mitani T, Tsuda K, Kozuka T, Wakasa K, Monden M. CT and MRI of siderotic regenerating nodules in hepatic cirrhosis. *J Comput Assist Tomogr* 1992; **16**: 578-582
19 **Ernst O**, Sergent G, Bonvarlet P, Canva-Delcambre V, Paris JC, L'Hermine C. Hepatic iron overload: diagnosis and quantification with MR imaging. *AJR Am J Roentgenol* 1997; **168**: 1205-1208
20 **Gandon Y**, Guyader D, Heautot JF, Reda MI, Yaouanq J, Buhe T, Brissot P, Carsin M, Deugnier Y. Hemochromatosis: diagnosis and quantification of liver iron with gradient-echo MR imaging. *Radiology* 1994; **193**: 533-538
21 **Ito K**, Mitchell DG, Gabata T, Hann HW, Kim PN, Fujita T, Awaya H, Honjo K, Matsunaga N. Hepatocellular carcinoma: association with increased iron deposition in the cirrhotic liver at MR imaging. *Radiology* 1999; **212**: 235-240
22 **Murakami T**, Kuroda C, Marukawa T, Harada K, Wakasa K, Sakurai M, Monden M, Kasahara A, Kawata S, Kozuka T. Regenerating nodules in hepatic cirrhosis: MR findings with pathologic correlation. *AJR Am J Roentgenol* 1990; **155**: 1227-1231
23 **Taylor AJ**, Carmody TJ, Quiroz FA, Erickson SJ, Varma RR, Komorowski RA, Foley WD. Focal masses in cirrhotic liver: CT and MR imaging features. *AJR Am J Roentgenol* 1994; **163**: 857-862
24 **Matsui O**, Kadoya M, Kameyama T, Yoshikawa J, Arai K, Gabata T, Takashima T, Nakanuma Y, Terada T, Ida M. Adenomatous hyperplastic nodules in the cirrhotic liver: differentiation from hepatocellular carcinoma with MR imaging. *Radiology* 1989; **173**: 123-126
25 **Nagasue N**, Yukaya H, Chang YC, Kimura N, Ota N, Nakamura T. Hepatocellular pseudotumour (regenerating nodule) in the cirrhotic liver mimicking hepatocellular carcinoma. *Br J Surg* 1988; **75**: 1124-1128
26 **Dodd GD 3rd**, Baron RL, Oliver JH 3rd, Federle MP. Spectrum of imaging findings of the liver in end-stage cirrhosis: Part II, focal abnormalities. *AJR Am J Roentgenol* 1999; **173**: 1185-1192
27 **Kim TK**, Choi BI, Han JK, Chung JW, Park JH, Han MC. Nontumorous arterioportal shunt mimicking hypervascular tumor in cirrhotic liver: two-phase spiral CT findings. *Radiology* 1998; **208**: 597-603
28 **Takayama T**, Makuuchi M, Hirohashi S, Sakamoto M, Okazaki N, Takayasu K, Kosuge T, Motoo Y, Yamazaki S, Hasegawa H. Malignant transformation of adenomatous hyperplasia to hepatocellular carcinoma. *Lancet* 1990; **336**: 1150-1153
29 **Nakanuma Y**, Terada T, Ueda K, Terasaki S, Nonomura A, Matsui O. Adenomatous hyperplasia of the liver as a precancerous lesion. *Liver* 1993; **13**: 1-9
30 **Brunati A**, Feruzi Z, Sokal E, Smets F, Fervaille C, Gosseye S, Clapuyt P, de Ville de Goyet J, Reding R. Early occurrence of hepatocellular carcinoma in biliary atresia treated by liver transplantation. *Pediatr Transplant* 2007; **11**: 117-119
31 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodes J. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430
32 **Llovet JM**, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907-1917
33 **Llovet JM**, Bru C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin Liver Dis* 1999; **19**: 329-338

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RAPID COMMUNICATION

Celecoxib-related gastroduodenal ulcer and cardiovascular events in a randomized trial for gastric cancer prevention

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Abstract

AIM: To evaluate the long-term risk of gastroduodenal ulcer and cardiovascular events induced by celecoxib in a population-based, randomized, double-blind, placebo-controlled study.

METHODS: From 2004 to 2006, a total of 1024 Chinese patients (aged 35 to 64 years) with severe chronic atrophic gastritis, intestinal metaplasia or dysplasia were randomly assigned to receive 200 mg of celecoxib twice daily or placebo in Linqu County (Shandong Province, China), a high-risk area of gastric cancer. All gastroduodenal ulcer and cardiovascular events occurred were recorded and the patients were followed up for 1.5 years after treatment. At the end of the trial, a systematic interview survey about other adverse events was conducted.

RESULTS: Gastroduodenal ulcer was detected in 19 of 463 (3.72%) patients who received celecoxib and 17 of 473 (3.31%) patients who received placebo, respectively (odds ratio = 1.13, 95% CI = 0.58-2.19). Cardiovascular (CV) events occurred in 4 patients who received celecoxib and in 5 patients who received placebo, respectively. Compared with those who received placebo, patients who received celecoxib had no significant increase in occurrence of CV events (hazard ratio = 0.84, 95% CI = 0.23-3.15). Among the adverse events acquired by interview survey, only the frequency of bloating was significantly higher in patients treated with celecoxib than in those treated with placebo.

CONCLUSION: Treatment of gastric cancer with celecoxib is not associated with increased risk of gastroduodenal ulcer and cardiovascular events.

Key words: Celecoxib; Gastroduodenal ulcer; Cardiovascular diseases; Adverse effects; Epidemiology; Randomized controlled trial

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INTRODUCTION

Celecoxib, approved by the US Food and Drug Administration (FDA) in 1998 for osteoarthritis and rheumatoid arthritis, is a cyclooxygenase-2 (COX-2) inhibitor. Owing to the selective inhibition of COX-2, this drug provides similar anti-inflammatory effects and a reduced risk of gastrointestinal complications in osteoarthritis and rheumatoid arthritis patients compared with nonsteroidal anti-inflammatory drugs (NSAIDs)[1,2], which inhibit both COX-1 and COX-2. In addition, celecoxib, a selective inhibitor of COX-2, can block tumor growth by its antiangiogenic and proapoptotic effects, suggesting that it can be used in the prevention and treatment of cancers[3-5].

However, it was reported that rofecoxib, also a COX-2 inhibitor, is associated with gastrointestinal toxic effects and cardiovascular (CV) events[6,7]; But, it has no gastrointestinal toxicity[8]. The conflicting results have raised the concern about the safety of celecoxib[9,10]. In 2005, the FDA Advisory Committee concluded that the adverse events of celecoxib are less than those of rofecoxib[11]. Therefore, we studied the safety issue of celecoxib. Gastroduodenal ulcer and CV events induced by celecoxib are reported in this paper.

MATERIALS AND METHODS

Study population

In 2004, a total of 1024 subjects, randomly selected from 12 villages of Linqu County (Shandong Province, China), participated in this study. Their age was 35-64 years. All subjects received a brief physical examination and their medical history was recorded. Subjects were ineligible if they had a history of stroke within two years, angina or congestive heart failure or myocardial infarction within one year, neoplastic diseases in the previous 10 years, esophageal or gastric surgery, inflammatory bowel disease, or bleeding diathesis, paracetamol allergy or hypersensitivity to aspirin, or other life-threatening illness. The remainders received ^{13}C-urea breath test (^{13}C-UBT) and gastroscopic examination with biopsies from 5 standard sites of the stomach. Only those who had *Helicobacter pylori (H pylori)* infection and a histological diagnosis of severe chronic atrophic gastritis (CAG), intestinal metaplasia (IM) or dysplasia (DYS) were enrolled in the intervention trial. A written informed consent was obtained from each participant and the trial was approved by the Institutional Review Board (IRB) of Peking University School of Oncology (PUSO).

Study design and randomization

Subjects were randomly assigned to received antibiotics and/or celecoxib or their placebo in a 2 × 2 factorial design. Finally, the subjects were divided into four groups. Group 1 received anti-*H pylori* treatment in the first week followed by 200 mg celecoxib twice daily for 24 mo, group 2 received anti-*H pylori* treatment in the first week followed by a look-alike celecoxib placebo for 24 mo, group 3 received a look-alike anti-*H pylori* placebo in the first week followed by celecoxib twice daily for 24 mo, group 4 received a look-alike anti-*H pylori* placebo in the first week followed by a look-alike celecoxib placebo for 24 mo. We only observed and evaluated the risk of cardiovascular and other adverse events in the celecoxib and placebo groups (Figure 1). Both the participants and investigators were blinded to the treatment. Randomization of treatment assignments was generated at Westat Inc. in the US after eligibility was determined.

From March 16 to 30, 2004, the eligible participants were given a triple therapy with 20 mg omeprazole, 1 g amoxicillin and 500 mg clarithromycin or placebo twice daily for 7 d to eradicate their *H pylori* infection. Then 200 mg of celecoxib or placebo twice daily was given orally from April 8, 2004 to May 6, 2006, except for April 2005 because of the interim gastroscopic examination.

Follow-up

During the period of study, labeled pill bottles of celecoxib or placebo were distributed to participants in each village by PUSO staff and trained field staff each month. The field staff visited each participant twice a month to monitor treatment-related events and to promote pill compliance in the entire duration of the study. The staff counted and recorded the number of pills remaining in each bottle before the new pill bottles were distributed each month. If a subject was not at home during the staff visit, an evening visit was scheduled. A subject was considered compliant if the pill bottle was empty at the end of that month. If a subject was unable to be contacted at the time of counting pills, he or she was considered non-compliant.

Adverse events

Gastroduodenal ulcer was detected in 2005 and 2006 by the same group of PUSO physicians and gastroenterologists. Gastroscopic procedures, including biopsy samples taken from seven standard sites of stomach and histopathologic criteria, have been described elsewhere[12]. The gastroenterologist and pathologist were blinded to the subjects' intervention.

The CV events were defined as fatal or nonfatal myocardial infarction, ischemic and hemorrhagic stroke as previously described[13]. When visiting the participants, investigators recorded the CV events and other complaints of the participants. While investigators were absent, participants-reported symptoms were recorded by doctors in village clinics. All the CV events were diagnosed in local hospitals.

Other non-adjudicated adverse events were acquired by an interview among all the subjects at the end of the trial in May 2006. All the subjects' symptoms in the past two years were inquired and recorded by the trained interviewers, checked and categorized by two physicians in a blinded fashion after completion of the survey.

If the symptoms were related to treatment, PUSO physicians and field staff paid a close attention to the

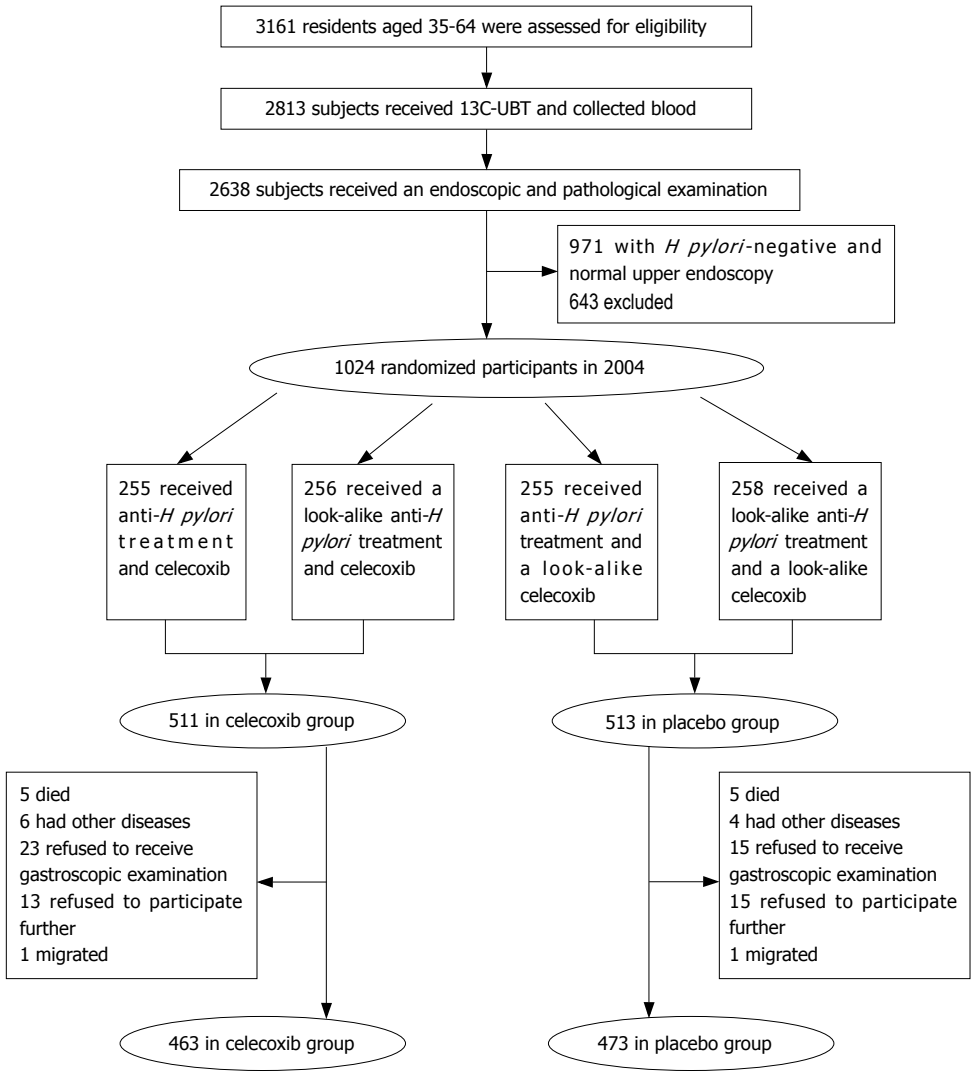

Figure 1 Trial profile.

subjects for at least 2 mo and these subjects received continuous treatment if the symptoms were aggravated.

Statistical analysis

This study was designed to achieve a significant level of 95% (< 0.05) and a power of 90% to detect a 20% regression of pre-malignant lesions, based on the background of 80% prevalence of gastric atrophy. At least 120 subjects were required in each group in order to detect a significant difference between the different treatment groups.

All data analyses were performed in a blinded fashion. The relative risks (with 95% confidence intervals) of gastroduodenal ulcer were analyzed using logistic regression by adjusting gender, age, smoking and drinking. The rate of CV events was determined and multivariate hazard ratio (HR) was calculated using the Cox proportional-hazard model. All *P* values were two-sided, and $P < 0.05$ was considered statistically significant. All analyses were performed with SAS software, version 8.2.

RESULTS

The 1024 participants were divided into celecoxib treatment group ($n = 511$) and placebo treatment group ($n = 513$). The baseline characteristics were balanced between the two groups (Table 1). During the two-year period of treatment, 88 participants who were relatively evenly distributed between the two groups withdrew from the study (Figure 1). The compliance rate was 90.61% in the celecoxib treatment group and 92.20% in the placebo treatment group, respectively.

From April 2004 to May 2006, gastroduodenal ulcer was detected in 19 of 463 (3.72%) participants of the celecoxib treatment group and in 17 of 473 (3.31%) participants of the placebo treatment group, respectively. The odds ratio (OR) was 1.13 (95% CI = 0.58-2.19, Table 2).

During the entire period of follow-up, CV events occurred in 4 participants of the celecoxib treatment group and in 5 participants of the placebo treatment group, respectively (Table 2). Compared with the placebo treatment group, the celecoxib treatment group had no

Table 1 Baseline characteristics of 1024 participants

	Celecoxib, *n* (%)	Placebo, *n* (%)	*P*
Male	238 (46.58)	235 (45.81)	0.81
Age (yr, means ± SD)	52.94 ± 6.51	52.93 ± 6.48	0.97
Smoking	146 (28.57)	142 (27.68)	0.75
Drinking	172 (33.66)	175 (34.11)	0.88
Hypertension	155 (30.33)	160 (31.19)	0.77

Table 2 Incidence and risk of side effects in two groups

	Celecoxib, *n* (%)	Placebo, *n* (%)	OR (95% CI)
Gastroduodenal ulcer	19 (3.72)	17 (3.31)	1.13 (0.58-2.22)
CV events	4 (0.86)	5 (1.06)	0.84 (0.23-3.15)
Main nonadjudicated side effects			
Abdominal pain	8 (1.73)	13 (2.75)	0.62 (0.26-1.52)
Bloating	19 (4.10)	7 (1.48)	2.85 (1.19-6.84)
Constipation	9 (1.94)	15 (3.17)	0.61 (0.26-1.40)
Diarrhea	24 (5.18)	24 (5.07)	1.02 (0.57-1.83)
Dizziness	25 (5.40)	36 (7.61)	0.69 (0.41-1.17)
Gastric spasmus	15 (3.24)	15 (3.17)	1.02 (0.49-2.12)
Headache	26 (5.62)	23 (4.86)	1.16 (0.65-2.07)
Heartburn	29 (6.26)	23 (4.86)	1.31 (0.75-2.30)
Loss of appetite	25 (5.40)	16 (3.38)	1.63 (0.86-3.10)
Muscle pain	55 (11.88)	70 (14.80)	0.78 (0.53-1.13)
Nausea	14 (3.02)	17 (3.59)	0.84 (0.41-1.72)
Pain in the chest	16 (3.46)	17 (3.59)	0.96 (0.48-1.92)
Palpitations	22 (4.75)	16 (3.38)	1.43 (0.74-2.75)

significant increase in occurrence of CV events (HR = 0.84, 95% CI = 0.23-3.15).

The main nonadjudicated side effects are listed in Table 2. Except for bloating (OR = 2.85, 95% CI = 1.19-6.84), there were no significant differences in the frequency of other nonadjudicated adverse events between the two groups.

DISCUSSION

In this study, gastroduodenal ulcer and CV events occurred in the subjects who took 200 mg celecoxib twice daily.

Two previous trials addressed the possibility that celecoxib has a lower rate of gastrointestinal complications than NSAIDs[14,15]. It was reported that the annual incidence rate of upper gastrointestinal complications and symptomatic ulcers is significantly lower in the celecoxib treatment group than in the combined diclofenac and ibuprofen treatment group (2.08% *vs* 3.54%; $P = 0.02$) after 6 mo of treatment[14]. It has been shown that the incidence rate of gastric ulcer in the celecoxib treatment group and diclofenac treatment group is 18% and 34%, respectively ($P < 0.001$), and the incidence rate of duodenal ulcer is 5% and 11%, in the celecoxib treatment group and diclofenac treatment group, respectively ($P < 0.009$)[15].

Although the distinct role of celecoxib in ulcer is still unclear[16], most studies suggested that celecoxib is not associated with gastric or duodenal ulcer[17-19]. Our trial compared the effects of celecoxib and placebo on gastroduodenal ulcer, and the risk of gastroduodenal ulcer was not increased after treatment with 200 mg celecoxib daily compared with placebo.

The association between celecoxib and CV events was still debatable in our study. It was reported that a single dose of 400 mg celecoxib daily and placebo does not induce excess CV risk[20]. However, it was reported that 800 mg celecoxib increases the risk of death due to cardiovascular disease, myocardial infarction, stroke, or heart failure[21].

The mechanism underlying the potential cardiovascular risk of COX-2 inhibitors is not fully understood. Although the imbalance caused by COX-2 inhibitors suppressing the COX-2 dependent prostacyclin production in endothelial cells without affecting the synthesis of platelet-derived thromboxane A_2, may promote thrombosis and increase the risk of CV events[22-24], the extent of instability to serum thromboxane and platelet function can be influenced by many factors, such as different doses of COX-2 inhibitors, variability among patients[16].

In this study, different dose-effects of celecoxib on cardiovascular risk were observed. The dose of 800 mg celecoxib daily could increase the CV risk. However, 400 mg celecoxib daily did not increase the CV risk, suggesting that it can be used in the treatment of gastric ulcer.

In the present study, the frequency of bloating was higher in the celecoxib treatment group than in the placebo treatment group. However, the CV events were mild and tolerable, and none of the participants withdrew from this trial.

In conclusion, increases in gastroduodenal ulcer and CV events do not occur in subjects who take 200 mg celecoxib twice daily for two years. Celecoxib can be used in prevention and treatment of gastric cancer.

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COMMENTS

Background

Celecoxib, a cyclooxygenase-2 inhibitor, is widely used as an analgesic and anti-inflammatory agent. In addition, it can prevent cancer. However, it is necessary to evaluate the risk of gastroduodenal ulcer and cardiovascular events, particularly in population-based studies.

Research frontiers

No increase in gastroduodenal ulcer and cardiovascular (CV) events were found in the subjects who took 200 mg celecoxib twice daily for two years.

Innovations and breakthroughs

This paper firstly reports an assessment of celecoxib-related gastroduodenal ulcer and cardiovascular events in Chinese population.

Applications

Celecoxib (200 mg twice daily for two years) can prevent and treat gastric cancer in Chinese population.

Peer review

The authors documented the absence of adverse effects of prolonged celecoxib administration at gastroduodenal and cardiovascular level in Chinese patients. The study was well designed and the results are reliable.

REFERENCES

1 **Bombardier C**, Laine L, Reicin A, Shapiro D, Burgos-Vargas R, Davis B, Day R, Ferraz MB, Hawkey CJ, Hochberg MC, Kvien TK, Schnitzer TJ. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N Engl J Med* 2000; **343**: 1520-1528, 2 p following 1528

2 **Singh G**, Fort JG, Goldstein JL, Levy RA, Hanrahan PS, Bello AE, Andrade-Ortega L, Wallemark C, Agrawal NM, Eisen GM, Stenson WF, Triadafilopoulos G. Celecoxib versus naproxen and diclofenac in osteoarthritis patients: SUCCESS-I Study. *Am J Med* 2006; **119**: 255-266

3 **Gasparini G**, Longo R, Sarmiento R, Morabito A. Inhibitors of cyclo-oxygenase 2: a new class of anticancer agents? *Lancet Oncol* 2003; **4**: 605-615

4 **Steinbach G**, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK, Levin B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* 2000; **342**: 1946-1952

5 **Harris RE**, Beebe-Donk J, Alshafie GA. Reduction in the risk of human breast cancer by selective cyclooxygenase-2 (COX-2) inhibitors. *BMC Cancer* 2006; **6**: 27

6 **Langman MJ**, Jensen DM, Watson DJ, Harper SE, Zhao PL, Quan H, Bolognese JA, Simon TJ. Adverse upper gastrointestinal effects of rofecoxib compared with NSAIDs. *JAMA* 1999; **282**: 1929-1933

7 **Bresalier RS**, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgan K, Lines C, Riddell R, Morton D, Lanas A, Konstam MA, Baron JA. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med* 2005; **352**: 1092-1102

8 **Bertagnolli MM**, Eagle CJ, Zauber AG, Redston M, Solomon SD, Kim K, Tang J, Rosenstein RB, Wittes J, Corle D, Hess TM, Woloj GM, Boisserie F, Anderson WF, Viner JL, Bagheri D, Burn J, Chung DC, Dewar T, Foley TR, Hoffman N, Macrae F, Pruitt RE, Saltzman JR, Salzberg B, Sylwestrowicz T, Gordon GB, Hawk ET. Celecoxib for the prevention of sporadic colorectal adenomas. *N Engl J Med* 2006; **355**: 873-884

9 **Psaty BM**, Potter JD. Risks and benefits of celecoxib to prevent recurrent adenomas. *N Engl J Med* 2006; **355**: 950-952

10 **Graham DJ**. COX-2 inhibitors, other NSAIDs, and cardiovascular risk: the seduction of common sense. *JAMA* 2006; **296**: 1653-1656

11 **Okie S**. Raising the safety bar--the FDA's coxib meeting. *N Engl J Med* 2005; **352**: 1283-1285

12 **You WC**, Li JY, Blot WJ, Chang YS, Jin ML, Gail MH, Zhang L, Liu WD, Ma JL, Hu YR, Mark SD, Correa P, Fraumeni JF Jr, Xu GW. Evolution of precancerous lesions in a rural Chinese population at high risk of gastric cancer. *Int J Cancer* 1999; **83**: 615-619

13 **Collaborative overview of randomised trials of antiplatelet therapy--I: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients.** Antiplatelet Trialists' Collaboration. *BMJ* 1994; **308**: 81-106

14 **Silverstein FE**, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, Burr AM, Zhao WW, Kent JD, Lefkowith JB, Verburg KM, Geis GS. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA* 2000; **284**: 1247-1255

15 **Emery P**, Zeidler H, Kvien TK, Guslandi M, Naudin R, Stead H, Verburg KM, Isakson PC, Hubbard RC, Geis GS. Celecoxib versus diclofenac in long-term management of rheumatoid arthritis: randomised double-blind comparison. *Lancet* 1999; **354**: 2106-2111

16 **Brinkmann G**, Burkhart C, Clausen M, Henze E. [The effect of Belzer and Bretschneider cardioplegia solutions on myocardial energy metabolism. A study with 31phosphorus magnetic resonance spectroscopy in an animal model] *Z Kardiol* 1992; **81**: 339-344

17 **Varas-Lorenzo C**, Maguire A, Castellsague J, Perez-Gutthann S. Quantitative assessment of the gastrointestinal and cardiovascular risk-benefit of celecoxib compared to individual NSAIDs at the population level. *Pharmacoepidemiol Drug Saf* 2007; **16**: 366-376

18 **Kasliwal R**, Layton D, Harris S, Wilton L, Shakir SA. A comparison of reported gastrointestinal and thromboembolic events between rofecoxib and celecoxib using observational data. *Drug Saf* 2005; **28**: 803-816

19 **Deeks JJ**, Smith LA, Bradley MD. Efficacy, tolerability, and upper gastrointestinal safety of celecoxib for treatment of osteoarthritis and rheumatoid arthritis: systematic review of randomised controlled trials. *BMJ* 2002; **325**: 619

20 **Arber N**, Eagle CJ, Spicak J, Racz I, Dite P, Hajer J, Zavoral M, Lechuga MJ, Gerletti P, Tang J, Rosenstein RB, Macdonald K, Bhadra P, Fowler R, Wittes J, Zauber AG, Solomon SD, Levin B. Celecoxib for the prevention of colorectal adenomatous polyps. *N Engl J Med* 2006; **355**: 885-895

21 **Solomon SD**, McMurray JJ, Pfeffer MA, Wittes J, Fowler R, Finn P, Anderson WF, Zauber A, Hawk E, Bertagnolli M. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med* 2005; **352**: 1071-1080

22 **Fitzgerald GA**. Coxibs and cardiovascular disease. *N Engl J Med* 2004; **351**: 1709-1711

23 **Hawkey CJ**. COX-2 inhibitors. *Lancet* 1999; **353**: 307-314

24 **Antman EM**, DeMets D, Loscalzo J. Cyclooxygenase inhibition and cardiovascular risk. *Circulation* 2005; **112**: 759-770

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RAPID COMMUNICATION

Radiofrequency ablation as a treatment for hilar cholangiocarcinoma

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Abstract

AIM: To explore the role of radio-frequency ablation (RFA) as a treatment for hilar cholangiocarcinoma.

METHODS: Eleven patients with obstructive cholestasis underwent Computed Tomography (CT) examination, occupying lesions were observed in the hepatic hilar region in each patient. All lesions were confirmed as cholangioadenocarcinoma by biopsy and were classified as type Ⅲ or Ⅳ by percutaneous transhepatic cholangiography. Patients were treated with multiple electrodes RFA combined with other adjuvant therapy. The survival rate, change of CT attenuation coefficient of the tumor and tumor size were studied in these patients after RFA.

RESULTS: In a follow-up CT scan one month after RFA, a size reduction of about 30% was observed in six masses, and two masses were reduced by about 20% in size, three of the eleven masses remained unchanged. In a follow-up CT scan 6 mo after RFA, all the masses were reduced in size (overall 35%), in which the most significant size reduction was 60%. The survival follow-up among these eleven cases was 18 mo in average. Ongoing follow-up showed that the longest survival case was 30 mo and the shortest case was 10 mo.

CONCLUSION: RFA is a microinvasive and effective treatment for hilar cholangiocarcinoma.

INTRODUCTION

Hilar cholangiocarcinoma (also known as Klatskin Tumor) was first reported by Gerald Klatskin in 1965. Since then, many therapeutic methods have been established to treat this type of tumor. For those patients presenting with type Ⅰ and type Ⅱ tumors, surgical resection is good and has a high 5-year survival rate[1-5]. However, for those tumors classified as type Ⅲ and Ⅳ tumors, the surgical prognosis is poor even when combined with local tumor resection and left or right hemi-lobectomy of the liver, which can itself lead to further complications[6-8]. Thus, finding a surgical approach for the treatment of type Ⅲ and Ⅳ Klatskin tumors is problematic. Percutaneous image-guided radiofrequency ablation (RFA) has received increasing attention as a promising technique for the treatment of liver tumors. This technique permits the destruction of tumors without necessitating their removal, and in many cases, can be used in place of more invasive and expensive surgical treatment. Initial attempts at tissue ablation with radiofrequency have been limited to the 1.6-cm diameter coagulation necrosis obtained from a single conventional electrode[9]. In order to achieve larger thermal necrosis, internally cooled, single or

clustered electrode technique, as well as expandable needle techniques has been introduced. Hence, from May, 2003 to December, 2005, we applied RFA therapy to a group of 11 patients with pathologically confirmed, type Ⅲ and Ⅳ cholangiocarcinoma after percutaneous transhepatic cholangic drainage (PTCD) in order to determine its safety, efficacy, and outcome.

MATERIALS AND METHODS

Patient demographics

Eleven patients enrolled in this study were all male, ranging in age from 42 to 74 years, with a mean of 52 years. All patients presented with jaundice, and underwent CT examination, Occupying lesions were observed in hepatic hilar region in all cases. All lesions were confirmed as cholangioadenocarcinoma by biopsy and were classified as type Ⅲ a (n = 4), type Ⅲ b (n = 2), type Ⅳ (n = 5) tumors by percutaneous transhepatic cholangiography. The average dimensions of the tumor masses were 3.4-4.5 cm.

Instruments

The Marconi CT-Twin flash, with the following parameters: volumetric scan with 5-10 mm thickness and pitch 1, WE7568 multiple electrode tumor RF ablator, 200W pulse output and 290 KHz pulse frequency, was applied (made by Beijing Welfare Electronic Ca.). A temperature sensor was installed in the electrode to monitor the temperature and the sensor deviation was ± 0.50℃. A WHK-4 multiple electrode tumor RFA electrode with side holes ablation needle was applied. These systems deploy an array of multiple curved stiff wires in the shape of an umbrella from a single 14-gauge or 16-gauge canula (Figure 1). This array can produce zones of coagulation necrosis of up to 4-5 cm compared to the bipolar electrode. Furthermore, there are tiny side holes in the distal end of the central electrode, which make it possible for saline infusion during RFA in order to avoid charring and improve the ablation effect.

Therapeutic strategy

All patients were hospitalized with proper blood analyses and preparations before synthetic interventional therapy. PTCD was performed for biliary drainage for 2 wk prior to RFA in order to preserve or improve hepatic function. The tumor size determined the number of sessions and duration of RFA.

PTCD (percutaneous transhepatic cholangic drainage)

By using a 22 gauge Chiba needle, PTC was performed from the right middle axillary line to the most dilated intrahepatic biliary duct according to a prior CT scan. When the needle punctured the biliary duct, we fixed the needle and injected contrast media to perform cholangiography. The contrast media allowed better depiction of the stenotic segment and determined whether the left and right hepatic ducts were involved, allowing the tumor to be staged further. A unilateral drainage catheter was applied for type Ⅲ a and type Ⅲ b. Bilateral kissing catheters were applied for type Ⅳ. The percutaneous drainage catheter was fixed onto the skin with stitches and connected with a drainage bag for daily observation of external bile drainage.

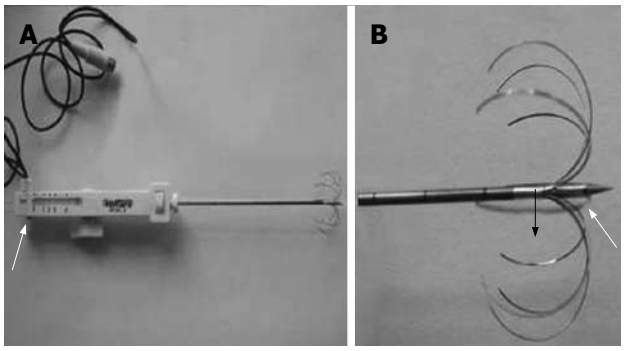

Figure 1 **A**: The white arrow indicates the injection hole; **B**: The white arrow indicates the side hole, and the black arrow indicates hooked array radiofrequency needles

RFA

After 2 wk of biliary drainage, depending on the patient's general condition and improvement of the hepatic function, RFA was applied by percutaneous puncture into the tumor mass with an ablation electrode needle under CT guiding (a density survey of the tumor mass was performed before the procedure). After optimal tumor puncture, we delivered the electrodes in appreciable diameter. The electrodes were distanced from the drainage catheter during the RFA procedure, thereby protecting the catheters from mechanical puncture and thermal damage. The duration of ablation was dependant on the size of the tumor mass, with 10 min for those ⩽ 3 cm, and 10-15 min for those around 3.1-4.0 cm. We prolonged the procedure time during which the temperature slowly increased. Injection of 1 mL of 10% saline from the side aperture of the needle every minute enhanced the excitement of ion vibrations, increasing the capability of the device. A CT scan was performed after delivering the electrodes to ensure that the entire lesion was fully covered within the region of RFA. This would provide complete tumor necrosis and eliminate the possible residual tumor infiltration in the adjacent area (Figures 2 and 3).

Follow up

After RFA, regular monthly abdominal CT scans including a plain scan, double-phase scan and delayed image were performed with biochemical blood analysis in the first 6 mo. For the next 6 mo, follow-up work was every 2 mo, and 1 year after RFA, follow-up was every 3 mo.

Evaluation of the therapeutic effect

One month after the RFA synthetic therapy, CT scans were compared to the baseline data. The treatment was counted as effective, in case where there was significant density attenuation of the tumor mass with no contrast

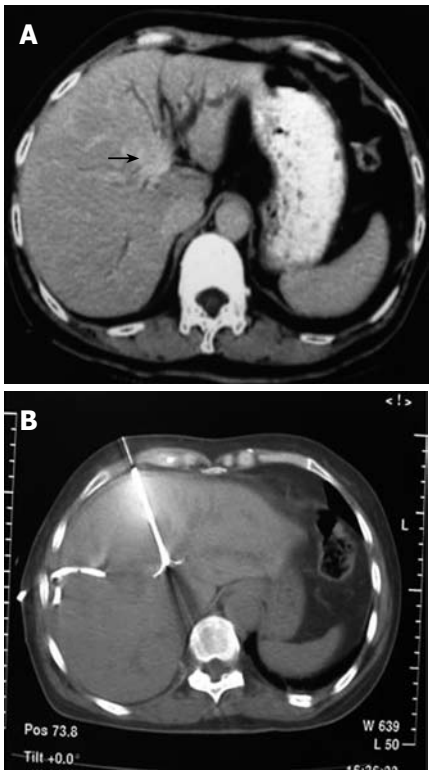

Figure 2 A case with type Ⅲb Klatskin tumor. **A**: Plain CT scan reveals that the tumor is in the main trunk of left hepatic duct with dilatation of branches; **B**: The patient underwent RFA under CT guidance after internal and external drainage for two weeks.

enhancement, regardless of whether the tumor size was reduced or remained unchanged. Conversely, cases were counted as non-effective, if the tumor mass was enlarged and there was no significant density attenuation and inhomogeneous enhancement by Ⅳ contrast.

RESULTS

Quantification of tumor density change

The average density of the 11 tumor masses was 44.23 Hu before RFA therapy. One month after the therapy, it decreased to 21.6 Hu with the most marked case being a decrease of about 40 Hu.

Quantification of tumor size

After RFA treatment, six masses had diminished in size by approximately 30%, two masses had been reduced by approximately 20% and no significant change in size was noted in three masses. These results were confirmed 1 mo later by a second CT scan. After 6 mo, the average size reduction in all the masses was approximately 35%. The most significant size reduction was 60%.

Quantification of biochemical blood analysis

One month after PTCD, the direct and indirect bilirubin levels returned to their normal range (direct bilirubin $\leqslant$ 8 μmol/L and indirect bilirubin $\leqslant$ 15 μmol/L) in nine cases. By 6 mo after RFA therapy, the bilirubin levels of all cases had returned to their normal range.

Quantification of complications

No severe complications occurred during or after each procedure with the exception of fever post RFA.

Survival analysis

The 11 patients were all alive at the end of the study. The survival period among these 11 cases was 18 mo on average. Ongoing follow-up showed that the longest survival case was 30 mo and the shortest case was 10 mo.

DISCUSSION

Gerald Klatskin[10] first described the specific entities of adenocarcinoma as confined below, at or above the confluence of the common hepatic duct in 1965. Afterwards, Bismuth and Corlette[11] classified the tumor into four types: Type Ⅰ: the lesion is confined to the common hepatic duct with no involvement of the left and right common ducts or confluence; Type Ⅱ: confluence of the hepatic ducts is involved, but the lesion does not extend to the left and right intrahepatic biliary ducts; Type Ⅲ a: the lesion spreads to the right hepatic duct; Type Ⅲ b: the lesion spreads to the left hepatic duct; Type IV: involvement of the left and right hepatic ducts and confluence[12-18].

For Type Ⅰ and Ⅱ tumors, surgical resection is the most effective form of therapy. Furthermore, a surgical approach is still possible in cases of local tumor recurrence[19,20]. However, for Type Ⅲ tumors, radical resection is an aggressive procedure that might involve removal of the left or right hepatic lobe and caudate lobe of the liver. A mortality rate of up to 10% has been encountered, and survival after surgical resection on this type of tumor has been disappointing[12,14,21-24]. Palliative resection of Type Ⅳ tumor is still controversial, but doctors tend to perform liver transplantation. Transartery chemoembolization (TACE) is not an ideal treatment for Type Ⅳ tumors. Since the tumor mass is hypo-vascular, only a faint tumor contrast dye will be observed by DSA (Digital Subtraction Angiography) resulting in inadequate lipiodol staining. Chemotherapy either general or intra-arterial does not improve survival[25-27]. Based on these reasons, we propose that RFA therapy should be used for the treatment of type Ⅲ and Ⅳ cholangiocarcinoma.

Due to the massive and wide range of invasion of the tumor, the majority of patients with Type Ⅲ and Ⅳ Klatskin's tumor present with serious obstructive jaundice on admission. Therefore, with the aid of DSA, PTCD is the first step of the sequential interventional therapy since it anticipates jaundice and decompensates liver function by using an internal and external drainage. Two weeks following the drainage, until jaundice diminishes and hepatic function is amended, RFA is carried out and that is the most critical step of this sequential interventional therapy. RFA is becoming a widely used tool for treatment of liver metastatic tumors especially since Rossi *et al* introduced new needle

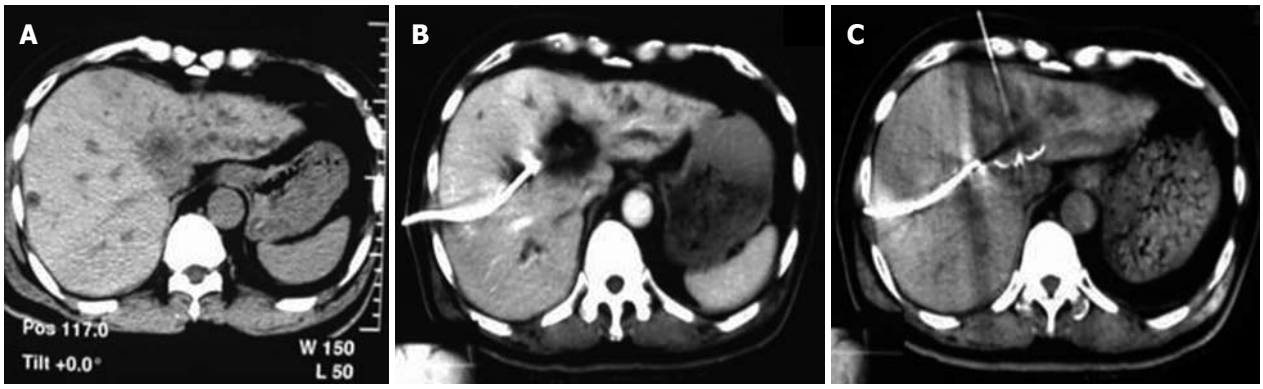

Figure 3 A case with type IV Klatskin tumor. **A**: The plain CT reveals that the tumor is in the portal hepatic region with dilatation of the left and right hepatic ducts; **B**: The patient underwent RFA under CT guidance after internal and external drainage for 2 wk; **C**: The plain CT scan reveals a liquidized and necrotic region of the tumor in the portal hepatic region without contrast enhancement 2 mo after RFA.

electrodes capable of increasing the diameter of tissue necrosis to 4-5 cm[28,29]. The tissue necrosis should include 5 mm of normal tissue around the lesion for oncological clearance.

The basic principle of RFA therapy is described below. Under CT or sonographic guidance, the electrode percutaneously punctures and is placed into the tumor tissue with single or multiple electrode probes. The cluster of electrodes at the end of the probe will emit median to high-frequency electromagnetic wave energy that may induce ionic vibrating friction of the target tissue cells resulting in the generation of heat. As the local temperature increases up to 80℃-90℃, it is sufficient to cause coagulation necrosis of the tumor tissue, and eventually, liquefaction or fibrosis. Coagulation of the peripheral vessel and tissue around the tumor will form a reactive zone. Thus, the tumor blood supplies are interrupted contributing to the prevention of metastasis.

The tumor size determines the number of sessions and duration of RFA. Furthermore, extending the range of RFA to 0.5 cm out of the normal tissue margin where possible will effectively eliminate potential minimal tumor infiltration in the normal liver tissue. This creates a free margin and may reduce the likelihood of tumor relapse.

RFA has a tendency to injure the biliary system when the lesions are near the porta hepatis. This injury may be a bile fistula or an obstruction of the biliary tract. In our study, none of these complications occurred, since PTCD was performed in all of our cases before RFA. The advantages of PTCD before RFA are that: (1) the probability of biliary tract injury decreases due to remission of the obstruction and dilation of the biliary tract after PTCD; and (2) the drain pipe which was detained in the biliary tract during PTCD can be used as a localization mark during RFA.

There are several large vessels near the porta hepatis. Thus, serious hemorrhage will occur if the puncture of the needle injures the large vessels. Hemorrhage will also occur if the dissepiment necrosis of the vessels happens during the RFA procedure due to the entry of the electrode. RFA treatment of all 11 cases in our study did not result in hemorrhagic complications. Our experience is that the radiologist who operates the RFA procedure must possess the imaging radiology and puncture technique, as well as being able to identify the large vessels in the porta hepatis and evade them. When the electrode enters the large vessels, the temperature remains low or the curve of the temperature offers crenate type and duty curve lasts high. At this time, the power source must be turned off immediately, and the location of the needle and electrode is adjusted. The cooling effect of the blood flow in the large vessels near the lesions results in lower efficacy in tumor necrosis. Our countermeasure is to inject 1 mL of 10% saline from the side aperture of the needle every minute to enhance ion vibration and excite the instrument to enhance the duty. It is also important to prolong the procedure time to ensure that the total time of effective temperature (higher than 70℃) lasts for 10-15 min.

Evaluation of the therapeutic effects of this treatment requires analysis of the changes in the density and size of the tumor mass by CT compared with base line data. One month follow-up after RFA showed that for all 11 cases, there were varying degrees of diminishing density and there was no enhancement in the contrast scans of the tumor mass. These effects were mainly due to the tumor coagulation and necrosis. In some cases, the lesion presented with an even lower density value because of the vacuum phenomenon resulting from long-term and multi-session therapy.

Six masses had diminished in size by approximately 30%, two masses had been reduced by approximately 20% and no significant change in size was noted in three masses.

Size reduction of the tumor mass was significantly noted in six out of 11 cases, two masses had been reduced by approximately 20%, and the other three lesions remained unchanged in other five patients in one month CT follow-up. However, 6 mo after synthetic sequential IR therapy, all 11 cases presented with varying degrees of size reduction. This was probably due to the slow progressive development of the coagulated necrosis, liquefaction and fibrosis of the lesion.

To date, all 11 patients are alive. A recent follow-up shows that the survival of a group of 29 cases of Klatskin tumor treated by surgical intervention. The mortality rate in the hospital (during and after surgery) was 17%, the average hospital stay was 71 d, and a survival rate of 1 year was observed in 50% of cases. Tsukada *et al* reported the survival after RFA therapy to be 18 mo on average, ranging between 10 mo and 30 mo so far. No complications among these 11 cases were observed during or after synthetic sequential interventional therapy. Parc *et al*[12] reported a group of 39 cases of Klatskin's tumor treated by surgical intervention. Radical resection was performed in 18 cases, and the remaining 21 cases underwent palliative operation. Among the 18 cases treated with radical resection, one had type Ⅰ, two had type Ⅱ, eight had type Ⅲ a, two had type Ⅲ b and five had type IV. Four of the 18 cases developed post-operative complications and the survival rate ranged between 1 (67%) and 5 (47%) years. Of the 21 cases treated with palliative operation, the post-surgery mortality was 14% and the mean survival range was only 7 mo. Nimura[30,31] performed a liver and bile duct resection combined with Whipple's operation in five patients with hilar tumors. Two of the five patients died after surgery and the three survivors died 8, 10 and 27 mo later.

Compared with surgical intervention, the RFA treatment that we used for type Ⅲ and Ⅳ Klatskin's tumor is less invasive and involves less complications. Furthermore, a higher mean survival rate (up to 18 mo) has been shown after RFA than surgical intervention. Thus, we believe that RFA is a less invasive, safe, effective and promising therapy for type Ⅲ and Ⅳ Klatskin tumors. The long-term curative effect requires further study.

COMMENTS

Background

Hilar cholangiocarcinoma (also known as Klatskin tumor) was first reported by Gerald Klatskin in 1965. Since then, many therapeutic methods have been established to treat this type of tumor. For those patients presenting with type I and type II tumors, surgical resection is good and has a high 5-year survival rate. However, for those tumors classified as type Ⅲ and Ⅳ tumors, the surgical prognosis is poor even when combined with local tumor resection and left or right hemi-lobectomy of the liver, which can itself lead to further complications. Thus, finding a surgical approach for the treatment of type Ⅲ and Ⅳ Klatskin tumors is problemtic.

Research frontiers

Percutaneous image-guided radiofrequency ablation (RFA) has received increasing attention as a promising technique for the treatment of liver tumors. This technique permits the destruction of tumors without necessitating their removal, and in many cases, can be used in place of more invasive and expensive surgical treatment. Initial attempts at tissue ablation with radiofrequency have been limited to the 1.6-cm diameter coagulation necrosis obtained from a single conventional electrode. In order to achieve larger thermal necrosis, internally cooled, single or clustered electrode technique, as well as expandable needle techniques have been introduced.

Innovations and breakthroughs

To date, all 11 patients are alive. A recent follow-up shows that the survival range after RFA therapy is 18 mo on average, ranging between 10 mo and 30 mo so far. No complications among these 11 cases were observed during or after synthetic sequential interventional therapy. Compared with surgical intervention, the RFA treatment that we used for type Ⅲ and Ⅳ Klatskin's tumor is less invasive and involves less complications.

Applications

RFA is a less invasive, safe, effective and promising therapy for type Ⅲ and Ⅳ Klatskin tumors. The long-term curative effect requires further study.

Peer review

According to the study, we know that a new therapy for type Ⅲ and Ⅳ Klatskin tumor, and RFA is less invasive, soft and have fewer complications than common surgical treatment. It is worth paying attention and we are looking forward to the better curative effect from RFA.

REFERENCES

1 **Becker T**, Lehner F, Bektas H, Meyer A, Luck R, Nashan B, Klempnauer J. [Surgical treatment for hilar cholangiocarcinoma (Klatskin's tumor)] *Zentralbl Chir* 2003; **128**: 928-935

2 **Slakey DP**. Radiofrequency ablation of recurrent cholangiocarcinoma. *Am Surg* 2002; **68**: 395-397

3 **Golfieri R**, Giampalma E, Fusco F, Galuppi A, Faccioli L, Galaverni C, Frezza G. Unresectable hilar cholangiocarcinoma: multimodality treatment with percutaneous and intraluminal plus external radiotherapy. *J Chemother* 2004; **16** Suppl 5: 55-57

4 **Lygidakis NJ**, Singh G, Bardaxoglou E, Dedemadi G, Sgourakis G, Nestoriois J, Malliotakis A, Pedonomou M, Safioleas M, Solomou EK, Grigorakos L, Vrachnos P. Changing trends in the management of Klatskin tumor. *Hepatogastroenterology* 2004; **51**: 689-696

5 **Launois B**, Terblanche J, Lakehal M, Catheline JM, Bardaxoglou E, Landen S, Campion JP, Sutherland F, Meunier B. Proximal bile duct cancer: high resectability rate and 5-year survival. *Ann Surg* 1999; **230**: 266-275

6 **Bismuth H**, Nakache R, Diamond T. Management strategies in resection for hilar cholangiocarcinoma. *Ann Surg* 1992; **215**: 31-38

7 **Liu CL**, Fan ST. Anterior approach for right hepatectomy for hilar cholangiocarcinoma. *J Hepatobiliary Pancreat Surg* 2003; **10**: 292-294

8 **Nagino M**, Kamiya J, Uesaka K, Sano T, Yamamoto H, Hayakawa N, Kanai M, Nimura Y. Complications of hepatectomy for hilar cholangiocarcinoma. *World J Surg* 2001; **25**: 1277-1283

9 **Goldberg SN**, Solbiati L, Hahn PF, Cosman E, Conrad JE, Fogle R, Gazelle GS. Large-volume tissue ablation with radio frequency by using a clustered, internally cooled electrode technique: laboratory and clinical experience in liver metastases. *Radiology* 1998; **209**: 371-379

10 **Klatskin G**. Adenocarcinoma of the hepatic duct at its bifurcation within the porta hepatitis. An unusual tumour with distinctive clinical and pathological features. *Am J Med* 1965; **38**: 241-256

11 **Bismuth H**, Castaing D, Traynor O. Resection or palliation: priority of surgery in the treatment of hilar cancer. *World J Surg* 1988; **12**: 39-47

12 **Parc Y**, Frileux P, Balladur P, Delva E, Hannoun L, Parc R. Surgical strategy for the management of hilar bile duct cancer. *Br J Surg* 1997; **84**: 1675-1679

13 **Gerhards MF**, van Gulik TM, Bosma A, ten Hoopen-Neumann H, Verbeek PC, Gonzalez Gonzalez D, de Wit LT, Gouma DJ. Long-term survival after resection of proximal bile duct carcinoma (Klatskin tumors). *World J Surg* 1999; **23**: 91-96

14 **Zovak M**, Doko M, Glavan E, Hochstadter H, Roic G, Ljubicic N. Klatskin tumor--results of surgical therapy. *Coll Antropol* 2004; **28**: 317-323

15 **Ruiz E**, Celis J, Sullon J. [Surgical treatment of Klatskin's tumor] *Rev Gastroenterol Peru* 1995; **15**: 167-175

16 **Santoro E**, Sacchi M, Carboni F, Santoro R, Scardamaglia F. Diagnostic and surgical features of Klatskin tumors. *Chir Ital* 1999; **51**: 1-7

17 **Tibble JA**, Cairns SR. Role of endoscopic endoprostheses in proximal malignant biliary obstruction. *J Hepatobiliary Pancreat Surg* 2001; **8**: 118-123
18 **Lillemoe KD**. Current status of surgery for Klatskin tumors. *Curr Opin Gen Surg* 1994; 161-167
19 **Acalovschi M**. Cholangiocarcinoma: risk factors, diagnosis and management. *Rom J Intern Med* 2004; **42**: 41-58
20 **Yi B**, Zhang BH, Zhang YJ, Jiang XQ, Zhang BH, Yu WL, Chen QB, Wu MC. Surgical procedure and prognosis of hilar cholangiocarcinoma. *Hepatobiliary Pancreat Dis Int* 2004; **3**: 453-457
21 **Iwatsuki S**, Todo S, Marsh JW, Madariaga JR, Lee RG, Dvorchik I, Fung JJ, Starzl TE. Treatment of hilar cholangiocarcinoma (Klatskin tumors) with hepatic resection or transplantation. *J Am Coll Surg* 1998; **187**: 358-364
22 **Robles R**, Parrilla P, Bueno FS, Ramirez P, Lujan JA, Rodriguez JM, Acosta F, Lopez J, Hernandez Q. Liver transplantation in the management of Klatskin's tumor. *Transplant Proc* 1999; **31**: 2494-2495
23 **Born P**, Rosch T, Bruhl K, Sandschin W, Weigert N, Ott R, Frimberger E, Allescher HD, Hoffmann W, Neuhaus H, Classen M. Long-term outcome in patients with advanced hilar bile duct tumors undergoing palliative endoscopic or percutaneous drainage. *Z Gastroenterol* 2000; **38**: 483-489
24 **Shimoda M**, Farmer DG, Colquhoun SD, Rosove M, Ghobrial RM, Yersiz H, Chen P, Busuttil RW. Liver transplantation for cholangiocellular carcinoma: analysis of a single-center experience and review of the literature. *Liver Transpl* 2001; **7**: 1023-1033
25 **McDougall NI**, Edmunds SE. An audit of metal stent palliation for malignant biliary obstruction. *J Gastroenterol Hepatol* 2001; **16**: 1051-1054
26 **Berr F**, Tannapfel A, Lamesch P, Pahernik S, Wiedmann M, Halm U, Goetz AE, Mossner J, Hauss J. Neoadjuvant photodynamic therapy before curative resection of proximal bile duct carcinoma. *J Hepatol* 2000; **32**: 352-357
27 **Wiedmann M**, Caca K, Berr F, Schiefke I, Tannapfel A, Wittekind C, Mossner J, Hauss J, Witzigmann H. Neoadjuvant photodynamic therapy as a new approach to treating hilar cholangiocarcinoma: a phase II pilot study. *Cancer* 2003; **97**: 2783-2790
28 **Gazelle GS**, Goldberg SN, Solbiati L, Livraghi T. Tumor ablation with radio-frequency energy. *Radiology* 2000; **217**: 633-646
29 **Wiedmann M**, Dietrich A, Mossner J, Witzigmann H, Caca K. Combined percutaneous transhepatic biliary drainage with port implantation for management of patients with malignant biliary obstruction. *Gastrointest Endosc* 2004; **60**: 117-120
30 **Nimura Y**, Hayakawa N, Kamiya J, Maeda S, Kondo S, Yasui A, Shionoya S. Combined portal vein and liver resection for carcinoma of the biliary tract. *Br J Surg* 1991; **78**: 727-731
31 **Tsukada K**, Yoshida K, Aono T, Koyama S, Shirai Y, Uchida K, Muto T. Major hepatectomy and pancreatoduodenectomy for advanced carcinoma of the biliary tract. *Br J Surg* 1994; **81**: 108-110

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RAPID COMMUNICATION

Delayed ethyl pyruvate therapy attenuates experimental severe acute pancreatitis *via* reduced serum high mobility group box 1 levels in rats

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Abstract

AIM: To investigate the effect of delayed ethyl pyruvate (EP) delivery on distant organ injury, survival time and serum high mobility group box 1 (HMGB1) levels in rats with experimental severe acute pancreatitis (SAP).

METHODS: A SAP model was induced by retrograde injection of artificial bile into the pancreatic ducts of rats. Animals were divided randomly into three groups (n = 32 in each group): sham group, SAP group and delayed EP treatment group. The rats in the delayed EP treatment group received EP (30 mg/kg) at 12 h, 18 h and 30 h after induction of SAP. Animals were sacrificed, and samples were obtained at 24 h and 48 h after induction of SAP. Serum HMGB1, aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (Cr) levels were measured. Lung wet-to-dry-weight (W/D) ratios and histological scores were calculated to evaluate lung injury. Additional experiments were performed between SAP and delayed EP treatment groups to study the influence of EP on survival times of SAP rats.

RESULTS: Delayed EP treatment significantly reduced serum HMGB1 levels, and protected against liver, renal and lung injury with reduced lung W/D ratios (8.22 ± 0.42 *vs* 9.76 ± 0.45, $P < 0.01$), pulmonary histological scores (7.1 ± 0.7 *vs* 8.4 ± 1.1, $P < 0.01$), serum AST (667 ± 103 *vs* 1 368 ± 271, $P < 0.01$), ALT (446 ± 91 *vs* 653 ± 98, $P < 0.01$) and Cr (1.2 ± 0.3 *vs* 1.8 ± 0.3, $P < 0.01$) levels. SAP rats had a median survival time of 44 h. Delayed EP treatment significantly prolonged median survival time to 72 h ($P < 0.01$).

CONCLUSION: Delayed EP therapy protects against distant organ injury and prolongs survival time *via* reduced serum HMGB1levels in rats with experimental SAP. EP may potentially serve as an effective new therapeutic option against the inflammatory response and multiple organ dysfunction syndrome (MODS) in SAP patients.

Key words: Severe acute pancreatitis; Ethyl pyruvate; High mobility group box 1; Multiple organ dysfunction syndrome; Survival time

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INTRODUCTION

Excessive activation of the inflammatory mediator cascade during severe acute pancreatitis (SAP) is a major cause of multiple organ dysfunction syndrome (MODS), which leads to a high mortality rate[1]. Therapeutic strategies targeting these inflammatory mediators are thought to be an ideal way to reduce the severity of SAP. This is difficult, however, because the cytokines, such as TNF-α and IL-1β, are released early in the development of a systemic inflammatory response[2]. This leaves a narrow therapeutic window for the administration of

therapeutics, and delayed delivery of anti-inflammatory therapeutics are not effective after the inflammatory mediator cascade has developed[2].

High mobility group box 1 (HMGB1) protein, which has been known as a ubiquitously expressed, intracellular DNA-binding protein for about 30 years, was recently identified as a late-acting mediator of endotoxin lethality[3]. It was reported that serum HMGB1 levels increased in patients with sepsis/endotoxemia[3-5], hemorrhagic shock[6], acute lung injury[7,8], rheumatoid arthritis[9] and disseminated intravascular coagulation[10]. HMGB1 is identified as a late mediator of endotoxin lethality because its systemic release during endotoxemia is delayed as compared with the rapid increase of the early proinflammatory cytokines, such as TNF-α and IL-1β. HMGB1 is released by endotoxin-stimulated macrophages only after a delay of 12-18 h. A similar delayed appearance of HMGB1 (8-32 h) was also observed in the serum of mice with endotoxemia after TNF-α had reached its peak and subsided[3]. Delayed anti-HMGB1 antibody dosing still confers significant protection against endotoxin lethality[3]. Strategies that target HMGB1 with specific antibodies or antagonists seem to have potential value for treating lethal systemic inflammatory diseases characterized by excessive HMGB1 release, and the delayed kinetics indicate that HMGB1 may provide a broader therapeutic window for treating those inflammatory disorders[11].

It has been recently demonstrated that the serum levels of HMGB1 were significantly elevated in patients with SAP on admission, and were correlated with the severity of the disease[12]. Early blockade of HMGB1 attenuates the development and associated organ dysfunction in experimental SAP[13]. These indicate that HMGB1 may be an effective therapeutic target of SAP. In a previous experimental study, we demonstrated that the serum levels of HMGB1 began to rise significantly at 12 h, and were maintained in high levels up to 48 h after induction of experimental SAP in rats[14]. Thus, we conceive that delayed therapeutic delivery targeting HMGB1 might attenuate SAP in rats.

Ethyl pyruvate (EP), a stable lipophilic pyruvate derivative, is an agent that can effectively protect animals from ischemia-reperfusion-induced tissue injury[15]. EP administration significantly improves the survival of lethal hemorrhagic shock in standard models[16,17], and significantly inhibits the systemic release of both early (TNF-α) and late (HMGB1) cytokines that mediate the lethality of sepsis and systemic inflammation, even when administered 24 h after cecal puncture[18]. We hypothesize that delayed EP administration could reduce the severity of SAP in rats through inhibiting the systemic release of HMGB1.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200-250 g were obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Before the experiment, the animals were kept in rooms at 20 ℃ ± 2 ℃ in 12-h light-dark cycles for at least 1 wk to acclimate to the surroundings with free access to water and standard rat chow.

Induction of pancreatitis

The animals were fasted with free access to water 12 h before surgery. The rats were then intra-abdominally anesthetized by 1% pentobarbital sodium (35 mg/kg body weight), and incised through a median incision of the abdomen. After the common bile duct was clamped in the hepatoduodenal ligament by a small bulldog clamp, the biliopancreatic duct was cannulated through mammary papilla from the anterior wall of the duodenum. 1 mL/kg body weight of 5% sodium taurocholate (Sigma, St. Louis, MO, USA) was injected by the cannula with an even speed of 0.1 mL/min, and the atraumatic vascular clamp was removed 10 min later. Finally, the abdominal incisions were closed in two layers. All procedures were performed using a sterile technique.

Study protocol

After the stabilization period, 96 male rats were randomly divided into three groups (each group n = 32), and each group was divided into two subgroups (each subgroup n = 16). Animals in a subgroup were sacrificed at either 24 h or 48 h after surgery. Rats in group Ⅰ (sham group) underwent laparotomy with manipulation of the pancreas (sham procedure) and received 40 mL/kg normal saline subcutaneously (single dose). Groups Ⅱ and Ⅲ underwent laparotomy with induction of SAP, and subsequently received saline every 6 h after induction of SAP. Rats in group Ⅱ (delayed EP treatment group) additionally received 30 mg/kg body weight EP intravenously at 12, 18 and 30 h after induction of SAP. EP (Sigma, St. Louis, MO, USA) was prepared in solution with sodium (130 mmol/L), potassium (4 mmol/L), calcium (2.7 mmol/L), chloride (139 mmol/L), and EP (28 mmol/L) (pH 7.0). Rats in group Ⅲ (SAP group) intravenously received the same volume of vehicle at the same time as in group Ⅱ. Twenty four hours after induction of SAP, the surviving rats were anesthetized with ether, and blood samples were taken from the inferior vena cava to measure serum HMGB1 levels and blood biochemical parameters. The animals were sacrificed, and the right lung was obtained to evaluate lung injury. 48 h after induction of SAP, only blood samples were taken in order to measure serum HMGB1 levels.

To investigate the effect of EP on the survival time of SAP rats, 48 male rats underwent laparotomy with induction of SAP, and were randomly divided into two groups (each group n = 24): SAP group and delayed EP treatment group. Rats in the EP treatment group received delayed EP delivery (30 mg/kg body weight) intravenously at 12, 18, 30 and 48 h after induction of SAP, and received

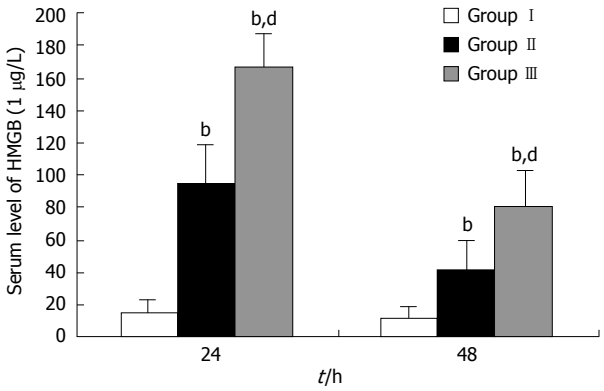

Figure 1 Serum HMGB1 levels of rats. Group Ⅰ: Sham group; Group Ⅱ: Delayed EP treatment group; Group Ⅲ: SAP group. $^{b}P < 0.01$ *vs* Group Ⅰ; $^{d}P < 0.01$ *vs* Group Ⅱ.

the above-mentioned fluid resuscitation. Rats in the SAP group received fluid resuscitation and the same volume of vehicle intravenously as in the EP treatment group. The number of surviving rats was recorded every 4 h after induction of SAP.

HMGB1 measurement

HMGB1 was analyzed by Western blot as described by Wang *et al*[3]. Briefly, serum was first filtered with Centricon YM-100 (Millipore) to clear the samples from cell debris and macromolecular complexes formed during clotting. Samples were then concentrated 15-fold with Centricon YM-30 and separated on 12% SDS-polyacrylamide gels. Protein was transferred to nitrocellulose membranes (Pall) and HMGB1 was analyzed by using polyclonal anti-HMGB1 antibodies (Santa Cruz) and secondary anti-goat alkaline phosphatase (Beijing Zhongshan Biotechnology). The intensity of the 30-kDa band was analyzed by densitometry. Standard curves were constructed using r-HMGB1 (Sigma, St. Louis, MO, USA).

Blood biochemical parameters measurements

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (Cr) levels were measured using a standard clinical automated analyzer.

Lung wet-to-dry-weight (W/D) ratios and histological scores

The right lung was wiped dry with filter paper and weighed. Then, it was dried to a constant weight at 60 ℃ for 72 h in an oven. W/D ratios could then be calculated. Routine paraffin sectioning was performed on the lung tissue. Pulmonary histological scores were graded using a Gloor score system (normal, 0; mild, 1; moderate, 2; severe, 3; overwhelming, 4) for intra-alveolar oedema, intra-alveolar haemorrhage, and neutrophil infiltration and these scores were then added to give a total score[19].

Statistical analysis

mean ± SD values for blood biochemical parameters, HMGB1 serum levels, lung W/D ratios and histological scores were determined. The differences between the two groups were further evaluated with the Mann-Whitney *U* test. Overall survival was calculated by the Kaplan-Meier Estimate. The log-rank test and the Breslow test were used to compare survival curves in the two groups. A P value < 0.05 was considered statistically significant.

Table 1 Blood biochemical parameters

Groups	Group Ⅰ	Group Ⅱ	Group Ⅲ
AST (IU/L)	154 ± 20	446 ± 91[b]	653 ± 98[b,d]
ALT (IU/L)	51 ± 9	667 ± 103[b]	1368 ± 271[b,d]
BUN (mg/dL)	26 ± 3	38 ± 4[b]	41 ± 4[b]
Cr (mg/dL)	0.4 ± 0.1	1.2 ± 0.3[b]	1.8 ± 0.3[b,d]

Blood samples were obtained 24 h after induction of SAP. Group Ⅰ: Sham group; Group Ⅱ: Delayed EP treatment group; Group Ⅲ: SAP group. $^{b}P < 0.01$ *vs* Group Ⅰ; $^{d}P < 0.01$ *vs* Group Ⅱ.

RESULTS

Serum HMGB1 levels

At 24 h and 48 h after induction of SAP, serum HMGB1 levels of SAP rats were higher than those of the sham group. Delayed EP administration significantly reduced the serum HMGB1 levels of SAP rats (Figure 1).

Lung injury

Lung W/D ratios were observed to evaluate the severity of pulmonary edema. At 24 h after induction of SAP, W/D ratios were elevated in the SAP group in comparison with the sham group (9.76 ± 0.45 *vs* 5.43 ± 0.21, $P < 0.01$). The lung W/D ratio of rats that received delayed EP administration was significantly lower than that of the SAP group ($P < 0.01$), although it was significantly higher than that of the sham group (8.22 ± 0.42 *vs* 5.43 ± 0.21, $P < 0.01$).

The pulmonary histological scores, an all round evaluation for lung injury, were lower in the EP treatment group than that in the SAP group (7.1 ± 0.7 *vs* 8.4 ± 1.1, $P < 0.01$), although they were significantly elevated in comparison with the sham group (*vs* 0.5 ± 0.1, $P < 0.01$).

Hepatic and renal dysfunction

EP treatment protected against liver and renal injury. 24 hours after induction of SAP, serum AST, ALT, BUN and Cr levels were significantly elevated in groups Ⅱ and Ⅲ. Delayed EP administration significantly attenuated the elevated AST, ALT and Cr levels (Table 1).

Survival analysis

SAP rats all died within 3 d without the delayed EP administration, and their median survival time was 44 h (95% confidence interval 29.6 h to 58.4 h). Delayed EP administration significantly prolonged the median survival time to 72 h (95% confidence interval 52.8 h to 91.2 h; Figure 2).

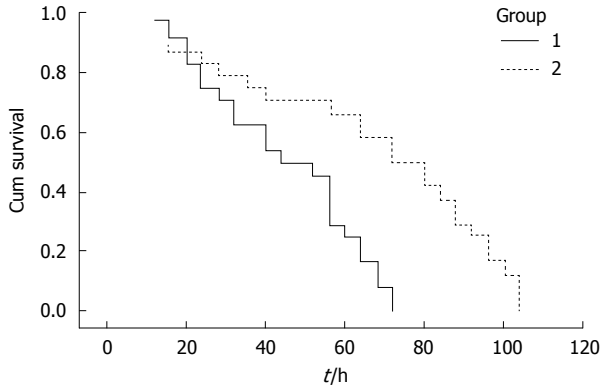

Figure 2 Kaplan-Meier survival curves of SAP rats. 1: SAP group; 2: Delayed EP treatment group. Log-rank test, $P = 0.0006$; Breslow test, $P = 0.0050$.

DISCUSSION

Extracellular HMGB1 was recently implicated as a late mediator of delayed endotoxin lethality. High serum HMGB1 levels in patients with sepsis are associated with increased mortality, and administration of HMGB1 produces acute inflammation in animal models of lung injury and endotoxemia. During lethal endotoxemia in mice, serum HMGB1 levels accumulate 8-32 h after LPS administration[3]. Passive immunization of mice with anti-HMGB1 antibodies attenuates LPS-induced lethality, even when antibody administration is delayed until after the onset of the early proinflammatory cytokine response (2 h after endotoxin administration)[3]. The delayed kinetics of HMGB1 appearance indicates that the therapeutic window may be significantly wider than for any previously described cytokine target.

In a previous experimental study, we demonstrated that the serum levels of HMGB1 began to increase significantly at 12 h after induction of SAP, after the TNF-α and IL-1β peak had already occurred. The delayed kinetics of HMGB1 release may provide a wider therapeutic window for SAP. It was shown that EP could inhibit HMGB1 release from macrophages and prevent the accumulation of serum HMGB1 levels in mice with lethal sepsis by inhibiting NF-κB and p38 MAPK signaling[18]. In this study, we administered SAP rats with EP intravenously. As a result, EP significantly reduced serum HMGB1 levels in rats with SAP, even though EP delivery began 12 h after induction of SAP.

In SAP, MODS, a consequence of the systemic inflammatory response syndrome, is a contributor to high mortality in the early phase[1]. It is conceivable that the release of mediators from the excess of activated macrophages/monocytes and neutrophils may lead to remote organ injury[1]. Serum HMGB1 levels are significantly elevated in patients with SAP on admission[12]. Purified rHMG-1 is lethal to both LPS-responsive (C3H/HeN, Balb/c) and LPS-resistant (C3H/HeJ) mice, indicating that HMG-1 mediates lethal toxicity in the absence of LPS signal transduction[3]. The cytokine activity of HMGB1 has been well-documented in many cell types. In cultured human primary macrophages/monocytes, HMGB1 stimulates the release of multiple proinflammatory cytokines, including TNF-α, IL-1α, IL-1β, IL-1RA, IL-6, IL-8, MIP-1α, and MIP-1β, but not IL-10 and IL-12[20]. In cultured human microvascular endothelial cells, addition of HMGB1 induces the expression of adhesion molecules, such as ICAM-1, VCAM-1, and RAGE, as well as the release of TNF-α and IL-8, MCP-1, PAI-1, and tPA[21]. HMGB1 also activates human neutrophils to produce proinflammatory mediators, such as TNF-α, IL-1β, and IL-8, suggesting an important role for HMGB1 in activation of neutrophils during inflammation[22]. HMGB1 also increases the permeability in cultured enterocytes *via* a nitric oxide (NO)-dependent pathway[23,24]. These indicate that HMGB1 is potent in augmenting the inflammatory response. A previous study showed the early blockade of HMGB1 attenuated organ dysfunction in experimental SAP[13]. This study indicated that HMGB1 might be a good target to prevent organ injury in SAP. In this study, to evaluate the degree of injury in distant organs, such as lung, liver and kidney, serum AST, ALT, BUN and Cr levels were measured, and lung W/D ratios were calculated. Serum AST, ALT and Cr levels, and lung W/D ratios were lower in the EP treatment group compared to those in the SAP group. This indicates that delayed EP treatment protected against distant organ injury. An additional study was performed to evaluate the effect of EP on survival times of SAP rats. As a result, delayed EP administration significantly prolonged the survival times of SAP rats.

These results give direct evidence that the beneficial effects of EP are due to downregulation of HMGB1, and reveal that EP may be a useful new therapeutic option against the inflammatory response and MODS in SAP rats, and may also be effective in SAP patients, even when anti-inflammatory therapy is delayed in the early phase. In this study, EP and reduction in serum HMGB1 levels actually reduces the severity of acute pancreatitis, and prolongs survival time of SAP rats. All rats finally die, however. Further studies should be performed to elucidate the role of HMGB1 in SAP and to determine whether a higher dose or earlier delivery of EP could improve the survival rates of SAP rats. Subsequently, clinical investigations could be carried out to study whether EP can be used for SAP patients.

COMMENTS

Background

Excessive activation of inflammatory mediator cascade during severe acute pancreatitis (SAP) is a major cause of the high mortality. Cytokines such as TNF-α and IL-1β are released early in the development of systemic inflammatory response. This leaves a narrow therapeutic window for administration of therapeutics and delayed delivery of that anti-inflammatory therapeutics is not effective after the inflammatory mediator cascade has developed.

Research frontiers

Extracellular high mobility group box 1 (HMGB1) was implicated as a late mediator of endotoxin lethality. The cytokine activity of HMGB1 has been well-documented in many cell types. It was reported that serum HMGB1 levels increased in patients with sepsis/endotoxemia, hemorrhagic shock, acute lung injury, rheumatoid arthritis and disseminated intravascular coagulation. It has been recently demonstrated that the serum levels of HMGB1 correlated with

the severity of SAP.

Innovations and breakthroughs

In a previous experimental study, the authors demonstrated that the serum levels of HMGB1 began to rise significantly at 12 h, and maintained at high levels up to 48 h after induction of experimental SAP in rats. The delayed kinetics indicates that HMGB1 may provide a broader therapeutic window for treating this lethal systemic inflammatory disease.

Applications

Ethyl pyruvate (EP), a stable lipophilic pyruvate derivatives, is a nontoxic food additive. According to this study, it is potential to be used as an effective and low-cost therapeutic remedy for SAP patients.

Peer review

This report is of interest and considerable potential importance, because it indicates that delayed treatment of rats with experimental SAP with EP is associated with a reduction of HMGB1 levels in blood; a decrease in lung, kidney, and liver injury; and prolonged survival. The "therapeutic window" for inhibiting this inflammatory mediator appears to be more favorable than for some other mediators which more rapidly reach a peak in the blood.

REFERENCES

1 **Lankisch PG**, Lerch MM. Pharmacological prevention and treatment of acute pancreatitis: where are we now? *Dig Dis* 2006; **24**: 148-159

2 **Tracey KJ**, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, Hariri RJ, Fahey TJ 3rd, Zentella A, Albert JD. Shock and tissue injury induced by recombinant human cachectin. *Science* 1986; **234**: 470-474

3 **Wang H**, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999; **285**: 248-251

4 **Yang H**, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, Susarla SM, Ulloa L, Wang H, DiRaimo R, Czura CJ, Wang H, Roth J, Warren HS, Fink MP, Fenton MJ, Andersson U, Tracey KJ. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc Natl Acad Sci USA* 2004; **101**: 296-301

5 **Sunden-Cullberg J**, Norrby-Teglund A, Rouhiainen A, Rauvala H, Herman G, Tracey KJ, Lee ML, Andersson J, Tokics L, Treutiger CJ. Persistent elevation of high mobility group box-1 protein (HMGB1) in patients with severe sepsis and septic shock. *Crit Care Med* 2005; **33**: 564-573

6 **Ombrellino M**, Wang H, Ajemian MS, Talhouk A, Scher LA, Friedman SG, Tracey KJ. Increased serum concentrations of high-mobility-group protein 1 in haemorrhagic shock. *Lancet* 1999; **354**: 1446-1447

7 **Abraham E**, Arcaroli J, Carmody A, Wang H, Tracey KJ. HMG-1 as a mediator of acute lung inflammation. *J Immunol* 2000; **165**: 2950-2954

8 **Ueno H**, Matsuda T, Hashimoto S, Amaya F, Kitamura Y, Tanaka M, Kobayashi A, Maruyama I, Yamada S, Hasegawa N, Soejima J, Koh H, Ishizaka A. Contributions of high mobility group box protein in experimental and clinical acute lung injury. *Am J Respir Crit Care Med* 2004; **170**: 1310-1316

9 **Taniguchi N**, Kawahara K, Yone K, Hashiguchi T, Yamakuchi M, Goto M, Inoue K, Yamada S, Ijiri K, Matsunaga S, Nakajima T, Komiya S, Maruyama I. High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. *Arthritis Rheum* 2003; **48**: 971-981

10 **Hatada T**, Wada H, Nobori T, Okabayashi K, Maruyama K, Abe Y, Uemoto S, Yamada S, Maruyama I. Plasma concentrations and importance of High Mobility Group Box protein in the prognosis of organ failure in patients with disseminated intravascular coagulation. *Thromb Haemost* 2005; **94**: 975-979

11 **Yang H**, Wang H, Czura CJ, Tracey KJ. HMGB1 as a cytokine and therapeutic target. *J Endotoxin Res* 2002; **8**: 469-472

12 **Yasuda T**, Ueda T, Takeyama Y, Shinzeki M, Sawa H, Nakajima T, Ajiki T, Fujino Y, Suzuki Y, Kuroda Y. Significant increase of serum high-mobility group box chromosomal protein 1 levels in patients with severe acute pancreatitis. *Pancreas* 2006; **33**: 359-363

13 **Sawa H**, Ueda T, Takeyama Y, Yasuda T, Shinzeki M, Nakajima T, Kuroda Y. Blockade of high mobility group box-1 protein attenuates experimental severe acute pancreatitis. *World J Gastroenterol* 2006; **12**: 7666-7670

14 **Yang ZY**, Wang CY, Xiong JQ, Tao J, Xu YQ, Liu T. Serum levels and roles of high mobility group box 1 protein in severe acute pancreatitis rats. *Huazhong Keji Daxue Xuebao* (Yixueban) 2004; **33**: 466-468

15 **Sims CA**, Wattanasirichaigoon S, Menconi MJ, Ajami AM, Fink MP. Ringer's ethyl pyruvate solution ameliorates ischemia/reperfusion-induced intestinal mucosal injury in rats. *Crit Care Med* 2001; **29**: 1513-1518

16 **Tawadrous ZS**, Delude RL, Fink MP. Resuscitation from hemorrhagic shock with Ringer's ethyl pyruvate solution improves survival and ameliorates intestinal mucosal hyperpermeability in rats. *Shock* 2002; **17**: 473-477

17 **Yang R**, Gallo DJ, Baust JJ, Uchiyama T, Watkins SK, Delude RL, Fink MP. Ethyl pyruvate modulates inflammatory gene expression in mice subjected to hemorrhagic shock. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G212-G221

18 **Ulloa L**, Ochani M, Yang H, Tanovic M, Halperin D, Yang R, Czura CJ, Fink MP, Tracey KJ. Ethyl pyruvate prevents lethality in mice with established lethal sepsis and systemic inflammation. *Proc Natl Acad Sci USA* 2002; **99**: 12351-12356

19 **Gloor B**, Blinman TA, Rigberg DA, Todd KE, Lane JS, Hines OJ, Reber HA. Kupffer cell blockade reduces hepatic and systemic cytokine levels and lung injury in hemorrhagic pancreatitis in rats. *Pancreas* 2000; **21**: 414-420

20 **Andersson U**, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, Janson A, Kokkola R, Zhang M, Yang H, Tracey KJ. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J Exp Med* 2000; **192**: 565-570

21 **Fiuza C**, Bustin M, Talwar S, Tropea M, Gerstenberger E, Shelhamer JH, Suffredini AF. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood* 2003; **101**: 2652-2660

22 **Park JS**, Arcaroli J, Yum HK, Yang H, Wang H, Yang KY, Choe KH, Strassheim D, Pitts TM, Tracey KJ, Abraham E. Activation of gene expression in human neutrophils by high mobility group box 1 protein. *Am J Physiol Cell Physiol* 2003; **284**: C870-C879

23 **Sappington PL**, Yang R, Yang H, Tracey KJ, Delude RL, Fink MP. HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in mice. *Gastroenterology* 2002; **123**: 790-802

24 **Li J**, Kokkola R, Tabibzadeh S, Yang R, Ochani M, Qiang X, Harris HE, Czura CJ, Wang H, Ulloa L, Wang H, Warren HS, Moldawer LL, Fink MP, Andersson U, Tracey KJ, Yang H. Structural basis for the proinflammatory cytokine activity of high mobility group box 1. *Mol Med* 2003; **9**: 37-45

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RAPID COMMUNICATION

Programmed death-1 expression is associated with the disease status in hepatitis B virus infection

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Abstract

AIM: To define the potential role of programmed death-1/programmed death-ligand (PD-1/PD-L) pathway in different hepatitis B virus (HBV) infection disease status; we examined the expression of PD-1 on antigen specific CD8+ T cells in peripheral blood of patients with chronic hepatitis B (CHB) and acute exacerbation of hepatitis B (AEHB) infection.

METHODS: The PD-1 level on CD8+ T lymphocytes and the number of HBV specific CD8+ T lymphocytes in patients and healthy controls (HCs) were analyzed by staining with pentameric peptide-human leukocyte antigen2 (HLA2) complexes combined with flow cytometry. Real-time quantitative polymerase chain reaction (PCR) was used to measure the serum HBV-DNA levels.

RESULTS: The level of PD-1 expression on total CD8+ T cells in CHB patients (13.86% ± 3.38%) was significantly higher than that in AEHB patients (6.80% ± 2.19%, $P < 0.01$) and healthy individuals (4.63% ± 1.23%, $P < 0.01$). Compared to AEHB patients (0.81% ± 0.73%), lower frequency of HBV-specific CD8+ T cells was detected in chronic hepatitis B patients (0.37% ± 0.43%, $P < 0.05$). There was an inverse correlation between the strength of HBV-specific CD8+ T-cell response and the level of PD-1 expression. Besides, there was a significant positive correlation between HBV viral load and the percentage of PD-1 expression on CD8+ T cells in CHB and AEHB subjects ($R = 0.541$, $P < 0.01$). However, PD-1 expression was not associated with disease flare-ups as indicated by alanine aminotransferase (ALT) levels ($R = 0.066$, $P > 0.05$).

CONCLUSION: Our results confirm previous reports that HBV specific CD8+ T-cell response in the peripheral blood is more intense in patients with AEHB than in chronic hepatitis B with persistent viral infection. Moreover, there is a negative correlation between the level of PD-1 and the intensity of virus specific CD8+ T cell response.

Key words: Chronic hepatitis B; Acute exacerbation of hepatitis B; Programmed death-1; Programmed death-ligand 1; Pentamer; Serum viral load; Blockade

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INTRODUCTION

Many individuals infected with hepatitis B virus (HBV) become chronic carriers and their liver disease may progress to chronic active hepatitis, cirrhosis, and hepatocellular carcinoma[1]. There is substantial evidence

to suggest that adaptive immunity has a central role in determining whether HBV infection is followed by recovery or viral persistence[2]. In particular, the cellular immune response plays a critical role in the ability of HBV-infected individuals to control viral replication[3]. Patients who develop chronic HBV infection have progressive low frequency and functional impairment of T helper cells (Th cells), both in the peripheral blood and the liver[4]. The cytotoxic T lymphocyte (CTL) response is also impaired in chronic HBV infection with high viral load[5]. However, the molecular mechanisms underlying the exhaustion of memory T-cell subsets have not yet been elucidated. Understanding the immunopathogenesis of HBV infection is crucial for the development of effective strategies to control HBV.

Our present knowledge suggests that the interaction between positive and negative costimulatory molecules expressed on T cells and antigen presenting cells is essential for the development of T cell responses[6,7]. Among the many costimulatory molecules, programmed death-1 (PD-1) and its ligands programmed death 1-ligand 1 (PD-L1) and PD-L2 constitute important pathways that regulate and fine-tune immune responses[8-10]. Recent evidence indicates that the exhausted virus-specific CD8+ and CD4+ T cells in chronic viral infection hyperexpress PD-1 molecule[11-14]. However, the infected cells that remain productive resist early apoptosis by down regulation of PD-1[15]. PD-L1 expression on monocytes and dendritic cells is also increased in HIV infected individuals[16]. These findings indicates that the PD-1/PD-L1 pathway plays a crucial role in inhibiting the function of virus-specific CD8+ T cells in chronic viral infections, in mice and humans[17] [Human immunodeficiency virus (HIV)[18-20], hepatitis C virus (HCV)[21,22] and HBV[23]]. These studies illustrated that the level of PD-1 expression correlates with the degree of HBV-specific T-cell impairment. Thus, induction of apoptosis may be a major mechanism employed by the PD-1/PD-L costimulatory pathway to affect the outcome of virus-specific T cell response. Such negative regulation of CD8+ T cell function by PD-1/PD-L system has also been observed in HBV infection[16].

It should be noted that the extent of HBV-specific T-cell exhaustion which influences the disease status of patients with HBV infection has thus far been analyzed only in small groups of patients, both those who were able to control an acute infection and those with established chronic infection[16]. However, little is known as to whether PD-1 expression on CD8+ T cells differs between patients with acute exacerbation of hepatitis B (AEHB) and chronic HBV infection.

To define the potential role of PD-1/PD-L pathway in acute exacerbation of HBV infection, we examined the expression of PD-1 on antigen specific CD8+ T cells in the peripheral blood in 32 patients with chronic untreated HBV infection and 11 patients with AEHB. The present study was performed by using pentamer technology combined with flow cytometry, allowing the direct visualization of HBV specific CD8+ T cells.

MATERIALS AND METHODS

Subjects

A total of 89 patients were enrolled in the study from either the outpatient clinic or the inpatient service of the Department of Infectious Diseases and Hepatology of the Union Hospital, Wuhan, China. The study group comprised of 66 chronic hepatitis B (CHB) patients [32 were human leukocyte antigen (HLA)-A2+], 23 AEHB (11 HLA-A2+) patients and 28 healthy donors. All subjects were anti-HBV treatment naive at the time of enrollment. Since APC-labeled HLA-A2 pentamer complexes specific for the HBV Core 18-27 (FLPSDFFPSU) can only identify HBV specific T cells in HLA-A2+ patients, all HBV-infected individuals subjected to fluorescent monoclonal antibodies and pentamer staining were first serologically identified as having the HLA-A2+ genotype. HLA typing was performed using flow cytometry by staining peripheral blood mononuclear cells (PBMCs) with a fluorescent anti-HLA-A2.01 antibody (Serotec). The criteria for the diagnosis of AEHB and CHB have been described previously in detail[24]. Based on the infection status, the subjects were divided into two groups: 11 patients had clinical, biochemical, and virologic evidence of AEHB infection [history of CHB, total bilirubin (TB) levels at least 10 times the upper limit of the normal, and plasma prothrombin activity (PTA) < 40%]. All patients were hepatitis B surface antigen (HBsAg) positive, and negative for antibodies against hepatitis C virus, hepatitis D virus (HDV), hepatitis E virus (HEV), HIV type-1 and HIV-2. Moreover, other causes of chronic liver disease were excluded. Patients with CHB patients had ALT levels ranging from 6 to 1485 U/liters and HBV DNA values from $< 10^3$ to 4.61×10^8 copies/mL. Patients with AEHB had TB levels ranging from 228 to 715.8 μmol/L, ALT from 36 to 174 U/liters, and HBV DNA from 4.3×10^4 to 8.9×10^7 copies/mL. A total of 28 HLA-A2+ uninfected age and sex matched healthy blood donors with normal liver functions were selected as healthy control (HC). Our study was approved by the local ethics committee, and all subjects gave a written informed consent.

Serum viral load and ALT determination

The presence or absence of HBsAg, hepatitis B "e" antigen (HBeAg), antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core antigen (anti-HBc), antibody to hepatitis B "e" antigen (anti-HBe), and antibodies to HCV, HDV, HIV-1, and HIV-2 were determined by using commercial enzyme immunoassay kits. Serum HBV viral load quantification was performed at the laboratory of Hepatology and Infectious Disease, Union Hospital by real-time polymerase chain reaction (PCR) (ABI PRISM 7300 Sequence Detector, PE Biosystems) based on the TaqMan technology using a commercial PCR diagnostic kit (Da An Gene Co. Ltd. of Zhong Shan University, Guangzhou, China). The viral DNA was extracted according to the manufacturer's protocol. Sequences of forward primer (5'-ATCCT

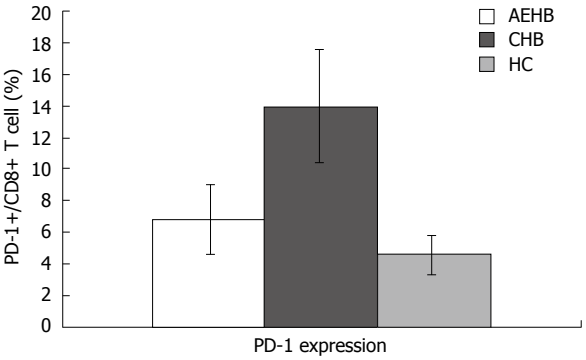

Figure 1 PD-1 expression on CD8+ T cells in patients with acute exacerbation of hepatitis B (AEHB), chronic hepatitis B (CHB) and healthy controls (HC).

GCTGCTATGCCTCATCTT-3'), reverse primer (5'-ACAGTGGGGGAAAGCCCTACGAA-3') and fluorogenic Taqman probes (5'-TGGCTAGTTTACTAG TGCCATTT-G3') were designed against a highly conserved region of the HBV genome overlapping the genes encoding the X-protein and DNA polymerase. The cycling program was: 50℃ for 2 min, 93℃ for 5 min, 40 cycles of 93℃ for 30 s, 53℃ for 30 s, 72℃ for 30 s, and 72℃ for 7 min. A serum sample quantified by b-DNA method was used as the standard to estimate the number of virus and as the quality control for HBV quantitative PCR. The internal control was estimated by commercialized β-actin kit (PE Biosystems) with reaction condition at 50℃ for 2 min first and 5 min at 93℃, followed by 40 cycles of denaturation at 93℃ for 30 s and annealing-extension at 65℃ for 1 min. The analysis was accomplished within 2 min automatically at the end of the run. The HBV DNA cut-off value was 1000 copies/mL.

The serum ALT levels were assayed by CL-7200 Fully-auto Chemistry Analyzer provided by Shimadzu Co. Ltd, at the Department of Clinical Laboratory, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China.

Isolation of PBMC

Using centrifugation on Ficoll-Hypaque density gradient (HaoYang Biological Manufacture, Tianjin, China) and RBC lysis Solution (Roche), PBMC were isolated from fresh heparinized blood (5 mL) collected from each patient, washed twice in phosphate-buffered saline and analyzed immediately.

Peptide-HLA class Ⅰ pentamer antibodies and cell surface staining

To measure the number of virus-specific CD8+ T cells and PD-1 expression on CD8+ T-cell subsets, cells were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and allophycocyanin (APC)-labeled monoclonal antibodies or pentamer, according to the manufacturers' instructions. Briefly, a total of 1×10^6 to 5×10^6 freshly isolated PBMC were incubated for 30 min at room temperature with APC-labeled HLA-A2 pentamer complexes specific for the HBV Core 18-27 (FLPSDFFPSU) (Proimmune Oxford, United Kingdom) in RPMI 1640 and 10% fetal calf serum. After a wash with phosphate-buffered saline-0.1% fetal calf serum, cell surface staining was performed for 15 min in the dark by simultaneously using anti-CD8 (PE-conjugated), anti-PD-1 (FITC-conjugated) and the corresponding IgG2a isotype controls (eBioscience San Diego, CA).

Flow cytometry

Cells were washed twice with PBS and gated on CD8+ T cells; 1×10^6 events in the lymphogate were collected by a BD Biosciences multi-color flow cytometer (LSR2, Becton Dickinson Biosciences). The data were analyzed by using the FACS Diva software. Pentamer-positive responses were reported as a percentage of pentamer-positive T cells among the total CD8+ T cell population. The frequency of pentamer-positive cells exceeding 0.02% of CD8+ T cells indicated a positive response.

Statistical analysis

We used SPSS 12.0 software to perform statistical analyses. Frequency of pentamer positive CD8+ T cells and levels of PD-1 expression in HCs and patients with different viremia levels were compared using the Mann-Whitney test. Spearman correlation analysis was used to evaluate the correlation between viral load, frequency of HBV-specific T cells and PD-1 expression. All tests were two-tailed and *P*-values less than 0.05 were considered significant.

RESULTS

PD-1 expression on PBMC in patients of different disease states

To determine PD-1 expression on CD8+ T cell population, we examined the frequency of PD-1-expression on CD8+ T-cell subsets using polychromatic flow cytometry in the three study groups: AEHB, CHB and HC. The PD-1 levels on the total CD8+ T cells in CHB patients (13.86% ± 3.38%) were significantly higher compared to AEHB patients (6.80% ± 2.19%, $P < 0.01$) and healthy individuals (4.63% ± 1.23%, $P < 0.01$) (Figure 1). Representative flow cytometry plots of PD-1 expression on CD8+ T cells from the peripheral blood of one health individual (5.8%) and two patients with CHB (12.71%) and AEHB (7.1%) are shown in Figure 2.

Comparison of HBV-specific CD8+ T cells upregulated in AEHB and CHB patients

The frequency of HBV-specific CD8+ T cells in the three study groups was assessed. The detection limit was 0.02% for CD8+ T cells specific for the core peptide spanning amino acids 18 to 27. None of the 28 healthy individuals showed a positive response with pentamer. Compared to AEHB patients (0.81% ± 0.73%), there was a lower frequency of HBV-specific CD8+ T cells (0.37% ± 0.43%) in CHB patients ($P < 0.05$) (Figure 3). Representative plots from two patients with CHB (0.07%) and AEHB (0.3%) infection indicating the number of HBV-specific CD8+ T cells are shown in Figure 4.

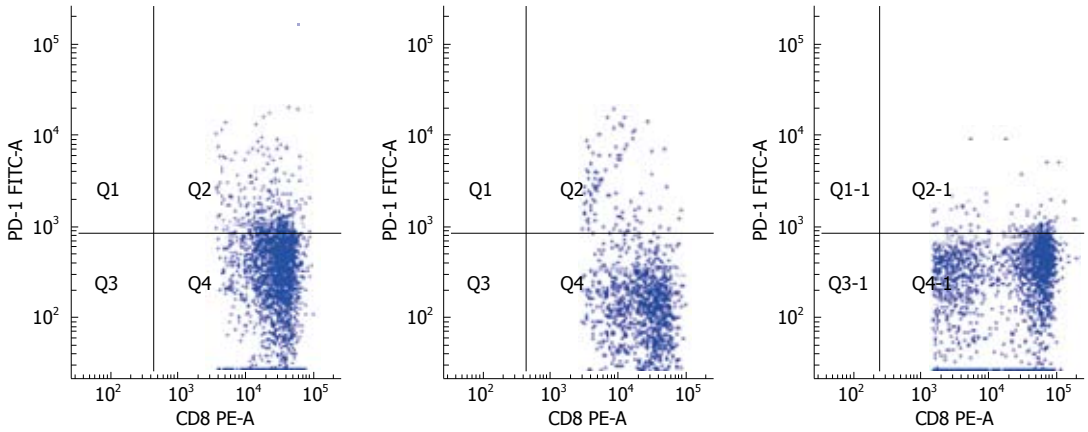

Figure 2 Comparison of PD-1 expression on CD8+ T cells in healthy controls (HC; 5.8%), chronic hepatitis B (CHB; 12.7%), and acute exacerbation of hepatitis B (AEHB; 7.1%).

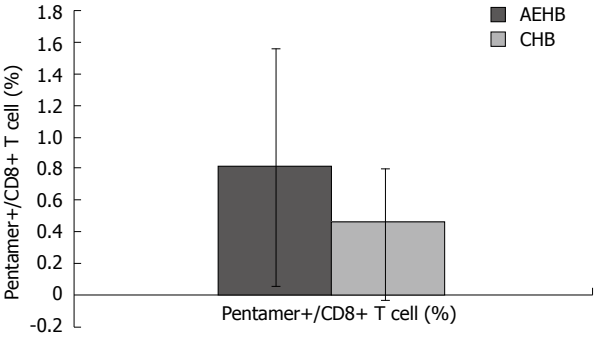

Figure 3 The number of HBV-specific CD8 T cells in CHB and AEHB patients.

PD-1 expression in relation to different HBV-specific CD8+ T cell responses

Using linear regression and Spearman's correlation analyses, we found an inverse correlation between HBV-specific CD8+ T-cell response and level of PD-1 expression in AEHB and CHB patients. In other words, the higher the PD-1 expression level, the weaker (or totally absent) HBV-specific CD8+ T-cell response was observed (Figure 5).

Correlation of PD-1 expression with serum HBV DNA load

In the present study, there was a significant positive correlation between HBV viral load and PD-1 expression on CD8+ T cells in CHB and AEHB subjects. These findings indicate that high plasma viral load correlates with PD-1 upregulation on the total CD8+ T cells in viremic individuals (Figure 6).

Correlation between PD-1 expression and serum ALT levels

We also assessed the relationship between PD-1 expression and serum ALT levels, another predictor of HBV disease progression. There was no correlation between PD-1 expression and serum ALT levels in the study patients (Figure 7).

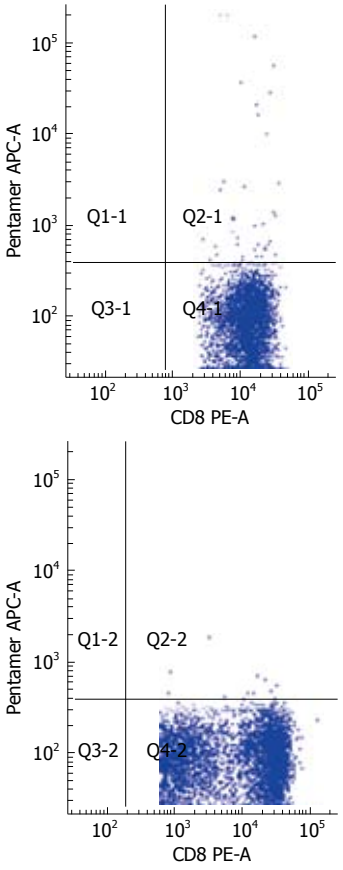

Figure 4 The number of HBV-specific CD8 T cells in two patients with CHB (0.07%) and AEHB (0.31%) respectively.

DISCUSSION

The development of chronic persistent HBV infection is usually associated with quantitative and qualitative exhaustion of functional T cells. Recent studies have shown that the negative costimulatory receptor PD-1 along with its ligand PD-L1 are upregulated on PBMC and virus specific T cells, to attenuate T cell responses which accounts for the impairment of their function[15,16]. However, under acute hepatitis B (AHB) conditions, the

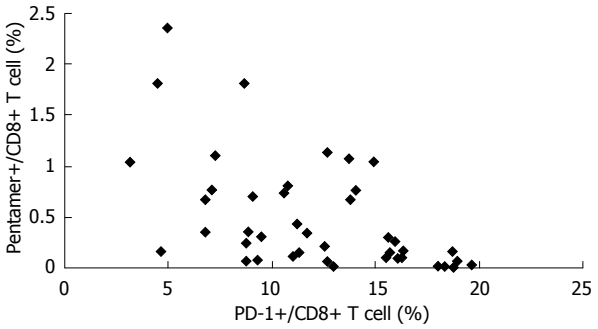

Figure 5 Significant inverse correlation between the number of HBV-specific CD8 T-cells and the level of PD-1 expression in patients with AEHB and CHB (R = 0.541, P < 0.01).

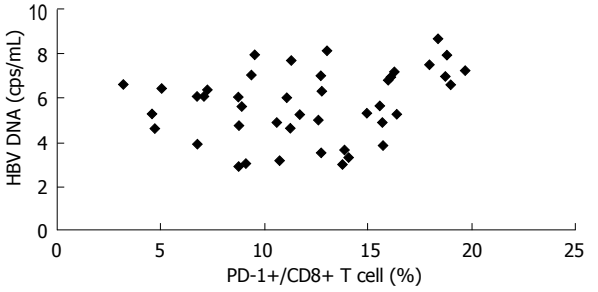

Figure 6 Positive correlation between serum viral load and level of PD-1 expression on CD8+ T cells in patients with CHB and AEHB (R = 0.272, P < 0.05).

levels of PD-1 are significantly down regulated on PBMC or on HBV specific CD8+ T cells compared to CHB patients. Several previous studies[10,25,26] have suggested that operating co-stimulatory pathways may provide a new method to restore the impaired function of virus specific T cells and control chronic virus infection. Blocking the PD-1/PD-L1 pathway by anti-PD-L1 mAb or applying soluble PD-1 results in improvement of T cell functions such as survival, proliferation and cytokine production[27-29]. This would result in better control of the virus infection. Thus, an accurate balance between positive and negative co-stimulatory regulation such as PD-1/ PD-L pathway may contribute to the outcome of disease progression in HBV infection.

However, the mechanism by which PD-1 affects the function of CD8+ T cells in AEHB is unclear. To clarify the correlation between PD-1 expression and different presentations of HBV infection, we investigated the role of PD-1 in AEHB and chronic HBV infection, using a MHC class I pentamer specific for 18-27 epitopes, using the flow cytometry technology. Consistent with previous studies, we found that peripheral blood CD8+ T cells showed a higher PD-1 expression in CHB patients compared to AEHB patients and HCs. There was a negative correlation with impaired HBV-specific CD8+ T cell function and a positive correlation with the plasma viral load. However, we did not find an association between PD-1 expression and hepatic inflammatory indicator: serum ALT levels. Our data indicates that similar to AHB patients, CD8+ T cells in AEHB patients expressed a low level of PD-1 and

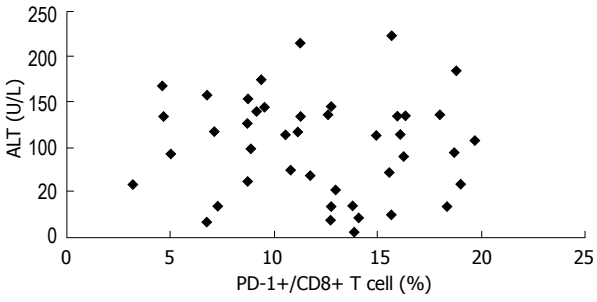

Figure 7 Lack of significant correlation between PD-1 expression and serum ALT levels in the study patients (R = 0.066, P > 0.05).

an enhanced level of HBV specific CD8+ T cells compared to CHB patients. These results are in line with previous reports which indicate that virus specific T cell function is upregulated during acute exacerbation of hepatitis B infection compared to during persistent CHB. Furthermore, the proportion of CD8+ T cells expressing PD-1 correlated negatively with the intensity of virus specific CD8+ T cell response.

The low levels of PD-1expression were associated with low levels of HBV viremia. However, we did not find any correlation between PD-1 expression and disease flare-up indicator, ALT. Recent studies have shown that persistent antigenic stimulation has a suppressive effect on functional T cells. Briefly, T cells up-regulate their surface receptors such as PD-1 to restrict positive TCR signaling and, therefore, avoid the development of a strong immune response, including autoimmune diseases. However, some microorganisms may utilize this *in vivo* protection system to escape the host immune system and result in persistent viremia. However, why this phenomenon is broken in patients with AEHB remains to be investigated.

Although PD-1/PD-L pathway plays a prominent role in the regulation of T cell dysfunction, other costimulatory molecules cannot be excluded. In a recent study, it was observed that apart from PD-1, several other gene expressions were involved in promoting exhausted CD8+ T cells during chronic viral infection[13]. However, recent data indicates that PD-1 is a major factor of apoptosis sensitivity over and above other factors[9]. Future studies should be addressed to clarify the intracellular mechanisms used by the PD-1/PD-L1 pathway to influence disease status during viral infection. Importantly, the use of highly active anti-retroviral therapy (HAART), accompanied by the recovery of the host immune response, has been found to down-regulate PD-1 expression in 'typical progressor' (TP) patients infected with HIV[11,30]. Therefore, it is important to determine whether direct suppression of PD-1/PD-L pathway could provide a potentially effective treatment for persistent viral infections.

COMMENTS

Background

Nearly, 2 billion people are infected with hepatitis B virus (HBV) worldwide. Persistent HBV infection of the liver is associated with end-stage liver diseases

including cirrhosis and hepatocellular carcinoma. During chronic HBV infection, the effector functions of virus specific T cells often show an impaired activity indicated by low levels of cytokine production and cytotoxic activity. As a result, viruses can persist and establish long-term residency *in vivo*. However, the underlying mechanisms responsible for the induction of T-cell tolerance are not completely understood.

Research frontiers

The aim of this study was to explore the potential role of programmed death-1/programmed death-ligand (PD-1/PD-L) pathway in antiviral immunity during HBV infection.

Innovations and breakthroughs

This is the first report on the differences in PD-1 expression on CD8+ T cells in acute exacerbation of hepatitis B (AEHB) and chronic HBV infection.

Applications

Blockade of PD-1/PD-L1 pathway may open a novel therapeutic strategy for restoring the function of the exhausted CD8+ T cells, and enhancing viral control during chronic viral infections. The perspective of future application: further studies to assess the intracellular mechanisms used by the PD-1/PD-L1 pathway to influence the disease status in viral infection.

Peer review

This is a requisite investigation. The levels of PD-1 on CD8+ T cells has until now been analyzed only in small groups of patients with acute infection and established chronic infection. The manuscript provides new information about PD-1 expression on CD8+ T cells in different disease states of HBV infection including AEHB and chronic hepatitis B (CHB).

REFERENCES

1 **Liu TT**, Fang Y, Xiong H, Chen TY, Ni ZP, Luo JF, Zhao NQ, Shen XZ. A case-control study of the relationship between hepatitis B virus DNA level and risk of hepatocellular carcinoma in Qidong, China. *World J Gastroenterol* 2008; **14**: 3059-3063

2 **Zerbini A**, Pilli M, Boni C, Fisicaro P, Penna A, Di Vincenzo P, Giuberti T, Orlandini A, Raffa G, Pollicino T, Raimondo G, Ferrari C, Missale G. The characteristics of the cell-mediated immune response identify different profiles of occult hepatitis B virus infection. *Gastroenterology* 2008; **134**: 1470-1481

3 **Radziewicz H**, Uebelhoer L, Bengsch B, Grakoui A. Memory CD8+ T cell differentiation in viral infection: a cell for all seasons. *World J Gastroenterol* 2007; **13**: 4848-4857

4 **Menne S**, Tennant BC. Unraveling hepatitis B virus infection of mice and men (and woodchucks and ducks) *Nat Med* 1999; **5**: 1125-1126

5 **Rehermann B**, Ferrari C, Pasquinelli C, Chisari FV. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. *Nat Med* 1996; **2**: 1104-1108

6 **Khoury SJ**, Sayegh MH. The roles of the new negative T cell costimulatory pathways in regulating autoimmunity. *Immunity* 2004; **20**: 529-538

7 **Nurieva R**, Thomas S, Nguyen T, Martin-Orozco N, Wang Y, Kaja MK, Yu XZ, Dong C. T-cell tolerance or function is determined by combinatorial costimulatory signals. *EMBO J* 2006; **25**: 2623-2633

8 **Muhlbauer M**, Fleck M, Schutz C, Weiss T, Froh M, Blank C, Scholmerich J, Hellerbrand C. PD-L1 is induced in hepatocytes by viral infection and by interferon-alpha and -gamma and mediates T cell apoptosis. *J Hepatol* 2006; **45**: 520-528

9 **Bowen DG**, Shoukry NH, Grakoui A, Fuller MJ, Cawthon AG, Dong C, Hasselschwert DL, Brasky KM, Freeman GJ, Seth NP, Wucherpfennig KW, Houghton M, Walker CM. Variable patterns of programmed death-1 expression on fully functional memory T cells after spontaneous resolution of hepatitis C virus infection. *J Virol* 2008; **82**: 5109-5114

10 **Zhang Z**, Zhang JY, Wherry EJ, Jin B, Xu B, Zou ZS, Zhang SY, Li BS, Wang HF, Wu H, Lau GK, Fu YX, Wang FS. Dynamic programmed death 1 expression by virus-specific CD8 T cells correlates with the outcome of acute hepatitis B. *Gastroenterology* 2008; **134**: 1938-1949, 1949. e1-e3

11 **Day CL**, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu B, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM, Goulder PJ, Klenerman P, Ahmed R, Freeman GJ, Walker BD. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006; **443**: 350-354

12 **Latchman YE**, Liang SC, Wu Y, Chernova T, Sobel RA, Klemm M, Kuchroo VK, Freeman GJ, Sharpe AH. PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. *Proc Natl Acad Sci USA* 2004; **101**: 10691-10696

13 **Okazaki T**, Honjo T. The PD-1-PD-L pathway in immunological tolerance. *Trends Immunol* 2006; **27**: 195-201

14 **Petrovas C**, Casazza JP, Brenchley JM, Price DA, Gostick E, Adams WC, Precopio ML, Schacker T, Roederer M, Douek DC, Koup RA. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J Exp Med* 2006; **203**: 2281-2292

15 **Venkatachari NJ**, Buchanan WG, Ayyavoo V. Human immunodeficiency virus (HIV-1) infection selectively downregulates PD-1 expression in infected cells and protects the cells from early apoptosis in vitro and in vivo. *Virology* 2008; **376**: 140-153

16 **Meier A**, Bagchi A, Sidhu HK, Alter G, Suscovich TJ, Kavanagh DG, Streeck H, Brockman MA, LeGall S, Hellman J, Altfeld M. Upregulation of PD-L1 on monocytes and dendritic cells by HIV-1 derived TLR ligands. *AIDS* 2008; **22**: 655-658

17 **Barber DL**, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; **439**: 682-687

18 **Day CL**, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu B, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM, Goulder PJ, Klenerman P, Ahmed R, Freeman GJ, Walker BD. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006; **443**: 350-354

19 **Petrovas C**, Casazza JP, Brenchley JM, Price DA, Gostick E, Adams WC, Precopio ML, Schacker T, Roederer M, Douek DC, Koup RA. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J Exp Med* 2006; **203**: 2281-2292

20 **Zhang JY**, Zhang Z, Wang X, Fu JL, Yao J, Jiao Y, Chen L, Zhang H, Wei J, Jin L, Shi M, Gao GF, Wu H, Wang FS. PD-1 up-regulation is correlated with HIV-specific memory CD8+ T-cell exhaustion in typical progressors but not in long-term nonprogressors. *Blood* 2007; **109**: 4671-4678

21 **Golden-Mason L**, Palmer B, Klarquist J, Mengshol JA, Castelblanco N, Rosen HR. Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. *J Virol* 2007; **81**: 9249-9258

22 **Urbani S**, Amadei B, Tola D, Massari M, Schivazappa S, Missale G, Ferrari C. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol* 2006; **80**: 11398-11403

23 **Boni C**, Fisicaro P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, Laccabue D, Zerbini A, Cavalli A, Missale G, Bertoletti A, Ferrari C. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* 2007; **81**: 4215-4225

24 **Chinese Society of Infectious Diseases and Parasitology and Chinese Society of Hepatology of Chinese Medical Association**. The program of prevention and cure for viral hepatitis. *Zhonghua Ganzangbing Zazhi* 2000; **8**: 324-329

25 **Bukczynski J**, Wen T, Wang C, Christie N, Routy JP, Boulassel MR, Kovacs CM, Macdonald KS, Ostrowski M, Sekaly RP, Bernard NF, Watts TH. Enhancement of HIV-specific CD8 T cell responses by dual costimulation with

CD80 and CD137L. *J Immunol* 2005; **175**: 6378-6389
26 **Yu Q**, Yue FY, Gu XX, Schwartz H, Kovacs CM, Ostrowski MA. OX40 ligation of CD4+ T cells enhances virus-specific CD8+ T cell memory responses independently of IL-2 and CD4+ T regulatory cell inhibition. *J Immunol* 2006; **176**: 2486-2495
27 **Lukens JR**, Cruise MW, Lassen MG, Hahn YS. Blockade of PD-1/B7-H1 interaction restores effector CD8+ T cell responses in a hepatitis C virus core murine model. *J Immunol* 2008; **180**: 4875-4884
28 **Ha SJ**, Mueller SN, Wherry EJ, Barber DL, Aubert RD, Sharpe AH, Freeman GJ, Ahmed R. Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. *J Exp Med* 2008; **205**: 543-555
29 **Onlamoon N**, Rogers K, Mayne AE, Pattanapanyasat K, Mori K, Villinger F, Ansari AA. Soluble PD-1 rescues the proliferative response of simian immunodeficiency virus-specific CD4 and CD8 T cells during chronic infection. *Immunology* 2008; **124**: 277-293
30 **Trautmann L**, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, Boulassel MR, Delwart E, Sepulveda H, Balderas RS, Routy JP, Haddad EK, Sekaly RP. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med* 2006; **12**: 1198-1202

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RAPID COMMUNICATION

Effect of admission hypertriglyceridemia on the episodes of severe acute pancreatitis

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Abstract

AIM: To investigate the effect of admission hypertriglyceridemia (HTG) on the episodes of severe acute pancreatitis (SAP).

METHODS: One hundred and seventy-six patients with SAP were divided into HTG group (n = 45) and control group (n = 131) according to admission triglyceride (TG) ⩾ 5.65 mmol/L and < 5.65 mmol/L, respectively. Demographics, etiology, underlying diseases, biochemical parameters, Ranson' s score, acute physiology and chronic heath evaluation Ⅱ (APACHE Ⅱ) score, Balthazar's computed tomography (CT) score, complications and mortality were compared. Correlation between admission TG and 24-h APACHE Ⅱ score was analyzed.

RESULTS: SAP patients with HTG were younger (40.8 ± 9.3 years *vs* 52.6 ± 13.4 years, P < 0.05) with higher etiology rate of overeating, high-fat diet (40.0% *vs* 14.5%, P < 0.05) and alcohol abuse (46.7% *vs* 23.7%, P < 0.01), incidence rate of hypocalcemia (86.7% *vs* 63.4%, P < 0.01) and hypoalbuminemia (84.4% *vs* 60.3%, P < 0.01), 24-h APACHE Ⅱ score (13.6 ± 5.7 *vs* 10.7 ± 4.6, P < 0.01) and admission serum glucose (17.7 ± 7.7 *vs* 13.4 ± 6.1, P < 0.01), complication rate of renal failure (51.1% *vs* 16.8%, P < 0.01), shock (37.9% *vs* 14.5%, P < 0.01) and infection (37.4% *vs* 18.3%, P < 0.01) and mortality (13.1% *vs* 9.1%, P < 0.01). Logistic regression analysis showed a positive correlation between admission TG and 24-h APACHE Ⅱ score (r = 0 .509, P = 0.004).

CONCLUSION: The clinical features of SAP patients with HTG are largely consistent with previous studies. HTG aggravates the episodes of SAP.

Key words: Clinical study; Hypertriglyceridemia; Severe acute pancreatitis; Clinical features; Outcome

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INTRODUCTION

Hypertriglyceridemia (HTG) is a rare but well-recognized cause for severe acute pancreatitis (SAP), which has been intensively studied since Speck noted the association between hyperlipidemia and acute pancreatitis (AP) in 1865[1-4]. HTG can be a primary cause for AP which occurs in 1.3%-3.8% of AP patients, or secondary to other factors prior to the increase of lipid levels, or both[5]. Clinically, mild to moderate hyperlipidemia in AP patients, particularly in alcoholic pancreatitis patients, is often observed. However, it is difficult for clinicians to distinguish mild to moderate hyperlipidemia secondary to AP from marked HTG that primarily causes AP.

It is generally believed that a serum triglyceride (TG) level of more than 1000 mg/dL (about 11.3 mmol/L) is needed to precipitate AP, the reduction of which to well below 1000 mg/dL may effectively prevent further episodes[6]. Animal studies showed that HTG intensifies the course of AP including both edematous and necrotizing pancreatitis[7,8]. It was reported that TG would worsen pancreatic injury induced by AP when it reaches 5.65 mmol/L, thus playing a worth-noting role in predisposing mild pancreatitis to the vicious episode[9]. Extreme elevation of TG in AP patients with familial HTG can cause the so-called "hyperlipidemic abdominal crisis"[10]. However, it was also reported that

SAP patients with HTG can have pancreatic necrosis, pseudocysts, abscesses and other complications that can be seen in other types of pancreatitis with a different clinical course from other forms of AP[11]. Thus, the correlation between HTG and the severity of AP episodes is still uncertain.

In this study, the clinical features and the effect of HTG on the episodes of SAP were investigated with HTG as a coexisting medical condition of SAP.

MATERIALS AND METHODS

Patients

The diagnostic criteria for SAP formulated at the Bangkok World Congress of Gastroenterology 2002 in Thailand[12] were employed and organ failure was defined according to the Criteria of Clinical Diagnosis and Classification System for AP formulated by the Pancreatic Surgical Society of Chinese Medical Association in 1997[13] in this study. From March 2003 to December 2004, all patients diagnosed with SAP and admitted to West China Hospital of Sichuan University within 72 h after onset of symptoms were included. Patients were excluded if they had hepatic dysfunction or renal failure prior to the development of SAP.

Methods

One hundred and seventy-six SAP patients admitted to our hospital were enrolled and divided into HTG group (n = 45) and control group (n = 131) according to admission TG $\geqslant$ 5.65 mmol/L and TG < 5.65 mmol/L, respectively. Blood samples were collected at admission for biochemical examinations in Department of Laboratory Medicine of our hospital.

All patients received standardized comprehensive treatments[12]. The main protocols of treatment throughout the study were intensive care, oxygen inhalation, fasting, intermittent gastrointestinal decompression and fluid infusion. The balance of internal environment was maintained, prophylactic antibiotics were used for 7-14 d, H_2 receptor antagonists or proton pump inhibitors were given for 7 d. When the patients developed respiratory failure, a respirator was employed to assist respiration. When the patients developed hypoalbuminaemia, 20% of human serum album in 50 mL was used daily until the serum albumin value became normal. When serum lipid value was decreased to normal, fat emulsion was added in parenteral nutrition. During hospitalization, microbiological tests of sputum, urine, feces, or blood were performed, when the following susceptible clinical symptoms or signs appeared: body temperature $\geqslant$ 38.5℃ and white blood cell (WBC) count $\geqslant 20 \times 10^9$ /L, signs of peritoneal irritation (area) in more than 2 quadrants, and intractable malnutrition. Contrast-enhanced computed tomography (CECT) was performed to determine necrotic infection of (peri) pancreas. For those who were unsuitable for CECT evaluated by the investigator, magnetic resonance imaging was alternatively eligible. Ultrasound-guided fine needle aspiration (FNA) was performed for microbiological testing when air bubbles appeared in necrotic tissue of the (peri) pancreas. Bacterial infection was confirmed by positive culture or smear examination. Fungal infection was confirmed by positive fungi in no less than 2 different specimens by culture or smear examination.

The sex, age, etiology, underlying diseases, biochemical parameters and incidence of complications including acute respiratory distress syndrome (ARDS), renal failure, acute hepatitis, shock, encephalopathy, infection rate, and mortality, were confirmed by one of the investigators using a standard data collection instrument. The Ranson's score, 24-h APACHE Ⅱ score and admission Balthazar's CT score were calculated by a single investigator.

Table 1 Clinical features of patients in HTG and control groups

	HTG group (n = 45)	Control group (n = 131)
Sex (Male/Female)	27/18	72/59
Age (mean ± SD, yr)	40.8 ± 9.3	52.6 ± 13.4
	(24-61)[a]	(22-82)
Etiology, *n* (%)		
Overeating and high fat diet	18 (40.0)[a]	19 (14.5)
Alcohol abuse	21 (46.7)[b]	31 (23.7)
Gallstones	5 (11.1)[b]	37 (28.2)
L-Asparaginase chemotherapy	2 (4.4)	0
Pregnancy	6 (13.3)	0
Underlying diseases, *n* (%)		
Hypertension	6 (13.3)	15 (11.5)
Coronary heart disease	3 (6.7)	7 (5.3)
Atherosclerosis	3 (6.7)	9 (6.9)
Familial hyperlipidemia	6 (13.3)	11 (8.4)
Admission biochemical		
Serum glucose (mmol/L)	17.7 ± 7.7[b]	13.4 ± 6.1
Hypoalbuminaemia (%)	38 (84.4)[b]	79 (60.3)
Hypocalcaemia (%)	39 (86.7)[b]	83 (63.4)
Hypopotassemia (%)	16 (35.6)	50 (38.2)
Hyponatremia (%)	26 (57.8)	59 (45.0)
Ranson's score (mean ± SD)	4.7 ± 1.9	4.9 ± 2.0
24-h APACHE Ⅱ score (mean ± SD)	13.6 ± 5.7[b]	10.7 ± 4.6
Balthazar's CT score (mean ± SD)	5.4 ± 2.3	6.3 ± 5.4

[a]P < 0.05, [b]P < 0.01 *vs* control group.

Statistical analysis

Data were expressed as mean ± SD or percentage. Data in normal distribution were analyzed using t-test. Data in abnormal distribution were analyzed using Wilcoxon rank sum test. Categorical data were analyzed using chi-square test. The correlation between serum TG and 24-h APACHE Ⅱ score was analyzed using Logistic regression. P < 0.05 was considered statistically significant.

RESULTS

Clinical features

The clinical features of the patients in the HTG and control groups are summarized in Table 1. There were no statistical differences in sex distribution and incidence

Table 2 Incidence of complications and mortality in HTG and control groups *n* (%)

	HTG group (n = 45)	Control group (n = 131)
Complications		
ARDS	29 (64.4)	61 (46.6)
Renal failure	23 (51.1)[b]	22 (16.8)
Acute hepatitis	20 (44.4)	38 (29.0)
Shock	17 (37.9)[b]	19 (14.5)
Encephalopathy	10 (22.2)	24 (18.3)
Infection	17 (37.4)[b]	24 (18.3)
Death	14 (31.1)[b]	12 (9.1)

[b]$P < 0.01$ *vs* control group.

rate of underlying diseases between the two groups. Patients in the HTG group were younger ($P < 0.05$) with a higher rate of overeating and a high fat diet ($P < 0.05$) and alcohol abuse ($P < 0.01$) and less gallstones ($P < 0.01$). The Ranson's score and initial Balthazar's CT score were not statistically different between the two groups, but the 24-h APACHE Ⅱ score was higher in the HTG group than in the control group ($P < 0.01$). The incidence rate of hypokalemia and hyponatremia had no difference and was higher in the HTG group than in the control group ($P < 0.01$), and admission serum glucose was higher in the HTG group than in the control group ($P < 0.01$).

Complications and mortality

There were no statistical differences in complications such as ARDS, acute hepatitis and encephalopathy between the two groups. However, more complications, such as renal failure, shock and infection occurred in the HTG group than in the control group ($P < 0.01$). The mortality was higher in the HTG group than in the control group ($P < 0.01$) (Table 2).

DISCUSSION

HTG-associated SAP is an uncommon, but potentially life-threatening disease[14]. HTG may be primary in origin (hereditary or sporadic genetic disorder of metabolism) or secondary (associated with an identifiable disease or condition and is reversible with control or eradication of that disease or condition) to other factors or both[15-17]. Although the exact pathogenesis of HTG-associated AP is not clear, it is thought to result from toxic injury to acinar cells and capillary endothelia by excessive free fatty acids from hydrolysis of TG[18,19]. Competitive oxidation of ethanol is also responsible for SAP by aggravating the level of serum lipids[20]. L-asparaginase-induced HTG is suggested as a possible mechanism of SAP[21,22]. HTG is the cause for gestational pancreatitis in 56% cases[23]. Elevated estrogen increases the synthesis of TG and depresses plasma postheparin lipolytic activities by inhibiting the removal efficiency of TG, and high-fat intake may be a cause for AP during pregnancy[24-27], which are largely consistent with the results of our study, showing that the patients with HTG were younger with more diet-associated etiologies including overeating, high-fat diet and alcohol abuse. SAP patients with HTG had a higher incidence of hypoalbuminaemia and hypocalcemia and a higher level of admission serum glucose, which may be associated with the aggravation of HTG, resulting from severe stress response and metabolic disorders[28].

The role of HTG in modulating disease course of AP is still controversial. Although studies demonstrated that HTG has no significant correlation to complications of disease or overall end-of-episode severity in AP patients[3,12]. It was reported that HTG is independently associated with the severity of AP and plays a role in the aggravation of acute necrotizing pancreatitis[29-31]. The results of our study show that the incidence of admission hypocalcaemia, a predictable index of SAP[32], and the 24-h APACHE Ⅱ score were higher in the HTG group than in the control group. The complications, such as renal failure, shock and infection, and the mortality were higher in the HTG group than in the control group, indicating that HTG aggravates SAP, leading to systemic complications and a high mortality rate of SAP.

In conclusion, the clinical features of HTG-associated SAP are largely consistent with previous studies. HTG aggravates SAP.

COMMENTS

Background

Hypertriglyceridemia (HTG) is associated with severe acute pancreatitis (SAP), which has long been recognized. HTG may be a primary etiology of SAP, and it is difficult for clinicians to identify it. Some previous studies suggest that HTG is independently associated with the severity of acute pancreatitis (AP) and plays a role in the aggravation of acute necrotizing pancreatitis. However, its mechanism underlying such a role is still controversial.

Research frontiers

HTG is a rare cause for pancreatitis. An elevated TG level is needed to precipitate the episode of AP, which is called "hyperlipidemic abdominal crisis". Moreover, recurrent pancreatitis may occur in patients with familial hyperlipoproteinemia. Which patients progress to the extremely dangerous state during SAP and specific strategy against its deterioration and recurrence are the major research field.

Innovations and breakthroughs

It is generally believed that the serum triglyceride (TG) level of more than 1000 mg/dL (about 11.3 mmol/L) is needed to precipitate AP, but the clinicians cannot identify mild to moderate HTG secondary to SAP with marked HTG that causes AP in the acute phase. This research used admission HTG as a coexisting factor for SAP patients at admission.

Applications

At present, the exact role of HTG in the development of SAP remains unclear. Our study, conducted in one of the largest institutions in China, showed that HTG could aggravate SAP and leads to a worse outcome of SAP patients, thus providing applicable and valuable evidence for prognostic evaluation of pancreatitis.

Terminology

AP is an acute inflammatory process in the pancreas involveing local or other organ systems. Severe AP is defined as the occurrence of organ failure. HTG is defined as an abnormal concentration of TG in the blood.

Peer review

This research investigated the effect of admission HTG on the severity of SAP, by regarding admission HTG as a coexisting factor for SAP. The study was well designed and its results are valuable.

REFERENCES

1 **Pfau J**. [Acute pancreatitis and hypertriglyceridemia] *Rev Med Chil* 1989; **117**: 907-909
2 **Ohmoto K**, Neishi Y, Miyake I, Yamamoto S. Severe acute pancreatitis associated with hyperlipidemia: report of two cases and review of the literature in Japan. *Hepatogastroenterology* 1999; **46**: 2986-2990
3 **Yadav D**, Pitchumoni CS. Issues in hyperlipidemic pancreatitis. *J Clin Gastroenterol* 2003; **36**: 54-62
4 **Gan SI**, Edwards AL, Symonds CJ, Beck PL. Hypertriglyceridemia-induced pancreatitis: A case-based review. *World J Gastroenterol* 2006; **12**: 7197-7202
5 **Fortson MR**, Freedman SN, Webster PD 3rd. Clinical assessment of hyperlipidemic pancreatitis. *Am J Gastroenterol* 1995; **90**: 2134-2139
6 **Toskes PP**. Hyperlipidemic pancreatitis. *Gastroenterol Clin North Am* 1990; **19**: 783-791
7 **Wang YJ**, Sun JB, Li F, Zhang SW. Hyperlipidemia intensifies cerulein-induced acute pancreatitis associated with activation of protein kinase C in rats. *World J Gastroenterol* 2006; **12**: 2908-2913
8 **Czako L**, Szabolcs A, Vajda A, Csati S, Venglovecz V, Rakonczay Z Jr, Hegyi P, Tiszlavicz L, Csont T, Posa A, Berko A, Varga C, Varga Ilona S, Boros I, Lonovics J. Hyperlipidemia induced by a cholesterol-rich diet aggravates necrotizing pancreatitis in rats. *Eur J Pharmacol* 2007; **572**: 74-81
9 **Oh RC**, Lanier JB. Management of hypertriglyceridemia. *Am Fam Physician* 2007; **75**: 1365-1371
10 **Miller A**, Lees RS, McCluskey MA, Warshaw AL. The natural history and surgical significance of hyperlipemic abdominal crisis. *Ann Surg* 1979; **190**: 401-408
11 **Balachandra S**, Virlos IT, King NK, Siriwardana HP, France MW, Siriwardena AK. Hyperlipidaemia and outcome in acute pancreatitis. *Int J Clin Pract* 2006; **60**: 156-159
12 **Toouli J**, Brooke-Smith M, Bassi C, Carr-Locke D, Telford J, Freeny P, Imrie C, Tandon R. Guidelines for the management of acute pancreatitis. *J Gastroenterol Hepatol* 2002; **17** Suppl: S15-S39
13 **The Pancreatology Academic Group of Surgical Branch of Chinese Medical Association**. The clinical diagnosis and grading standard for acute pancreatitis. *Zhonghua Waike Zazhi* 1997; **35**: 773-775
14 **Athyros VG**, Giouleme OI, Nikolaidis NL, Vasiliadis TV, Bouloukos VI, Kontopoulos AG, Eugenidis NP. Long-term follow-up of patients with acute hypertriglyceridemia-induced pancreatitis. *J Clin Gastroenterol* 2002; **34**: 472-475
15 **Dominguez-Munoz JE**, Junemann F, Malfertheiner P. Hyperlipidemia in acute pancreatitis. Cause or epiphenomenon? *Int J Pancreatol* 1995; **18**: 101-106
16 **Yuan G**, Al-Shali KZ, Hegele RA. Hypertriglyceridemia: its etiology, effects and treatment. *CMAJ* 2007; **176**: 1113-1120
17 **Havel RJ**. Pathogenesis, differentiation and management of hypertriglyceridemia. *Adv Intern Med* 1969; **15**: 117-154
18 **Saharia P**, Margolis S, Zuidema GD, Cameron JL. Acute pancreatitis with hyperlipemia: studies with an isolated perfused canine pancreas. *Surgery* 1977; **82**: 60-67
19 **Kimura W**, Mossner J. Role of hypertriglyceridemia in the pathogenesis of experimental acute pancreatitis in rats. *Int J Pancreatol* 1996; **20**: 177-184
20 **Morita Y**, Yoshikawa T, Takeda S, Matsuyama K, Takahashi S, Yoshida N, Clemens MG, Kondo M. Involvement of lipid peroxidation in free fatty acid-induced isolated rat pancreatic acinar cell injury. *Pancreas* 1998; **17**: 383-389
21 **Top PC**, Tissing WJ, Kuiper JW, Pieters R, van Eijck CH. L-asparaginase-induced severe necrotizing pancreatitis successfully treated with percutaneous drainage. *Pediatr Blood Cancer* 2005; **44**: 95-97
22 **Alvarez OA**, Zimmerman G. Pegaspargase-induced pancreatitis. *Med Pediatr Oncol* 2000; **34**: 200-205
23 **Chang CC**, Hsieh YY, Tsai HD, Yang TC, Yeh LS, Hsu TY. Acute pancreatitis in pregnancy. *Zhonghua Yi Xue Za Zhi (Taipei)* 1998; **61**: 85-92
24 **Yamauchi H**, Sunamura M, Takeda K, Suzuki T, Itoh K, Miyagawa K. Hyperlipidemia and pregnancy associated pancreatitis with reference to plasma exchange as a therapeutic intervention. *Tohoku J Exp Med* 1986; **148**: 197-205
25 **Achard JM**, Westeel PF, Moriniere P, Lalau JD, de Cagny B, Fournier A. Pancreatitis related to severe acute hypertriglyceridemia during pregnancy: treatment with lipoprotein apheresis. *Intensive Care Med* 1991; **17**: 236-237
26 **Hsia SH**, Connelly PW, Hegele RA. Pancreatitis, hyperlipidemia, and pregnancy in two sisters. *Ann Intern Med* 1997; **126**: 88-89
27 **Kraus J**, Krska Z, Krausova V, Horejs J, Zak A, Souckova M. [Severe necrotizing pancreatitis during pregnancy] *Rozhl Chir* 2005; **84**: 621-625
28 **Xue P**, Huang ZW, Li YH, Guo J, Wang ZC, Zhao JL, You Z. [Clinical study on severe acute pancreatitis associated with hypoalbuminemia in early stage] *Zhongxiyi Jiehe Xuebao* 2005; **3**: 443-445
29 **Chen CH**, Dai CY, Hou NJ, Chen SC, Chuang WL, Yu ML. Etiology, severity and recurrence of acute pancreatitis in southern taiwan. *J Formos Med Assoc* 2006; **105**: 550-555
30 **Dominguez-Munoz JE**, Malfertheiner P, Ditschuneit HH, Blanco-Chavez J, Uhl W, Buchler M, Ditschuneit H. Hyperlipidemia in acute pancreatitis. Relationship with etiology, onset, and severity of the disease. *Int J Pancreatol* 1991; **10**: 261-267
31 **Navarro S**, Cubiella J, Feu F, Zambon D, Fernandez-Cruz L, Ros E. [Hypertriglyceridemic acute pancreatitis. Is its clinical course different from lithiasic acute pancreatitis?] *Med Clin* (Barc) 2004; **123**: 567-570
32 **Harrison DA**, D'Amico G, Singer M. The Pancreatitis Outcome Prediction (POP) Score: a new prognostic index for patients with severe acute pancreatitis. *Crit Care Med* 2007; **35**: 1703-1708

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RAPID COMMUNICATION

Correlation between expression and differentiation of endocan in colorectal cancer

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Abstract

AIM: To investigate the expression frequency of endocan in colorectal cancer and analyze the relationship between endocan expression and clinical parameters and to study the role of endocan in colorectal carcinogenesis.

METHODS: Expression of endocan in 72 tumor tissue samples of colorectal cancer as well as in 27 normal mucous membrane tissue samples was analyzed using in situ hybridization, immunohistochemistry on tissue microarray, Western blot and reverse-transcript polymerase chain reaction (RT-PCR).

RESULTS: The expression of endocan was higher in normal colon and rectum tissue samples than in cancerous tissue samples (mRNA = 92.6%, protein = 36%), and was lower in colorectal cancer tissue samples (mRNA = 70.4%, protein = 36.1%). No correlation was found between staining intensity and clinical parameters such as sex, age, tumor size and TNM stage. However, the expression of endocan was positively correlated with the tissue differentiation in colorectal cancer.

CONCLUSION: The expression of endocan is down-regulated in colorectal cancer and is positively correlated with the tissue differentiation in colorectal cancer, suggesting that the expression of endocan is associated with development and differentiation of colorectal cancer.

Key words: Endocan; Colorectal cancer; Differentiation; Expression; In situ hybridization

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INTRODUCTION

Colon and rectum cancers accounted for about 1 million new cases in 2002 (9.4% of the world total)[1], There is at least a 25-fold variation in the occurrence of colorectal cancer around the world. The incidence of colorectal cancer increases rather rapidly in countries where the overall risk was formerly low (especially in Japan, but also elsewhere in Asia)[2]. Although it has been found that many factors are correlated with genesis and development of colon and rectum cancers, it cannot explain all the clinical and pathological manifestations. It is critical to investigate new factors which are intimately correlated with initiation and development of colorectal cancer.

Endocan, previously called endothelial cell-specific molecule-1 (ESM-1)[3], is over expressed in human tumors, and its serum levels are elevated in late-stage lung cancer and experimental tumor, as measured by enzyme-linked immunoassay or by

immunohistochemistry. mRNA level of endocan is also recognized as one of the most significant molecular signatures with a poor prognosis of several types of cancer including lung cancer. Over expression of this dermatan sulphate proteoglycan is also directly involved in tumor progression as observed in mouse models of human tumor xenografts. These results suggest that endocan is a biomarker of inflammatory disorders and tumor progression as well as a validated therapeutic target in cancer.

We studied the expression of endocan in colon and rectum tissue samples. The results of this study indicate that endocan expression is down-regulated in colorectal cancer and positively correlated with the differentiation of colorectal cancer. Changes in endocan expression represent an important step in development and differentiation of colorectal cancer.

MATERIALS AND METHODS

Patients and samples

Seventy-two colorectal cancer patients, who consecutively underwent radical surgical resection at Anhui Medical University Hospital from the year 2001 to 2003, were recruited into this study. Tumor and mucosa samples were embedded in paraffin after 16 h formalin fixation. None of the patients (23 males, 49 females, mean age 54 years, range 17-87 years) received any anticancer therapy. According to the TNM classification[4], 43 cases were at stages Ⅰ and Ⅱ, 29 cases at stages Ⅲ and Ⅳ. Well- and moderately- differentiated adenocarcinoma was found in 57 patients and poorly-differentiated adenocarcinoma was observed 15 patients[5], and strong lymphoid infiltrate including lymphoid follicles with germinal centers was demonstrated in 39 patients.

In situ hybridization

cRNA probe labeling: The sequences of specific primers for endocan are as follows: sense, 5'-AGCTGGAATTCCATGAAGAG (20 bp) and antisense, 5'-TCTCTCAGAAAGCTTAGCCG (20 bp)[3]. PCR was performed to amplify endocan DNA, and the PCR product was ligated into the pGEM-T-Vector to get the recombinant plasmid pGEM-T-endocan. The recombinant plasmid was transformed into *E.coli*, amplified and digested with the restriction endonuclease (*Eco*RI and *Hin*dⅢ). The objective gene (V-gene) was purified using a DNA gel extraction kit to gobtain the probes for the following digoxigenin-labeling and detected according to the manufacturer's instructions.

Hybridization: All specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. A series of 5-μm thick sections were cut for analysis. In situ hybridization was performed as previously described[6] with certain modifications, using digoxigenin-labeled antisense cDNA probes. Briefly, the sections were dried at 60℃ for 4 h, dewaxed, rehydrated and pretreated with DEPC-treated PBS containing 100 mmol/L glycine and 0.3% Triton X-100, respectively. The sections were then permeabilized with 20 μg/mL RNase-free proteinase K (boster, Wuhuan, China) for 20 min, incubated at 37℃ for at least 20 min with prehybridization buffer. Each section was overlaid with 30 μL hybridization buffer containing a 10 ng digoxigenin-labeled cDNA probe and incubated at 42℃ overnight. After hybridization, the section was incubated with digoxigenin antibody (75 mU/mL) for 2 h. The positive signal for endocan mRNA was detected using DAB as a substrate. The presence of brown staining in the cytoplasm was considered positive.

Protein extraction from paraffin- fixed tissue

Paraffin-fixed tissue was cut into 50 5-μm thick sections for protein extraction and mounted onto plain glass slides. Three 5-μm thick sections for protein extraction were deparaffinized in xylene, rehydrated in graded ethanol, immersed in distilled water, and air-dried. To exclusively collect 5 mm × 5 mm cancer tissues, the targeted areas were cut microscopically with a fine needle for observation of the morphology of HE-stained sections under a microscope. After the tissue sections on the glass slide were immersed in distilled water, only the targeted areas of cancer tissue were separated from the glass slide and recovered. Adenoma tissue was also cut into sections and collected in the same manner. Normal mucosa was recovered from 5 cm-long sections of full-depth colorectal wall with a fine needle as previously described[7,8].

Immunohistochemistry

The pathology of colorectal carcinoma was performed on 5-μm thick sections of 10% formalin-embedded samples with a S-P kit. Slides were boiled in 10 mmol/L citrate buffer (pH 6.0) for 10 min to allow antigen retrieval before a 12-h incubation at 4℃ with primary antibody against endocan (Santa Cruz). The mean percentage of positive tumor cells was determined in ten areas at a high magnification (× 400) and graded from 0 to 4 (0 ⩽ 5% positive cells, 1 = 6%-25%, 2 = 26%-50%, 3 = 51%-75%, and 4 = 76%-100%, 0 = negative, 1-4= positive). Negative controls were obtained by omitting the primary antibody. Each normal mucosa sample, as an internal positive control, was simultaneously analyzed. Slides were read by two observers blinded to the clinical data.

Reverse-transcript polymerase chain reaction (RT-PCR)

Two micrograms of total RNA was prepared from colon and rectal tissues, randomly primed, and reverse transcribed with Superscript Ⅱ (Gibco). The sequences of specific primers used for endocan are as follows: sense, 5'-CTCAGGCATGGATGGCATGAAGTG-3'; antisense, 5'-GAGACCCGGCAGCATTCTCTTTCA-3'; and β-actin: sense: 5'-ACTCTTCCAGCCTTCCTTC-3' and antisense: 5'-ATCTCCTTCTGCATCCTGTC-3'. After a hot start at 94℃, 35 PCR cycles were performed, each cycle consisting of annealing at 57℃ for 45 s and extension at 72℃ for 45 s.

Western blot analysis

Twenty micrograms of protein was incubated in a

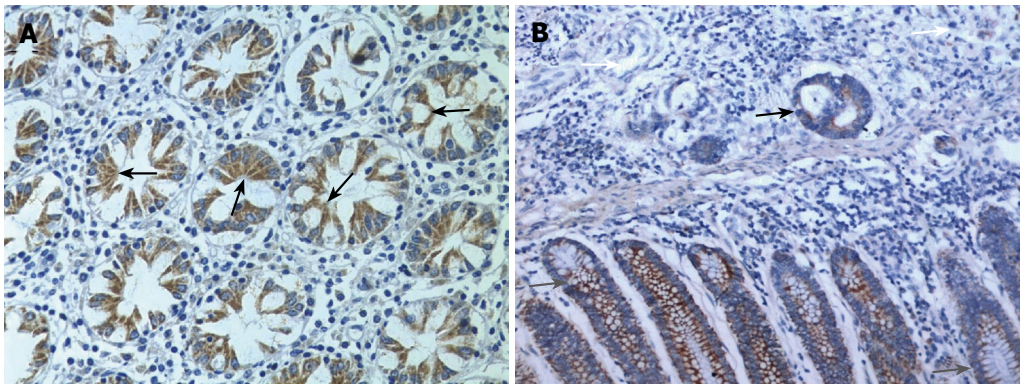

Figure 1 Expression of endocan in normal mucous membrane and cancer tissues of colon and rectum tissues. **A**: Endocan was expressed in the cytoplasm of the epithelial cell and it had a high polarity under the nucleus; **B**: The expression of endocan in colon and rectum tissues. This section show that it had a high expression in mucous membrane by the side of carcinoma tissue (gray arrow), also in well and moderately-differentiated colon carcinoma tissues (black arrow), but a weak expression in poorly differentiated carcinoma tissues (white arrow).

Table 1 Differential expression of *endocan* mRNA and protein in normal and colon cancer tissues

Type	*n*	Expression of *endocan* mRNA		Positive (%)	*P*
		+	-		
Normal mucous membrane	27	25	2	92.6	0.001
Colon carcinoma tissue	72	24	48	33.3	(χ^2 = 25.266)

Table 2 Differential expression of endocan protein in normal and colorectal cancer tissues

Type	*n*	Expression of endocan protein		Positive (%)	*P*
		+	-		
Normal mucous membrane	27	19	8	70.4	0.005
Colon carcinoma tissue	72	26	46	36.1	(χ^2 = 7.965)

loading buffer (125 mmol/L Tris-HCl, pH 6.8, 10% β-mercapto-ethanol, 4.6% SDS, 20% glycerol and 0.003% bromophenol blue) for 5 min at 100℃, separated by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and electroblotted to PVDF membrane (BioRad). After non-specific binding sites were blocked for 1 h with 5% nonfat milk in TPBS (PBS contained 0.05% Tween 20), the membrane was incubated overnight at 4℃ with primary antibody. After washing 3 times in TPBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 2 h at room temperature, and washed twice with TPBS. Immunoblot was detected by autoradiography using an enhanced chemoluminescence detection kit.

Statistical analysis

Chi-square test and *F*-test were used to compare the categorical data. SPSS 11.0 was used to analyze the data.

RESULTS

Expression of endocan in colon mucous membrane and colorectal cancer specimens

In situ hybridization analysis showed that endocan mRNA was expressed in the cytoplasm of epithelial cells in mucous membrane of colon and rectum and in well- and moderately-differentiated colorectal cancer. However, endocan mRNA expression was down-regulated in poorly-differentiated colorectal cancer (Figures 1 and 2).

Meanwhile, we performed immunohistochemical staining for endocan protein with a monoclonal antibody against human endocan. The endocan protein expression was concordantly regulated by mRNA.

The statistical results demonstrated that endocan was differentially expressed in normal colon mucosa and carcinoma tissue samples. The expression rate of endocan was 92.6% (25/27) in normal colon mucosa tissue samples and 36.1% (24/72) in colorectal cancer tissue samples, and was significantly lower in cancerous tissue samples than in normal tissue samples (P = 0.001, Table 1). Endocan protein was identically expressed as mRNA; The expression rate was 70.4% (19/27) in normal colon and rectum mucosa tissue samples and 36.1% (26/72) in colorectal cancer tissue samples. Endocan was also differently expressed in normal and colorectal cancer tissue samples (P = 0.005, Table 2).

Correlation between expression of endocan and differentiation of colorectal cancer

The expression of mRNA and protein in colorectal cancer tissue samples was not correlated with age, gender, clinical stage, tumor size or lymph node metastasis, but positively correlated with the differentiation of tumors (Table 3).

RT-PCR and Western blot were performed to further observe the relationship between the expression levels of endocan and differentiation of colorectal cancer (Figure 3). Both endocan transcript and translation were detected in colon mucous membrane and in well- and moderately-differentiated colon carcinoma, but scarely detected in poorly-differentiated carcinoma.

DISCUSSION

Endocan was originally cloned from a human endothelial

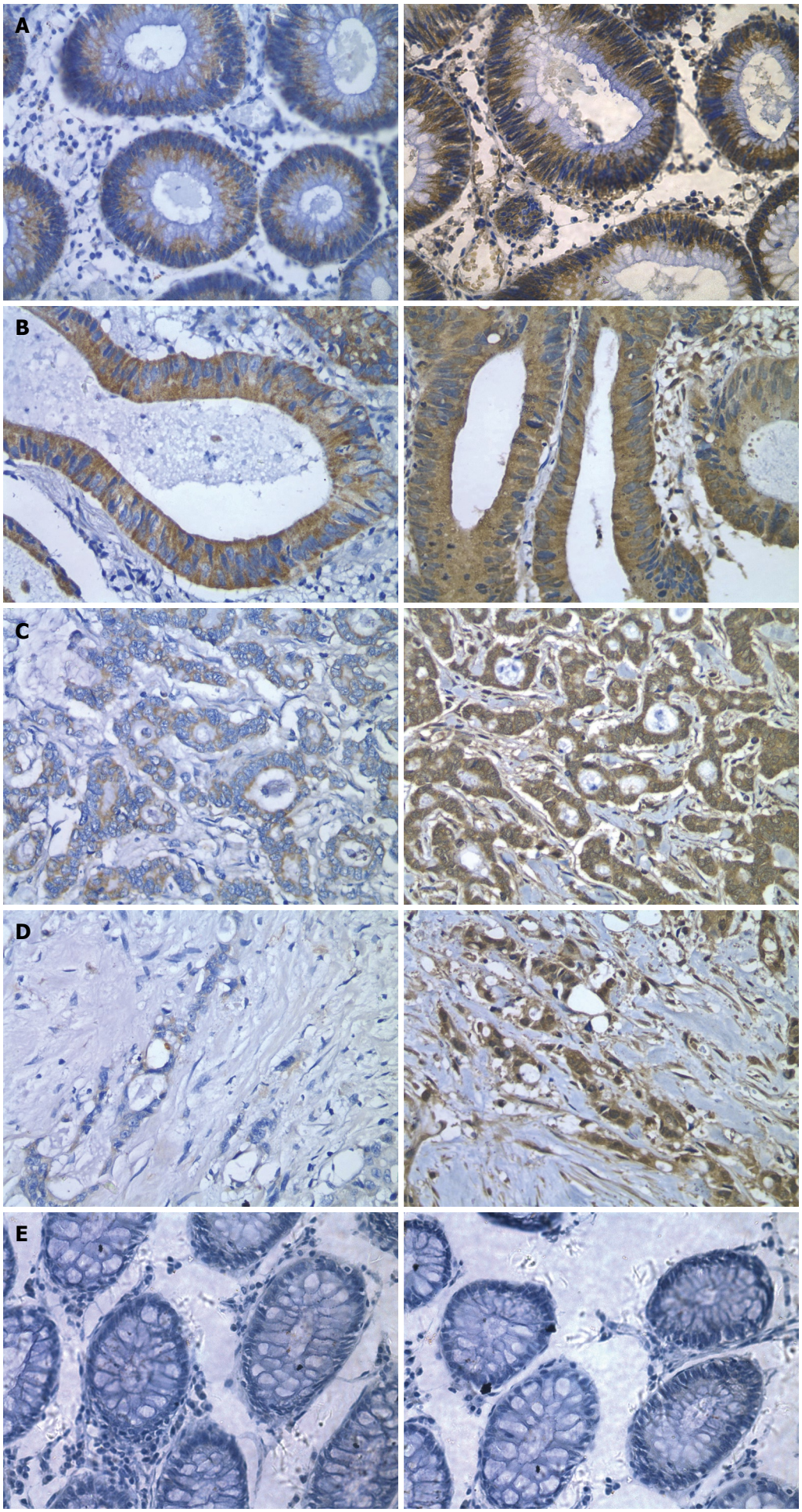

Figure 2 The expression of endocan in normal mucous membrane and different differentiation colon carcinoma tissues. It had a high expression in well and moderately-differentiated colon carcinoma tissues, but a weak expression in poorly differentiated carcinoma tissues. **A**: The expression of endocan in normal mucous membrane; **B**: The expression of endocan in well-differentiation colon carcinoma tissue; **C**: The expression of endocan in well-differentiation colon carcinoma tissue; **D**: The expression of endocan in poorly differentiated colon carcinoma tissue; **E**: Negative control. Left: *in situ* hybridization; Right: immunohistochemistry.

Table 3 Correlation of *endocan* mRNA and protein expression with clinical pathological parameters

Group		*n*	*Endocan* mRNA				Endocan protein			
			+	-	Positive (%)	*P*	+	-	Positive (%)	*P*
Age	≤ 54	34	10	24	29.4	0.676 (χ^2 = 0.174)	11	23	32.4	0.702 (χ^2 = 0.146)
	> 54	38	14	24	36.8		15	23	39.5	
Sex	Male	23	9	14	39.1	0.919 (χ^2 = 0.01)	10	13	43.5	0.530 (χ^2 = 0.395)
	Female	49	17	32	34.7		16	33	32.7	
Size	≤ 3	11	3	8	27.3	0.643[a]	5	6	45.5	0.483[a]
	> 3	61	21	40	34.4		21	40	34.4	
Infiltration	Full-thickness	64	21	43	32.8	0.791[a]	21	43	32.8	0.099[a]
	Non-full-thickness	8	3	5	37.5		5	3	62.5	
Metastasis	Nonmetastatic tumor	27	8	19	29.6	0.796 (χ^2 = 0.067)	9	18	33.3	0.899[a]
	Metastatic tumor	45	16	29	35.6		17	28	37.8	
Grade	Differentiated (well + moderately)	57	23	34	40.4	0.031 (χ^2 = 4.642)	26	31	45.6	0.003 (χ^2 = 8.824)
	Poorly differentiated	15	1	14	6.7		1	14	6.7	
TNM stage	Ⅰ and Ⅱ	43	18	25	41.9	0.324 (χ^2 = 0.973)	18	25	41.9	0.324 (χ^2 = 0.973)
	Ⅲ and Ⅳ	29	8	21	27.6		8	21	27.6	

[a]$P < 0.05$ *vs* controls.

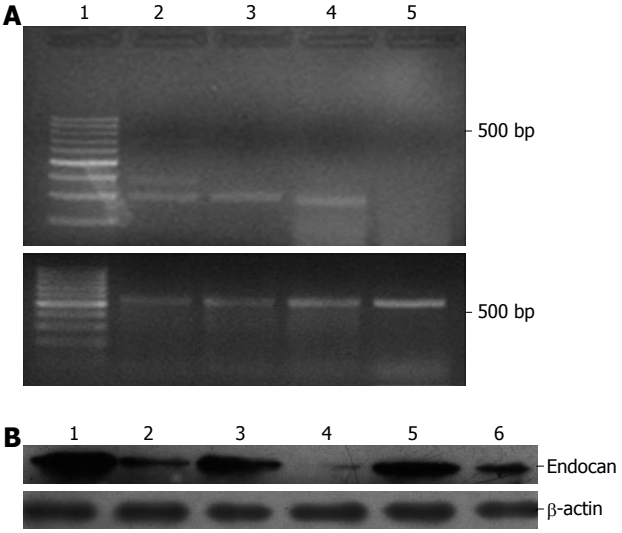

Figure 3 The expression of endocan in colon and rectum tissues. **A**: RT-PCR analysis of *endocan* mRNA in colon and rectum tissues. *Endocan* mRNA was highly expressed in normal colon and rectum tissue and well and mid-differentiated colorectal cancer tissues, but was down regulated in poorly differentiated colorectal cancer tissues. Endocan expression (up) and β-actin expression (down). 1: 100 bp Marker; 2: Normal colon and rectum mucous membrane; 3: Well-differentiated differentiated colorectal cancer tissue; 4: Moderately colorectal cancer tissue; 5: Poorly differentiated colorectal cancer tissue. **B**: Expression of endocan by Western blot. Endocan was detected at high expression levels in normal colorectal and well-differentiated tissues; Moreover, there was a down regulation in the poorly differentiated colorectal cancer tissues. Lane 1, 3, 5: Normal mocusa, well-differentiated tissues; lane 2, 4, 6: Poorly-differentiated tissues.

cell cDNA library by Lassalle and collaborators in 1996[3]. This molecule is the product of a single gene, localized on human chromosome 5 at the position 5q11.2, that is organized into 3 exons separated by 2 introns. It encodes for a soluble proteoglycan of 50 kDa containing a mature polypeptide of 165 amino acids and a single dermatan sulphate chain, covalently linked to the serine residue at position 137[9].

Endocan, as a proteoglycan, plays an important role in several pathophysiological processes including inflammatory disorders[10-15] and tumor progression, and in the control of fundamental cellular processes, such as adhesion[16], migration and angiogenesis. Inflammatory cytokines, such as TNF-α and LPS[17], and pro-angiogenic growth factors, such as VEGF[18], HGF/SF[19-22] and FGF-2[23,24], strongly stimulate the expression and secretion of endocan in human endothelial cells.

Endocan has been identified as a potential novel endothelial cell marker and a new target for cancer therapy. It was reported that high endocan mRNA levels correlate with a poor prognosis and metastasis of several types of cancer, including breast, renal and lung cancer[1,25,26]. A study of 78 breast cancer patients, with the aim to define the optimal prognosis classifier, was performed on 70 genes according to standard prognostic criteria, showing that endocan over expression in breast cancer is associated with a higher risk of metastasis and death within 5 years[27]. Furthermore, 1234 genes that have been identified are differentially expressed in renal cell carcinoma, and endocan mRNA level is 3-fold higher in renal cell carcinoma samples than in normal tissue samples[28]. This up-regulation of endocan expression also correlates with increased tumor vasculature and inflammation in renal cancer, which is actually the ninth most common malignancy in Western countries, and no effective treatment is available for it. Similarly, a recent extensive hybridization study showed that the endocan gene is one of the most highlighted genes, with at least a 2-fold increase in all the 8 renal cell carcinoma samples analyzed, compared to normal tissue samples[29]. Interestingly, a parallel up-regulation was also revealed for VEGF and c-Met proto-oncogene receptor for HGF/SF, both of which are heavily implicated in angiogenesis. A comparable study, by dot blotting and hybridization showed that endocan is dramatically up-regulated in several (5/14) renal cell carcinoma biopsies, and is correlated with both VEGF and VEGF receptor gene expressions[30]. A gene profiling study of tissues from 23 lung cancer patients demonstrated that endocan is one of the significant poor prognosis classifiers among the 42

genes associated with a high risk of cancer-related death.

Endocan was less reported in colon and rectum tissues. Moreover, little is known about its molecular mechanism. We mapped the regulation of endocan expression in normal membrane mucosa and colorectal cancer tissue samples. Our results reveal that endocan was significantly expressed at transcriptional and translational level in normal colorectal mucous membrane and in well- and moderately-differentiated colorectal cancer, but weakly expressed in poorly-differentiated colorectal cancer. Meanwhile, RT-PCR and Western blot also showed that the expression of endocan was upregulated in normal colon and rectum tissue samples, and down-regulated in poorly-differentiated colorectal cancer tissue samples.

All these data show that endocan is differently expressed in colon and rectum tissue and other tissues. According to the previous results, endocan is almost not expressed in normal human tissue except in lung tissue. Our study showed that endocan was also expressed in normal colon and rectum tissue, but its expression was down-regulated in colorectal cancer, suggesting that regulation may be complex in colon and rectum. As we know, there are a lot of germs in human colon. Most of the outer germs are killed by gastric acid when they get into the stomach through the mouth. In the upper part of the small intestine, the number of germs is also small. However, this number increases gradually at the end of the ileum and reaches its maximum in the colon, where the contents is neutral or alkaline and movement is slower, thus making the germs propagate at a fast pace. There are 10^9-10^{11} germs per gram of colon contents. However, these germs can decompose protein, which is called degradation. In this process, the germs also produce some virulent substances, amino acids, peptide, amine, and hydrogen sulphide and proper indole, all of which can activate macrophages and monocytes to secrete a large number of cell factors, such as IL-1 and TNF-α, which can stimulate expression of endocan. That is why we can detect a high expression of endocan in normal colon and rectum tissue. However, the expression of endocan was down-regulated in poorly-differentiated colorectal cancer, suggesting that endocan may be closely related with differentiation and development of colorectal cancer.

COMMENTS

Background

Endocan plays a key role in the regulation of certain processes, such as cell adhesion, inflammatory disorders and tumor progression and correlates with poor prognosis and metastasis in several types of cancer, including breast, renal and lung cancer, indicating that endocan may also play a role in the pathology of cancer cells and/or may be a tumor marker. However, few studies are available on endocan expression in colorectal tissue. This study was to map endocan expression in colorectal tissue and analyze the relationship between endocan expression and tumor differentiation.

Research frontiers

Colorectal cancer accounted for about 1 million new cases in 2002 and its incidence increases. The results of this study indicate that endocan may be used as a special molecule in the early diagnosis and treatment of colorectal cancer.

Innovations and breakthroughs

The results of this study show that endocan expression plays a role in the pathogenesis of colorectal cancer.

Applications

The expression level of endocan plays an important role in the pathogenesis of colorectal cancer. Endocan may be used in the treatment of colorectal cancer in clinical practice.

Peer review

The authors showed that the expression level of endocan was lower in colon cancer tissue than in normal colon tissue. The study was well-designed and the data are original and informative.

REFERENCES

1 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74-108

2 **Parkin DM**, Bray FI, Devesa SS. Cancer burden in the year 2000. The global picture. *Eur J Cancer* 2001; **37** Suppl 8: S4-S66

3 **Lassalle P**, Molet S, Janin A, Heyden JV, Tavernier J, Fiers W, Devos R, Tonnel AB. ESM-1 is a novel human endothelial cell-specific molecule expressed in lung and regulated by cytokines. *J Biol Chem* 1996; **271**: 20458-20464

4 **Sobin LH**, Fleming ID. TNM Classification of Malignant Tumors, fifth edition (1997). Union Internationale Contre le Cancer and the American Joint Committee on Cancer. *Cancer* 1997; **80**: 1803-1804

5 **Mostofi FK**. International histologic classification of tumors. A report by the Executive Committee of the International Council of Societies of Pathology. *Cancer* 1974; **33**: 1480-1484

6 **Tang KF**, Pantoja CR, Redman RM, Lightner DV. Development of in situ hybridization and RT-PCR assay for the detection of a nodavirus (PvNV) that causes muscle necrosis in Penaeus vannamei. *Dis Aquat Organ* 2007; **75**: 183-190

7 **Ikeda K**, Monden T, Kanoh T, Tsujie M, Izawa H, Haba A, Ohnishi T, Sekimoto M, Tomita N, Shiozaki H, Monden M. Extraction and analysis of diagnostically useful proteins from formalin-fixed, paraffin-embedded tissue sections. *J Histochem Cytochem* 1998; **46**: 397-403

8 **Chung JY**, Braunschweig T, Baibakov G, Galperin M, Ramesh A, Skacel M, Gannot G, Knezevic V, Hewitt SM. Transfer and multiplex immunoblotting of a paraffin embedded tissue. *Proteomics* 2006; **6**: 767-774

9 **Bechard D**, Gentina T, Delehedde M, Scherpereel A, Lyon M, Aumercier M, Vazeux R, Richet C, Degand P, Jude B, Janin A, Fernig DG, Tonnel AB, Lassalle P. Endocan is a novel chondroitin sulfate/dermatan sulfate proteoglycan that promotes hepatocyte growth factor/scatter factor mitogenic activity. *J Biol Chem* 2001; **276**: 48341-48349

10 **Iozzo RV**, San Antonio JD. Heparan sulfate proteoglycans: heavy hitters in the angiogenesis arena. *J Clin Invest* 2001; **108**: 349-355

11 **Timar J**, Lapis K, Dudas J, Sebestyen A, Kopper L, Kovalszky I. Proteoglycans and tumor progression: Janus-faced molecules with contradictory functions in cancer. *Semin Cancer Biol* 2002; **12**: 173-186

12 **Sanderson RD**, Yang Y, Suva LJ, Kelly T. Heparan sulfate proteoglycans and heparanase--partners in osteolytic tumor growth and metastasis. *Matrix Biol* 2004; **23**: 341-352

13 **Reed CC**, Waterhouse A, Kirby S, Kay P, Owens RT, McQuillan DJ, Iozzo RV. Decorin prevents metastatic spreading of breast cancer. *Oncogene* 2005; **24**: 1104-1110

14 **Grant DS**, Yenisey C, Rose RW, Tootell M, Santra M, Iozzo RV. Decorin suppresses tumor cell-mediated angiogenesis. *Oncogene* 2002; **21**: 4765-4777

15 **Xiong JX**, Wu ZS, Wu Q, Zhou Q, Gui SY and Wang Y. Expression of endothelial cell specific molecular-1 in renal

cell carcinoma. *Bulletin of Chinese Cancer* 2004; **13**: 309-312
16 **Scherpereel A**, Depontieu F, Grigoriu B, Cavestri B, Tsicopoulos A, Gentina T, Jourdain M, Pugin J, Tonnel AB, Lassalle P. Endocan, a new endothelial marker in human sepsis. *Crit Care Med* 2006; **34**: 532-537
17 **Bu LJ**, Zuo L, Ren CL, Zhang SM, Zhou Q, Gui SY, Wang Y. LPS effects on the growth of human umbilical vein endothelial cells and expression of ESM-1. *Acta Universitatis Medicinalis Anhui* 2005; **40**: 7-9
18 **Abid MR**, Yi X, Yano K, Shih SC, Aird WC. Vascular endocan is preferentially expressed in tumor endothelium. *Microvasc Res* 2006; **72**: 136-145
19 **Sarrazin S**, Adam E, Lyon M, Depontieu F, Motte V, Landolfi C, Lortat-Jacob H, Bechard D, Lassalle P, Delehedde M. Endocan or endothelial cell specific molecule-1 (ESM-1): a potential novel endothelial cell marker and a new target for cancer therapy. *Biochim Biophys Acta* 2006; **1765**: 25-37
20 **Seidel C**, Borset M, Hjertner O, Cao D, Abildgaard N, Hjorth-Hansen H, Sanderson RD, Waage A, Sundan A. High levels of soluble syndecan-1 in myeloma-derived bone marrow: modulation of hepatocyte growth factor activity. *Blood* 2000; **96**: 3139-3146
21 **Delehedde M**, Sergeant N, Lyon M, Rudland PS, Fernig DG. Hepatocyte growth factor/scatter factor stimulates migration of rat mammary fibroblasts through both mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways. *Eur J Biochem* 2001; **268**: 4423-4429
22 **Bhowmick NA**, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature* 2004; **432**: 332-337
23 **Aitkenhead M**, Wang SJ, Nakatsu MN, Mestas J, Heard C, Hughes CC. Identification of endothelial cell genes expressed in an in vitro model of angiogenesis: induction of ESM-1, (beta)ig-h3, and NrCAM. *Microvasc Res* 2002; **63**: 159-171
24 **Penc SF**, Pomahac B, Winkler T, Dorschner RA, Eriksson E, Herndon M, Gallo RL. Dermatan sulfate released after injury is a potent promoter of fibroblast growth factor-2 function. *J Biol Chem* 1998; **273**: 28116-28121
25 **Xiong JX**, Wu Q, Zhou Q, Wu ZS, Gui SY , Wang Y. Expression and relation between endothelial cell specific molecule-1 mRNA and protein in renal cell carcinoma. *J Clin Exp Pathol* 2004; **20**: 187-189
26 **Perey L**, Benhattar J, Peters R, Jaunin P, Leyvraz S. High tumour contamination of leukaphereses in patients with small cell carcinoma of the lung: a comparison of immunocytochemistry and RT-PCR. *Br J Cancer* 2001; **85**: 1713-1721
27 **van't Veer LJ**, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002; **415**: 530-536
28 **Lenburg ME**, Liou LS, Gerry NP, Frampton GM, Cohen HT, Christman MF. Previously unidentified changes in renal cell carcinoma gene expression identified by parametric analysis of microarray data. *BMC Cancer* 2003; **3**: 31
29 **Amatschek S**, Koenig U, Auer H, Steinlein P, Pacher M, Gruenfelder A, Dekan G, Vogl S, Kubista E, Heider KH, Stratowa C, Schreiber M, Sommergruber W. Tissue-wide expression profiling using cDNA subtraction and microarrays to identify tumor-specific genes. *Cancer Res* 2004; **64**: 844-856
30 **Borczuk AC**, Shah L, Pearson GD, Walter KL, Wang L, Austin JH, Friedman RA, Powell CA. Molecular signatures in biopsy specimens of lung cancer. *Am J Respir Crit Care Med* 2004; **170**: 167-174
31 **Harris M**. Monoclonal antibodies as therapeutic agents for cancer. *Lancet Oncol* 2004; **5**: 292-302

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RAPID COMMUNICATION

Diagnosis and treatment of spontaneous colonic perforation: Analysis of 10 cases

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Abstract

AIM: To investigate the etiology, diagnosis and treatment of spontaneous perforation of the colon.

METHODS: The clinical data of 10 cases of spontaneous perforation of the colon, observed at Fuding hospital from January 2004 to December 2007, were analyzed retrospectively.

RESULTS: The mean age at onset was 65 years (range from 45 to 73). Seven patients had a history of chronic constipation. All patients complained of sudden lower abdominal pain. The perforation occurred after coloclysis and administration of senna leaves in two patients. Nine patients had signs of peritoneal irritation. Seven cases underwent abdominal paracentesis, which was diagnostic in six. Only one case was definitely diagnosed prior to surgery. One patient underwent neoplasty of the colon, another a partial resection of colon, six a neoplasty of the colon plus sigmoid colostomy, and two underwent Hartmann surgery. All perforation sites were opposite to the mesenteric edge. The perforation sites were located on descending colon in one case, sigmoid colon in three cases, and rectosigmoid colon in six cases. In five patients, surgical pathological examination was consistent with the microscopical changes of colonic perforation caused by feces. Three patients died after surgery.

CONCLUSION: Spontaneous perforation of the colon most commonly occurs among the elderly with chronic constipation. Abdominal paracentesis is helpful for the diagnosis. The perforation site is located opposite to the mesenteric edge. Sigmoid colon and rectosigmoid colon are the most frequent locations. Neoplasty of the colon and sigmoid colostomy are the most frequent treatment. The prognosis is bad and the mortality rate after surgery is high.

Key words: Spontaneous; Perforation; Colon; Treatment; Surgery

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Yang B, Ni HK. Diagnosis and treatment of spontaneous colonic perforation: Analysis of 10 cases. *World J Gastroenterol* 2008; 14(28): 4569-4572 Available from: URL: http://www.wjgnet.com/1007-9327/14/4569.asp DOI: http://dx.doi.org/10.3748/wjg.14.4569

INTRODUCTION

Spontaneous perforation of the colon is defined as a sudden perforation of the normal colon in the absence of diseases such as tumors, diverticulosis or external injury[1]. It is rare, often misdiagnosed and has a high mortality rate. It is seldom reported in the literature. In this study, we collected 10 cases of such patients during 2004 to 2007, and analyzed their clinical features in order to improve the understanding of this disease. The present study may be helpful for the diagnosis and treatment of spontaneous colonic perforation.

METHODS AND MATERIALS

Ten cases of spontaneous perforation of the colon were collected at Fuding hospital from January 2004 to December 2007. The clinicopathological data, including gender, age, past history, symptoms, physical examination, diagnostic assays, pathological examination and surgical information as well as outcome were analyzed retrospectively to assess the diagnosis and treatment.

RESULTS

Clinical data

There were 8 males and 2 females. The mean age at onset of the disease was 65 years (range, 45 to 73).

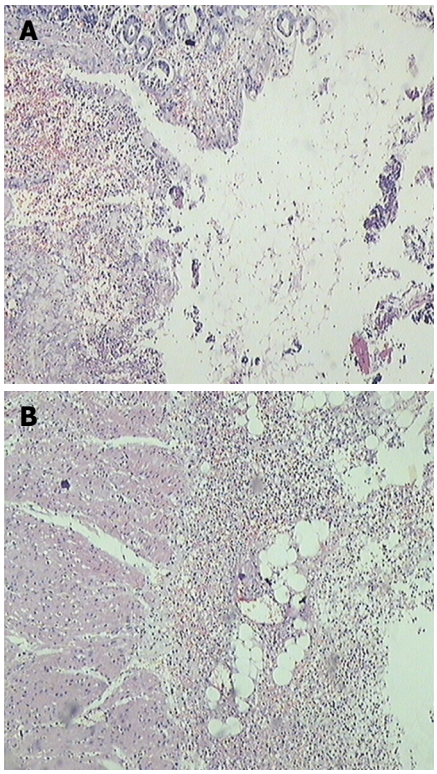

Figure 1 **A**: Inflammatory sphacelus and abundant neutrophil infiltration at the edge of the ulcer (HE, x 40); **B**: Numerous neutrophils infiltrate the muscular layer of the colon and the disrupted smooth muscle (HE, x 40).

Seven patients had a history of chronic constipation. All patients reported a sudden lower abdominal pain. Three patients had a history of oral administration of nonsteroid anti-inflammatory drugs (NSAIDs) to relieve abdominal pain. Five patients had possible correlated factors: in three, onset occurred after defecation and in the other two it did after coloclysis and administration of senna leaves. Three patients had symptoms compatible with shock prior to surgery. Physical examination showed signs of peritoneal irritation in 9 cases, with tension of all abdominal muscles, tenderness and rebound tenderness. The abdominal X-ray did not reveal any abnormal findings in 4 patients. Seven patients underwent diagnostic abdominal paracentesis. Feculent material was aspirated in six patients. Only one patient was definitely diagnosed before surgery. Five patients were misdiagnosed as having gastric or duodenal perforation, colorectal tumor in two other cases, acute gangrenous perforating appendicitis in one case and acute pancreatitis in one case.

Surgery

All patients underwent emergency surgical intervention. The time interval between onset and surgery ranged from 15 to 96 h. Seven patients underwent surgery 6 h after hospital admission. One patient underwent neoplasty of the colon, one a partial resection of the colon, six a neoplasty of the colon plus a sigmoid colostomy, and two had Hartmann surgery. All perforation sites were opposite to the mesenteric edge and were located on the descending colon in one case, the sigmoid colon in three, and the rectosigmoid colon in the remaining six.

Pathological examination

Five patients had surgical pathological examination. Macroscopic examination showed that the perforation sites were all located opposite to the mesenteric edge, had circular shape, and ranged from 2 to 3 cm in diameter. Microscopic examination showed necrosis of the whole wall of the colon. The perforation site was characterized at its surface by inflammatory sphacelus. Granulation tissue grew from the bottom. Submucosal edema, diffuse hemorrhage and abundant neutrophil infiltration were found in the surrounding colonic mucosa, consistent with the microscopical changes typical of colonic perforation caused by feces (Figure 1).

Prognosis

Three patients died after surgery because of multiple organ failure caused by septic shock. The other seven patients survived and six patients who received neoplasty and sigmoid colostomy underwent another surgical intervention to close the stoma 3 months thereafter.

DISCUSSION

Cause of spontaneous colonic perforation

The cause of spontaneous colonic perforation is usually unclear. In general, colonic perforation caused by feces is the most frequent occurrence. The disease has often been seen in patients with chronic constipation. In these cases, the solid feculent mass compresses the colonic wall, diminishes the blood supply and leads to ischemia and necrosis of colonic mucosa, which forms marked feculent ulcer changes. The ulcer might lead to colonic rupture in some cases[2-5]. Maurer *et al*[3] have proposed the diagnostic criteria of feculent colonic perforation: (1) Rounded shape, more than 1 cm in diameter; (2) The colon is full of stool, which diffuses to the abdominal cavity through the perforation; (3) Ischemia and necrosis of colonic mucosa leading to feculent ulcer and acute inflammatory reaction surrounding the perforation site can be seen at microscopical examination; (4) External injury or other diseases such as obstruction, tumors and diverticulosis must be excluded. All five cases with pathological examination were consistent with the above criteria. Maurer *et al*[3] also proposed that the feculent ulcer may present at multiple sites. The proportion of cases with multiple ulcers in the same colonic segment is about 28%.

Another cause is idiopathic colonic perforation. The pressure within the colonic lumen increases and distributes asymmetrically, leading to an excess pressure increase at the level of the angle[6]. The colonic wall is hyperdilated, becomes excessively thin and the perforation occurs. Compared to feculent perforation, idiopathic colonic perforation has the following features: (1) The perforation is linear; (2) Feculent ulcer cannot be seen at microscopic examination. The mucosal edge is clear and does not extend to the serosa. The broken

ends of the muscular layer are regular[7]. Although these two conditions are different both macroscopically and microscopically, they are occasionally difficult to distinguish at surgery. Surgical pathological examination is necessary to make a definite diagnosis[7].

The most frequent sites of spontaneous colonic perforation

The most frequent location is opposite to the mesenteric edge of the sigmoid colon and recto-sigmoid colon. Maurer *et al*[3] reported that 52 out of 81 cases (64%) of feculent perforation occurred at the above sites. In the study of Kasahara *et al*, 68% (44/65) of idiopathic colonic perforation were located at those sites[7]. In the present study, 9 patients had the same characteristics. This phenomenon may be due to the special physiological and anatomical features of sigmoid colon. There is no ramus anastomoticus between the lowest branch of sigmoid arteries and the superior rectal artery, which causes a physiological ischemia. When some stiff stool goes through sigmoid colon, the colonic wall is compressed and leads to the hindrance of blood supply. The blood supply to the opposite side of the mesentery is poor. The stool is more likely to stay in the rectosigmoid colon because of the confined colonic cavity. The smooth muscle contracts, which leads to the increase of the pressure of colonic cavity[8-12].

This disease is more frequent in the elderly and the mean age at onset is more than 60. About 61% to 81% of patients had constipation history[2,4]. It is often misdiagnosed because doctors are unaware about this disease. Only 10% of patients are definitely diagnosed prior to surgery[6]. In the present study, only one patient (1/10) was definitely diagnosed before surgery. It is very important to increase the awareness about this disease in order to improve the accuracy of diagnosis. We think that the possibility of this disease should be taken into consideration in elderly patients who have chronic constipation, when they have a history of induction of increased intra-abdominal pressure, present with sudden abdominal pain spreading to the whole abdomen and have peritoneal irritation signs[13,14]. In this study, the abdominal paracentesis was diagnostic in 6 out of 7 patients. Therefore, abdominal paracentesis is a valuable tool for the diagnosis of patients with this complication[15].

Surgical treatment of spontaneous colonic perforation

The mortality rate of this disease is as high as 35% to 47%[2,7]. In case of perforation, innumerable bacteria spread from the colon into the abdominal cavity and cause acute diffuse peritonitis. Bacterial toxins are absorbed and lead to infectious shock and then multiple organ failure. So, patients should undergo surgery as soon as the disease is definitely diagnosed[16-20]. The types of surgery are different depending on the time of onset, degree of peritonitis, general physical condition and lesion of the colon. The following types of surgery are common: neoplasty, colostomy, neoplasty plus proximal colostomy, Hartmann surgery[21-23]. Neoplasty plus proximal colostomy is the most popular since it is safe and time-sparing. Six patients underwent neoplasty plus proximal colostomy in the present study. Serpell *et al*[2] found that the mortality and complication rates after Hartmann surgery were lower than in case of other operations because Hartmann surgery dissects the affected colon. Maurer *et al*[3] proposed that feculent ulcer had multiple origins and, therefore, other segments of the colon should be explored during the operation. If the colonic wall is dilated or thinner, it should be resected. Subtotal colectomy may be essential for some cases, which can spare time-consuming coloclysis during the operation and avoid possible later re-perforation of the affected colon[24,25].

Spontaneous colonic perforation is noteworthy due to its high mortality rate. The possibility of this disease should be taken into consideration in elderly patients having chronic constipation and bed-ridden for long periods of time. Doctors should be careful when administering enema and cathartics.

COMMENTS

Background

Spontaneous perforation of the colon most commonly occurs in the elderly, is usually misdiagnosed before surgery and leads to a high mortality rate after surgery. Early correct diagnosis, early surgery and appropriate surgical treatment options are the key to improve the prognosis. Effective measures should be carried out to prevent this disease in high-risk patients. This study may help the surgeon to recognize this rare entity.

Research frontiers

This article focuses on a rare disease, i.e. acute abdomen due to spontaneous perforation of the colon. It is helpful to increase awareness about spontaneous perforation of the colon.

Innovations and breakthroughs

It is very important to increase the knowledge about this disease in order to improve the diagnostic accuracy. The authors think that the diagnosis of this disease should be considered in elderly patients with chronic constipation, who induce an increase of the intra-abdominal pressure, present with sudden abdominal pain spreading to the whole abdomen and have signs of peritoneal irritation. In this study, the results of abdominal paracentesis were positive for 6 out of 7 patients. Therefore, abdominal paracentesis is valuable for the diagnosis of patients with this complication.

Applications

The spontaneous colonic perforation should be correctly diagnosed, due to its high mortality rate. Doctors should be careful when administering enema and cathartics.

Peer review

This is an interesting series of patients with a rare condition. The authors performed a single study center report of 10 patients. They analyzed the clinicopathological data retrospectively and assessed the diagnostic procedures and the treatment. In addition, they point out to surgeons the importance of recognizing this rare cause of acute abdomen with a high mortality rate.

REFERENCES

1 **Zhang MJ**, Wu JB. Treatment of spontaneous perforation of the large intestine: a report of 9 cases. *Zhongguo Putong Waike Zazhi* 2002; **32**: 836-839
2 **Serpell JW**, Nicholls RJ. Stercoral perforation of the colon. *Br J Surg* 1990; **77**: 1325-1329
3 **Maurer CA**, Renzulli P, Mazzucchelli L, Egger B, Seiler CA, Buchler MW. Use of accurate diagnostic criteria may increase incidence of stercoral perforation of the colon. *Dis Colon Rectum* 2000; **43**: 991-998

4 **Haddad R**, Bursle G, Piper B. Stercoral perforation of the sigmoid colon. *ANZ J Surg* 2005; **75**: 244-246
5 **Tokunaga Y**, Hata K, Nishitai R, Kaganoi J, Nanbu H, Ohsumi K. Spontaneous perforation of the rectum with possible stercoral etiology: report of a case and review of the literature. *Surg Today* 1998; **28**: 937-939
6 **Bin Xu**, Shao WD, Shen HW, Wang QB. Emergence treatment of idiopathic rupture of sigmoid colon. *Zhonghua Jizhen Yixue Zazhi* 2004; **13**: 564-566
7 **Kasahara Y**, Matsumoto H, Umemura H, Shirafa S, Kuyama T. Idiopathic perforation of the sigmoid colon in Japan. *World J Surg* 1981; **5**: 125-130
8 **Huang WS**, Wang CS, Hsieh CC, Lin PY, Chin CC, Wang JY. Management of patients with stercoral perforation of the sigmoid colon: report of five cases. *World J Gastroenterol* 2006; **12**: 500-503
9 **Matsuo S**, Eguchi S, Azuma T, Kanetaka K, Itoh S, Yamaguchi S, Obata S, Kanematsu T. An unusual perforation of the colon: report of two cases. *Surg Today* 2002; **32**: 836-839
10 **Hao WX**. Analysis of the etiology and treatment for 18 cases with colonic perforation. *Henan Waikexue Zazhi* 2004; **10**: 46-47
11 **Wang ZQ**. Analysis of diagnosis and treatment of spontaneous enterorrhexis. *Xinjiang Yixue* 2006; **4**: 58-59
12 **Zhang LX**, Mao GJ, Shao AP. Diagnosis and treatment of spontaneous colonic perforation for 16 cases. *Zhonghua Weichang Waike Zazhi* 2006; **9**: 249
13 **Shao ZJ**, Zhang CD, Su ZG. Analysis on spontaneous colonic perforation for 11 patients. *Guangxi Yixue* 2006; **28**: 512
14 **Xu G**. Diagnosis and treatment for 7 old men with spontaneous colon rupture. *Zhongguo Zhongxiyi Jiehe Waike Zazhi* 2005; **11**: 528
15 **Li MJ**, Xiang JB, Chen LL. Emergency treatment for colonic perforation(29 patients reported). *Zhongguo Linchuang Yixue* 2001
16 **Li WZ**, Xiao SX, Zeng D. Surgery for 22 old men with colonic perforation. *Zhongguo Xiandai Shoushuxue Zazhi* 2004; **8**: 46-47
17 **Zhe ZF**, Sun SH. Analysis on 8 cases with spontaneous colon sigmoideum perforation. *Zhongguo Wuzhenxue Zazhi* 2004; **4**: 1124
18 **Jin MH**, Pan F. Analysis on spontaneous colon rupture (3 cases reported). *Zhongguo Yishi Jinxiu Zazhi* 2003; **11**: 350
19 **Chen HK**. Analysis on 9 patients with spontaneous colon rupture. *Hebei Yixue* 2003; **9**: 643
20 **Li XJ**, Yang CY. Spontaneous intestinal perforation for 3 patients. *Guangxi Yixue* 2002; **4**: 212
21 **Guo ZS**, Chen RZ. Treatment of 13 old men with Spontaneous colon sigmoideum. *Linchuang Yiyaoxue* 2001; **3**: 145
22 **Zheng XB**, Sun SH. Analysis on 15 patients with spontaneous colon sigmoideum. *Zunyi Yixueyuan Xuebao* 2001; **3**: 243
23 **Xu M**. Clinical manifestation and etiology analysis for 6 cases with spontaneous colon sigmoideum. *Hebei Yixue* 2003; **7**: 431
24 **Yi HY**, Wang L, Lin Y, Che MZ, Zhang ZS. Aggregate analysis on 103 cases with spontaneous intestinal perforation. *Zhongguo Zhongxiyi Jiehe Waike Zazhi* 2004; **6**: 367
25 **Wang FL**, Gao LF, Feng JX. Acute peritonitis induced by intestinal perforation for 30 cases. *Heilongjiang Yixue* 2000; **4**: 376

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CASE REPORT

Anabolic steroid abuse causing recurrent hepatic adenomas and hemorrhage

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Abstract

Anabolic steroid abuse is common among athletes and is associated with a number of medical complications. We describe a case of a 27-year-old male bodybuilder with multiple hepatic adenomas induced by anabolic steroids. He initially presented with tumor hemorrhage and was treated with left lateral hepatic segmentectomy. Regression of the remaining tumors was observed with cessation of steroid use. However, 3 years and a half after his initial hepatic segmentectomy, he presented with recurrent tumor enlargement and intraperitoneal hemorrhage in the setting of steroid abuse relapse. Given his limited hepatic reserve, he was conservatively managed with embolization of the right accessory hepatic artery. This is the first reported case of hepatic adenoma regrowth with recidivistic steroid abuse, complicated by life-threatening hemorrhage. While athletes and bodybuilders are often aware of the legal and social ramifications of steroid abuse, they should continue to be counseled about its serious medical risks.

Key words: Anabolic steroids; Adenoma; Liver; Hemorrhage

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INTRODUCTION

Anabolic steroid abuse is common among athletes and is associated with a number of medical complications[1-3]. Reported hepatic complications include cholestasis, elevation of aminotransferases, jaundice, benign hepatic adenomas, and rare cases of hepatocellular carcinoma[4-6]. Histologic findings include peliosis hepatis, a lesion characterized by hepatic sinusoidal dilatation that is often cystic[7,8]. Rupture of these cysts can cause fatal internal hemorrhage[9]. We report the first case of adenoma regrowth and hemorrhage after relapse of androgen abuse.

CASE REPORT

A 27-year-old man with a 5-year history of anabolic steroid abuse presented to the emergency room with 2 d of midepigastric pain and nausea. His only medications were oral androstenedione and intramuscular nandrolone. He was a police officer and competitive bodybuilder. He denied use of alcohol, tobacco, and intravenous drugs. Physical examination disclosed midepigastric tenderness and tender hepatomegaly. Laboratories were notable for 2.2 mg/dL total bilirubin, 1.3 mg/dL direct bilirubin, 2457 U/L ALT, 431 U/L AST, and 275 U/L alkaline phosphatase. Hematocrit was 50.5%. Abdominal computed tomography (CT) on admission showed a round, heterogeneous-appearing 9.9 cm × 9.6 cm mass in the left lobe of the liver. Magnetic resonance imaging (MRI) with gadolinium contrast demonstrated multiple hepatic masses, the largest of which measured 10.6 cm × 10.6 cm. The largest mass had an enhancing capsule and demonstrated signal heterogeneity, characteristic of an adenoma with intralesional hemorrhage (Figure 1A). The patient underwent left lateral hepatic segmentectomy with open cholecystectomy. Pathologic examination revealed an adenoma with peliosis hepatis, 25 cm in diameter. The patient was instructed to discontinue steroid use. On MRI 3 mo later, the adenomas appeared 40% smaller (Figure 1B). The patient subsequently

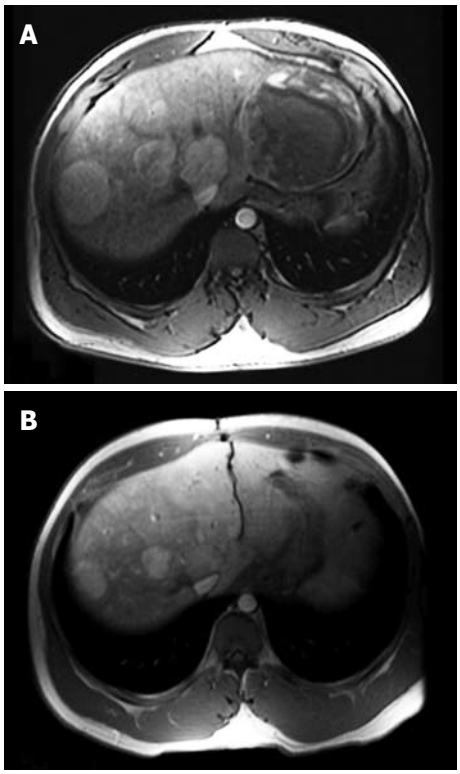

Figure 1 T1-weighted magnetic resonance imaging (MRI) of multiple hepatic adenomas. **A**: MRI at initial presentation demonstrates a heterogeneous-appearing, well-circumscribed mass measuring 10.6 cm x 10.6 cm in segments 2 and 3 of the liver and several smaller masses in the right liver. The largest mass has an enhancing capsule and demonstrates areas of internal T1 hyperintensity and hypointensity, as well as T2 hyperintensity, characteristic of an adenoma with intralesional hemorrhage; **B**: Three months after left lateral segmentectomy and steroid cessation, the lesions in the right liver appear approximately 40% smaller and enhance more homogeneously on T1-weighted MRI, indicating regression. Images have been electronically adjusted to illustrate lesions.

resumed oral androstenedione only.

Approximately 3 years and a half after his first presentation, the patient returned to the emergency room with sudden-onset, right-upper quadrant pain in the setting of recurrent injection nandrolone use 6 wk earlier. Vital signs were within normal limits, and there was tender hepatomegaly. Laboratories were notable for ALT 625 U/L, AST 398 U/L, and normal alkaline phosphatase and total bilirubin. Prothrombin time (PT) was 13.1 s; international normalized ratio (INR) was 1.1. The hematocrit was 38.7%. Abdominal CT revealed several lesions in the right lobe of the liver, the largest of which had increased in size to 7.7 cm × 7.2 cm and demonstrated intralesional hemorrhage, accompanied by a subcapsular hematoma and intraperitoneal hemorrhage. CT angiogram on hospital day 2 showed no contrast extravasation, but the hematocrit dropped to 24.9%. On hospital day 3, the right upper quadrant pain worsened, and he became tachycardic. Repeat abdominal CT showed expansion of the hematoma, with new anterior subcapsular and subphrenic components (Figure 2). The hematocrit was 24.4%. Because of his limited hepatic reserve and ongoing steroid abuse, he was felt to be a poor candidate for either hepatic resection or liver transplantation. He, therefore, underwent angiographic embolization of the accessory right hepatic artery. Four units of packed red blood cells were transfused. The serum ALT exceeded 10000 U/L after the procedure but declined over several days. After transient oliguric renal failure, he was discharged to home on post-procedural day 5.

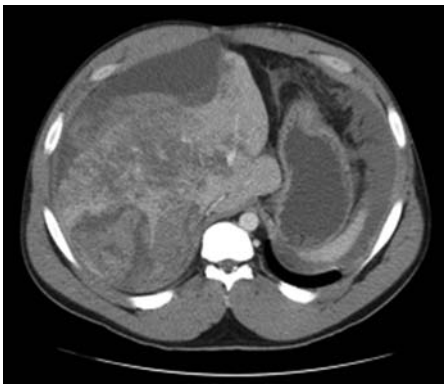

Figure 2 Abdominal CT at second presentation with abdominal pain after resumption of steroid abuse. A heterogeneous-appearing, right hepatic mass measuring 7.2 cm x 7.7 cm and a large subcapsular hematoma are seen, indicating that one of the hepatic adenomas has enlarged since the previous presentation and has hemorrhaged spontaneously. Image has been electronically altered.

DISCUSSION

We report a rare case of hepatic adenoma regrowth with recidivistic steroid abuse, complicated by life-threatening hemorrhage. This case underscores the potentially life-threatening complications of anabolic steroid abuse, and calls for a high index of suspicion among health care providers for hepatic complications if a history of steroid use is elicited.

The risk of androgen-associated liver tumors appears to correlate with the cumulative androgen dose and the potency of the steroid used[10]. Our patient self-administered both oral androstenedione, which has relatively weak androgenic potential, and parenteral nandrolone, which is particularly potent due to C10 hydroxylation. Since androstenedione has not been associated with liver tumors, it is likely that the nandrolone promoted development of his hepatic adenomas. This is consistent with the recurrence of his symptoms soon after resumption of nandrolone.

Both nandrolone and androstenedione have been classified as Schedule Ⅲ controlled substances in recognition of their abuse potential[11,12]. Despite these legal restrictions, anyone can still obtain these drugs with little difficulty over the Internet.

By resuming anabolic steroid consumption after his first hospitalization, our patient clearly demonstrated a pattern of substance abuse. Risk factors for anabolic steroid abuse in male bodybuilders include body-image disturbances, history of childhood conduct disorder, and poor father-son relationships[13]. Had our patient's condition deteriorated and necessitated consideration

of liver transplantation, he would have been required to demonstrate a commitment to abstinence from steroids, in a manner analogous to the alcoholic patient[14].

Patients and physicians must be reminded that the sequelae of anabolic steroid abuse are life threatening. While athletes and bodybuilders are often aware of the legal and social ramifications of steroid abuse, they should also be counseled about its serious medical risks. In the context of an addictive behavior pattern, assiduous surveillance for neoplasms should also be undertaken.

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REFERENCES

1 **Casavant MJ**, Blake K, Griffith J, Yates A, Copley LM. Consequences of use of anabolic androgenic steroids. *Pediatr Clin North Am* 2007; **54**: 677-690, x
2 **Curry LA**, Wagman DF. Qualitative description of the prevalence and use of anabolic androgenic steroids by United States powerlifters. *Percept Mot Skills* 1999; **88**: 224-233
3 **Korkia P**, Stimson GV. Indications of prevalence, practice and effects of anabolic steroid use in Great Britain. *Int J Sports Med* 1997; **18**: 557-562
4 **Kafrouni MI**, Anders RA, Verma S. Hepatotoxicity associated with dietary supplements containing anabolic steroids. *Clin Gastroenterol Hepatol* 2007; **5**: 809-812
5 **Socas L**, Zumbado M, Perez-Luzardo O, Ramos A, Perez C, Hernandez JR, Boada LD. Hepatocellular adenomas associated with anabolic androgenic steroid abuse in bodybuilders: a report of two cases and a review of the literature. *Br J Sports Med* 2005; **39**: e27
6 **Gorayski P**, Thompson CH, Subhash HS, Thomas AC. Hepatocellular carcinoma associated with recreational anabolic steroid use. *Br J Sports Med* 2008; **42**: 74-75; discussion 75
7 **Soe KL**, Soe M, Gluud C. Liver pathology associated with the use of anabolic-androgenic steroids. *Liver* 1992; **12**: 73-79
8 **Nadell J**, Kosek J. Peliosis hepatis. Twelve cases associated with oral androgen therapy. *Arch Pathol Lab Med* 1977; **101**: 405-410
9 **Hayward SR**, Lucas CE, Ledgerwood AM. Recurrent spontaneous intrahepatic hemorrhage from peliosis hepatis. *Arch Surg* 1991; **126**: 782-783
10 **Gelfand MM**, Wiita B. Androgen and estrogen-androgen hormone replacement therapy: a review of the safety literature, 1941 to 1996. *Clin Ther* 1997; **19**: 383-404; discussion 367-368
11 **Schedules of controlled substances.** Code of federal regulations, Title 21, Vol. 9, ChapterII, Part 1308. U.S. Food and Drug Administration, 2005.Available from URL: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=130 &show FR=1
12 **FDA warning on androstenedione (andro).** National Center for Complementary and Alternative Medicine, 2004. Available from URL: http://nccam.nih.gov/health/alerts/androstenedione/consumeradvisory.htm
13 **Kanayama G**, Pope HG, Cohane G, Hudson JI. Risk factors for anabolic-androgenic steroid use among weightlifters: a case-control study. *Drug Alcohol Depend* 2003; **71**: 77-86
14 **Steinman TI**, Becker BN, Frost AE, Olthoff KM, Smart FW, Suki WN, Wilkinson AH. Guidelines for the referral and management of patients eligible for solid organ transplantation. *Transplantation* 2001; **71**: 1189-1204

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CASE REPORT

Cerebral venous thrombosis and heparin-induced thrombocytopenia in an 18-year old male with severe ulcerative colitis

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Abstract

The risk of thromboembolism is increased in inflammatory bowel disease and its symptoms may be overlooked. Furthermore, its treatment can be complex and is not without complications. We describe a case of an adolescent boy who developed a cerebral sinus venous thrombosis during a relapse of his ulcerative colitis and who, while on treatment with heparin, developed heparin-induced thrombocytopenia (HIT). The treatment was then switched to fondaparinux, a synthetic and selective inhibitor of activated factor X.

Key words: Inflammatory bowel disease; Ulcerative colitis; Cerebral venous thrombosis; Heparin-induced thrombocytopenia; Fondaparinux

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) with unknown aetiology, which is localized in the colon. It affects both adults and children, and approximately 10% of the patients are diagnosed during childhood[1]. Drugs used to induce its remission consist mainly of 5-aminosalicylic acid (5-ASA) and steroids, and 5-ASA as its maintenance therapy[2] is often used. In more severe or steroid refractory cases, immune modulating therapy with azathioprine or 6-mercaptopurine, may be used, although the evidence for this is less convincing as compared to the treatment of Crohn's disease (CD)[3]. Despite intensive pharmacological treatment, relapse is not uncommon. Extra intestinal manifestations are reported to occur in about 40% of adult patients with UC[4]. Figures for children are lower.

There are several risk factors for cerebral venous thrombosis (CVT), such as hormones (e.g. contraceptives and pregnancy), different kinds of hereditary thrombophilia and local factors including tumors[5]. In childhood, other risk factors such as local head/neck infections, sepsis and dehydration due to systemic illness have been described[6]. CVT is associated with a significant morbidity and mortality in children, and antithrombotic therapy with heparin in the acute phase followed by warfarin is recommended both in

children and in adults[5,7]. However, CVT in children and adolescents with IBD is, only sparsely described. Hence, we describe a case of an adolescent boy who developed a cerebral sinus venous thrombosis during a relapse of his UC, and who, while on treatment with heparin, developed heparin-induced thrombocytopenia (HIT).

Written consent was received from the patient.

CASE REPORT

The patient was an 18-year old male who presented with extensive ulcerative colitis at the age of 12.5 years. He was initially treated with steroids and olsalazine. He went into remission within 3 mo. During the following years, it was complicated with several relapses, which were treated with repeated courses of prednisolone. Thus, two years after diagnosis, azathioprine (AZA) was started at a dosage of 1 mg/kg. His clinical condition was quite stable until the age of 17 years when an upper respiratory infection induced another relapse with worsening of the patient's general condition and a pronounced weight loss. A high dose of prednisolone (i.e., 40 mg daily) was restarted and the AZA dose was further increased (approximate 2 mg/kg). One month after the introduction of steroids, the patient developed unilateral peritonsillitis and was admitted to hospital for incision and intravenous penicillin G treatment. The dose of prednisolone was by then tapered to 10 mg daily. The pharyngeal symptoms resolved and the patient was discharged on the third day. However, 5 d after discharge, he was readmitted due to a three-day history of severe headache, accompanied with nausea and vomiting. His colitis was clinically improved, apart from continuing daily episodes of loose stools.

The vital signs were normal. Neurological examination did not show any abnormalities or clinical signs of meningitis. During the first days after admission, the patient's headache deteriorated. A CT-scan of the head was performed and interpreted as normal. Hence, a lumbar puncture performed surprisingly revealed an intralumbar pressure of 49 cmH_2O (< 20). The spinal fluid analyses were otherwise normal. An eye fundoscopy revealed bilateral papilloedema and a diagnosis of pseudotumor cerebri induced by the steroid treatment was suspected. In order to reduce the cerebrospinal pressure, 10 mL of cerebrospinal liquid was withdrawn. After an asymptomatic period of about 14 h, the patient's general appearance deteriorated with recurring severe headache.

Repeated neurological examinations did not reveal any focal symptoms. A magnetic resonance imaging of the brain (MRI) showed a CVT in the right sinus transversum and confluence area (Figure 1). D-dimer was clearly elevated. Treatment with intravenous heparin infusion was initiated with a loading dose of 5000 IU, followed by a continuous heparin infusion adjusted according to aPTT (approximately 27 IU/kg per hour was required to maintain aPTT 2-3 times the baseline value). Despite an ongoing active colitis, anticoagulant therapy was considered safe.

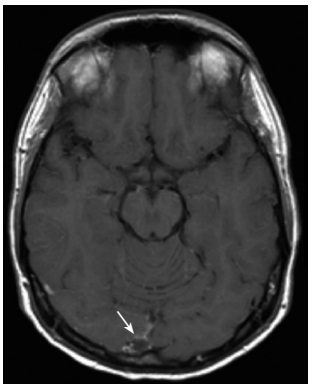

Figure 1 T1 contrast enhanced transverse image showing a focal dark area (arrow) in the right transverse sinus and confluence, which are consistent with a cerebral sinus thrombosis.

In the following 7 d, the patient's general condition improved, although his appetite was poor and the blood-stained diarrhoea continued. Mild iron deficiency anaemia was noted, but vitamin B12 and homocysteine levels were normal. Parenteral nutrition was given as a nutritional support.

On day 7, warfarin was introduced under concomitant continuous heparin infusion. A rapid platelet count fall of 80% in 48 h was noted. Hence, azathioprine was temporarily stopped due to its known risk of thrombocytopenia. Heparin-induced thrombocytopenia (HIT) was suspected, warfarin was stopped and heparin infusion was switched to fondaparinux (Arixtra®, GlaxoSmithKline). The starting dose of fondaparinux was 7.5 mg, which was tapered to 5 mg after 3 d. The diagnosis was later confirmed by the detection of IgG-antibodies against heparin with consistent criteria for HIT[8]. The patient received a total of 6 units of platelet transfusion over a period of 4 d when the platelet count was $< 18 \times 10^9$/L, due to the high expected risk of bleeding in this patient with colitis. Despite this, he had blood in his stools on several occasions during this period. The platelet count started to rise 7 d after heparin was stopped. When the platelet count reached $> 80 \times 10^9$/L, azathioprine and warfarin were reintroduced and after 2 d of therapeutic INR between 2 and 3, fondaparinux was stopped after 9 d of treatment. The course of thrombocytopenia and medical intervention are presented in Figure 2.

During the course of CVT and HIT, a low dose of prednisolone and olsalazine was continued.

A follow-up CT scan 6 mo after the CVT diagnosis showed normal blood flow in all cerebral venous sini with no clinical neurological sequels.

A thorough work-up 7 mo after discharge showed that antithrombin, fibrinogen, lipoprotein (a), factor V gene mutation, prothrombin gene mutation, PAI-I, antiphospholipid antibodies, homocysteine, were all normal. Warfarin treatment was stopped after 8 mo of treatment. Thereafter, protein S, protein C, lupus anticoagulants, and FVIII were tested normal.

The colitis eventually went into clinical and biochemical remission. The patient was hesitant to undergo another colonoscopy until 14 mo later, when the biopsies showed a mild diffuse inflammatory activity and some architectural mucosal changes.

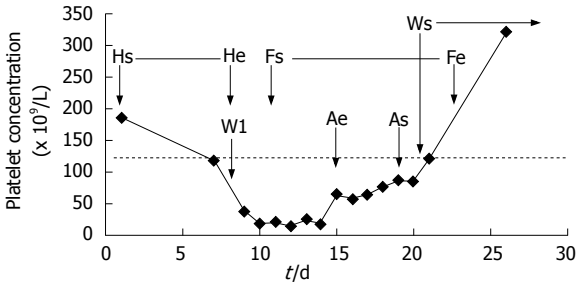

Figure 2 Platelet count expressed in 10^9/L blood (normal range, 125-340) from readmission until discharge showing the progress of thrombocytopenia. The letters denote start or end of heparin (Hs and He), fondaparinux (Fs and Fe), warfarin (Ws), and azatioprine (Ae and As). W1 denotes only one dose. Dotted line denotes a level of thrombocytes lower than their normal range.

DISCUSSION

The increased risk of thromboembolism (TE) in patients with active IBD is well established[9-13] with a 6.5% incidence of thrombosis or a 3-4 fold higher risk than in the general population[14]. TE can also occur in the pediatric age group[15]. The potential mechanisms underlying the prothrombotic state in IBD are hypercoagulation (elevated FⅧ, fibrinogen, decrease in antithrombin, protein S and protein C), hypofibrinolysis [elevated PAI-1 and lipoprotein (a)], platelet abnormalities, endothelial dysfunction (increased von Willebrand factor) and immunological abnormalities (antiphosphlipid antibodies)[14,16]. The common genetic risk factors for TE factor V mutation (Factor V Leiden) and prothrombin mutation (G20210A) are not overrepresented in patients with TE and IBD compared to other patients with TE[16].

It has been suggested that hyperhomocysteinemia is a risk factor for vascular disease and a mediator of TE in adults and occurs more commonly in both pediatric and adult IBD patients than in healthy controls[17,18], which probably reflects the nutritional status with depleted levels of folate, cobalamin (B12) and pyridoxine (B6)[19]. The increased risk of TE in IBD associated with hyperhomocysteinemia is, however, questioned[19]. Other acquired risk factors for TE that can affect patients with IBD are steroid treatment, surgery, immobilisation, dehydration, central venous catheters[16] iron deficiency and infections[6].

In our patient, the risk factors for the development of CVT were relapse of the disease, treatment with high doses of steroids and development of peritonsillitis, surgical incision, dehydration, and iron deficiency anemia. Despite the risk factors, the diagnosis of CVT was delayed mainly due to this complication which is rather rare. It is important to raise the question about sinus thrombosis in the request form to the radiology department since not only unenhanced CT, but also contrast CT needs to be performed for most cases.

Cerebral venous thrombosis is a rare, but serious complication of IBD and seems to be more common in UC than in CD patients[13]. Its prognosis is variable with a high risk of residual symptoms in affected children and adolescents. Even death has been reported[13]. The most frequent symptom is headache, which occurs in 75%-96% of patients. The headache is often severe and diffuse, usually preceding the appearance of neurological deficits. A combination of focal deficits, headache, seizures and altered consciousness is very suggestive of CVT[5]. Apart from supportive care, anticoagulation therapy with heparin is the first line therapy for mild to moderate cases. Thrombolysis using recombinant tissue-type plasminogen activator (rt-PA) or urokinase has also been tried with various successes[20]. Due to the potential risks, expert guidelines recommend that local thrombolysis using urokinase[20] or (rt-PA)[5] should be restricted to comatose patients or patients who deteriorate despite anticoagulant therapy.

HIT is defined as an immune-mediated side effect of heparin therapy, which causes a drop in platelet count of $\geqslant$ 50%[21]. HIT usually occurs after 5-10 d of heparin treatment, but a faster drop may be seen if heparin has been given earlier. Apart from thrombocytopenia, venous or arterial thrombosis can occur, even before thrombocytopenia. Other symptoms observed in children with HIT are acute thoracic pain, respiratory distress, anaphylactic shock and prolonged fever[8]. An auto-immune response to platelet surface factor 4 (PF4) in complex with heparin is the major pathophysiological factor[22]. These complexes lead to cellular activation and development of thrombocytopenia or thrombosis[22]. In children, this is probably as common as in adults. In a recent review by Risch and co-workers, the incidence of HIT in children is estimated to be 0%-2.3%[21]. Approximately 90 pediatric HIT cases have been reported in the literature until now[21]. Children or neonates treated in intensive care units after cardiac surgery and adolescents treated with unfractionated heparin after thromboembolism constitute the highest risk of developing HIT in the pediatric population[21]. Although a rapid increase in platelet levels after cessation of heparin is observed, stopping heparin, as a sole treatment, is not recommended since unfavourable outcome has been reported in over 40% of patients due to the risk of thrombosis. Hence, alternative anticoagulant therapy is recommended. Danaparoid (heparinoid) inhibits mainly factor Xa, and to a lesser extent prothrombin, lepirudin (thrombin inhibitor), and argatroban (thrombin inhibitor) are the recommended drugs for the treatment of HIT. Treatment of HIT with danaparoid has a risk of cross reactivity. Leptirudin and argatroban are expensive, administered as continuous intravenous infusion, and require frequent aPTT testing. However, for our patient, fondaparinux was used as anti-coagulant therapy. Fondaparinux is a synthetic and selective inhibitor of activated factor X (Xa), administered as subcutaneous injection once daily. The antithrombotic effect is achieved by a selective binding of fondaparinux to antithrombin, which increases 300-fold the endogenic neutralisation by antithrombin on factor Xa. Hence, it inhibits the production of thrombin and the development of thrombosis[23]. Fondparinux does not appear to interact with HIT-related antibodies to induce platelet activity and aggregation according to *in vitro* tests and small clinical trails[24].

For our patient, all potential future surgical

procedures and longer periods of immobilization should be accompanied with anti-embolic prophylactic treatment. However, due to his episode of HIT, heparin and low molecular weight heparin (LMWH) is contraindicated and alternative anticoagulant treatment must be used.

Patients with severe IBD are frequently treated with AZA, which may cause a problem during anticoagulant therapy for TE. Scarce reports are available on the AZA treatment which may interact with warfarin by diminishing its effect. Hence, the dose of warfarin may have to be increased 3-4 fold in order to achieve an optimal PK INR level[25].

Thromboembolic complications of IBD are not uncommon. If headache occurs during severe relapse of thromboembolism, CVT must be excluded. A CT scan with and without contrast is recommended as the first initial investigation. Platelet levels should be closely monitored during heparin treatment. When heparin is used, LMWH should be considered due to its lower risk of HIT than unfractionated heparin[26]. However, heparin has advantages in patients with a high risk of bleeding due to shorter T½. More studies concerning fondaparinux in treatment of patients with HIT are needed. It is necessary to evaluate the screening methods for identification of IBD patients with a high risk of thromboembolic complications. Furthermore, the efficacy and safety of prophylactic antithrombotic treatment of children with severe IBD must be evaluated in controlled clinical trials.

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REFERENCES

1 **Sawczenko A**, Sandhu BK, Logan RF, Jenkins H, Taylor CJ, Mian S, Lynn R. Prospective survey of childhood inflammatory bowel disease in the British Isles. *Lancet* 2001; **357**: 1093-1094
2 **Sutherland L**, Macdonald JK. Oral 5-aminosalicylic acid for induction of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2006; (2): CD000543
3 **Sands BE**. Immunosuppressive drugs in ulcerative colitis: twisting facts to suit theories? *Gut* 2006; **55**: 437-441
4 **Ozdil S**, Akyuz F, Pinarbasi B, Demir K, Karaca C, Boztas G, Kaymakoglu S, Mungan Z, Besisik F, Cakaloglu Y, Okten A. Ulcerative colitis: analyses of 116 cases (do extraintestinal manifestations effect the time to catch remission?). *Hepatogastroenterology* 2004; **51**: 768-770
5 **Masuhr F**, Mehraein S. Cerebral venous and sinus thrombosis: patients with a fatal outcome during intravenous dose-adjusted heparin treatment. *Neurocrit Care* 2004; **1**: 355-361
6 **Barnes C**, Deveber G. Prothrombotic abnormalities in childhood ischaemic stroke. *Thromb Res* 2006; **118**: 67-74
7 **Manco-Johnson MJ**. How I treat venous thrombosis in children. *Blood* 2006; **107**: 21-29
8 **Risch L**, Fischer JE, Herklotz R, Huber AR. Heparin-induced thrombocytopenia in paediatrics: clinical characteristics, therapy and outcomes. *Intensive Care Med* 2004; **30**: 1615-1624
9 **Derdeyn CP**, Powers WJ. Isolated cortical venous thrombosis and ulcerative colitis. *AJNR Am J Neuroradiol* 1998; **19**: 488-490
10 **Hasegawa H**, Yokomori H, Tsuji T, Hirose R. Hemorrhagic cerebral sinus thrombosis in a case of controlled ulcerative colitis. *Intern Med* 2005; **44**: 155
11 **Musio F**, Older SA, Jenkins T, Gregorie EM. Case report: cerebral venous thrombosis as a manifestation of acute ulcerative colitis. *Am J Med Sci* 1993; **305**: 28-35
12 **Tsujikawa T**, Urabe M, Bamba H, Andoh A, Sasaki M, Koyama S, Fujiyama Y, Bamba T. Haemorrhagic cerebral sinus thrombosis associated with ulcerative colitis: a case report of successful treatment by anticoagulant therapy. *J Gastroenterol Hepatol* 2000; **15**: 688-692
13 **Umit H**, Asil T, Celik Y, Tezel A, Dokmeci G, Tuncbilek N, Utku U, Soylu AR. Cerebral sinus thrombosis in patients with inflammatory bowel disease: a case report. *World J Gastroenterol* 2005; **11**: 5404-5407
14 **Twig G**, Zandman-Goddard G, Szyper-Kravitz M, Shoenfeld Y. Systemic thromboembolism in inflammatory bowel disease: mechanisms and clinical applications. *Ann N Y Acad Sci* 2005; **1051**: 166-173
15 **Kao A**, Dlugos D, Hunter JV, Mamula P, Thorarensen O. Anticoagulation therapy in cerebral sinovenous thrombosis and ulcerative colitis in children. *J Child Neurol* 2002; **17**: 479-482
16 **Danese S**, Papa A, Saibeni S, Repici A, Malesci A, Vecchi M. Inflammation and coagulation in inflammatory bowel disease: The clot thickens. *Am J Gastroenterol* 2007; **102**: 174-186
17 **Nakano E**, Taylor CJ, Chada L, McGaw J, Powers HJ. Hyperhomocystinemia in children with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2003; **37**: 586-590
18 **Romagnuolo J**, Fedorak RN, Dias VC, Bamforth F, Teltscher M. Hyperhomocysteinemia and inflammatory bowel disease: prevalence and predictors in a cross-sectional study. *Am J Gastroenterol* 2001; **96**: 2143-2149
19 **Oldenburg B**, Fijnheer R, van der Griend R, vanBerge-Henegouwen GP, Koningsberger JC. Homocysteine in inflammatory bowel disease: a risk factor for thromboembolic complications? *Am J Gastroenterol* 2000; **95**: 2825-2830
20 **Renowden S**. Cerebral venous sinus thrombosis. *Eur Radiol* 2004; **14**: 215-226
21 **Risch L**, Huber AR, Schmugge M. Diagnosis and treatment of heparin-induced thrombocytopenia in neonates and children. *Thromb Res* 2006; **118**: 123-135
22 **Poncz M**, Rauova L, Cines DB. The role of surface PF4: glycosaminoglycan complexes in the pathogenesis of heparin-induced thrombocytopenia (HIT). *Pathophysiol Haemost Thromb* 2006; **35**: 46-49
23 **Kuo KH**, Kovacs MJ. Fondaparinux: a potential new therapy for HIT. *Hematology* 2005; **10**: 271-275
24 **Savi P**, Chong BH, Greinacher A, Gruel Y, Kelton JG, Warkentin TE, Eichler P, Meuleman D, Petitou M, Herault JP, Cariou R, Herbert JM. Effect of fondaparinux on platelet activation in the presence of heparin-dependent antibodies: a blinded comparative multicenter study with unfractionated heparin. *Blood* 2005; **105**: 139-144
25 **Havrda DE**, Rathbun S, Scheid D. A case report of warfarin resistance due to azathioprine and review of the literature. *Pharmacotherapy* 2001; **21**: 355-357
26 **Begelman SM**, Hursting MJ, Aghababian RV, McCollum D. Heparin-induced thrombocytopenia from venous thromboembolism treatment. *J Intern Med* 2005; **258**: 563-572

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CASE REPORT

Septic thrombophlebitis of the porto-mesenteric veins as a complication of acute appendicitis

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Abstract

Pylephlebitis, a rare complication of acute appendicitis, is defined as thrombophlebitis of the portal venous system. Pylephlebitis usually occurs due to secondary infection in the region drained into the portal system. We report a case of pylephlebitis caused by acute appendicitis. The patient was transferred from a private clinic 1 wk after appendectomy with the chief complaints of high fever and abdominal pain. He was diagnosed with pylephlebitis of the portal vein and superior mesenteric vein by CT-scan. The patient was treated with antibiotics and anticoagulation therapy, and discharged on the 25th day and follow-up CT scan showed a cavernous transformation of portal thrombosis.

Key words: Acute appendicitis; Pylephlebitis; Antibiotics; Anti-coagulation therapy

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INTRODUCTION

Pylephlebitis is defined as thrombophlebitis of the portal venous system associated with intraperitoneal septic conditions, such as colonic diverticulitis, acute appendicitis, and cholangitis. Pylephlebitis is considered a seriously lethal condition.

Recent advances in antibiotic therapy have made the occurrence of pylephlebitis very rare. However, the mortality rate remains high because non-specific symptoms and low index of suspicion usually delay the diagnosis of pylephlebitis. The early use of optimal diagnostic modalities and surgical interventions are essential to ensure the survival of patients.

We describe a case of thrombophlebitis of the portal vein and superior mesenteric vein as a complication of acute appendicitis, which was successfully treated with antibiotics and anticoagulation therapy.

CASE REPORT

A 26-year-old man was transferred to the emergency department with the complaints of high fever and severe abdominal pain for 4 d. He underwent appendectomy for acute appendicitis 1 wk earlier. During the hospital stay, he had persistent fever up to 40℃ and diarrhea, and was treated with antibiotics, fluids and anti-pyretics.

On arrival, he had an elevated temperature of 39.7℃ and complained of severe epigastric pain. His blood pressure was 140/80 mmHg, his pulse rate was 114/min, and the respiration rate was 24/min. Physical examination noted epigastric pain and tenderness, but could not find rebound tenderness and muscle guarding. Laboratory results showed mild leukocytosis (11500/mm^3), anemia (9.9 g/dL hemoglobin), and elevated bilirubin (1.6/0.9 mg/dL total/direct bilirubin), alkaline phosphatase (213 U/L), and γ-GT (73 U/L).

Abdominal CT scan demonstrated thrombus formation in the portal vein extending from the superior mesenteric vein (SMV; Figure 1). He was admitted to the intensive care unit and treated with systemic Ⅳ antibiotics (the 3rd generation cephalosporin and metronidazole, chosen by empirical therapy) and anticoagulation therapy (a subcutaneous injection of low molecular heparin, 1 mg/kg every 12 h). He was given nothing by mouth and parenteral nutrition was initiated to decrease the portal blood flow from the mesenteric vein.

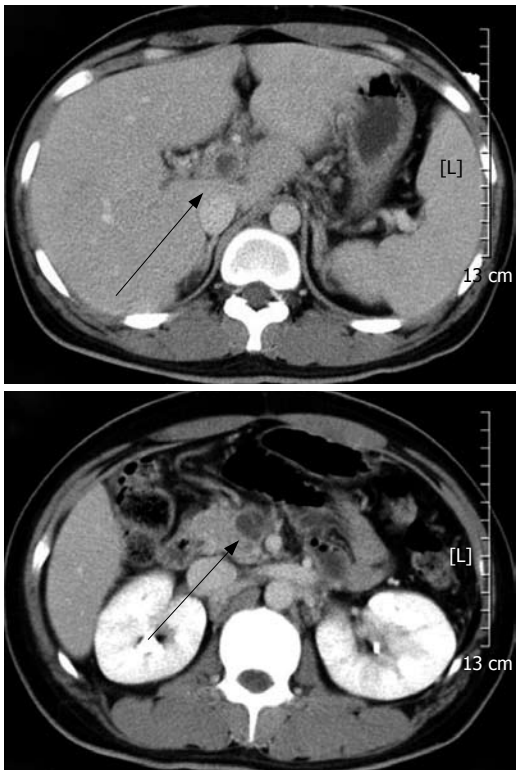

Figure 1 CT scan showing total occlusion of the portal vein with a thrombus (arrow) extending to the superior mesenteric vein.

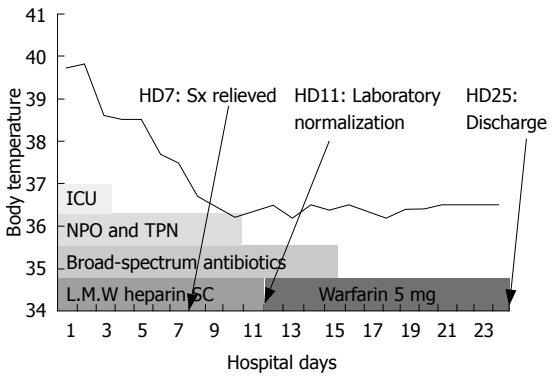

Figure 2 Clinical progress and treatment course of the patient. ICU: Intensive care unit; NPO: Non per os; TPN: Total parenteral nutrition; HD: Hospital day; L.M.W. heparin SC: Low molecular weight heparin subcutaneous injection.

By the 3rd hospital day, his symptoms were gradually improved and the liver dysfunction and leukocytosis were normalized on the 11th day. Thereafter, oral feeding was started. No microorganisms were identified in his blood culture specimen. On the 12th hospital day, low molecular heparin was replaced with warfarin (5 mg/d) and continued until one month after discharge (Figure 2).

The follow-up CT scan taken 2 wk after admission showed a cavernous transformation of portal vein thrombus and improved SMV thrombosis (Figure 3).

The patient was discharged on the 25th day without complications. A follow-up CT scan after 6 mo showed a slightly increased cavernous transformation of the portal vein and marked improvement of the SMV thrombosis.

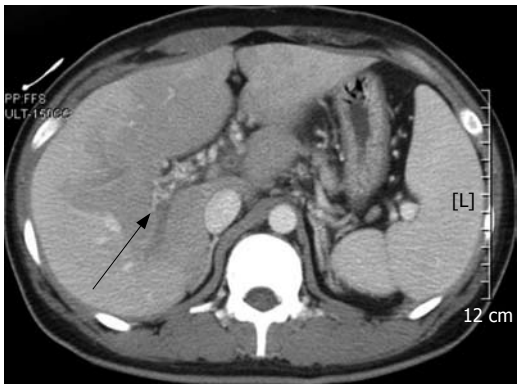

Figure 3 Arrow indicates the cavernous transformation of the portal vein on follow-up CT scan.

He appeared healthy and had no clinical and laboratory abnormalities on follow-up.

DISCUSSION

Although pylephlebitis is a rare complication derived from septic conditions of the portal drainage area most commonly caused by colonic diverticulitis, it occurs in association with acute appendicitis, inflammatory bowel disease, suppurative pancreatitis, acute cholangitis, bowel perforation, and pelvic infection[1-3].

This disease entity occurred in 0.4% of patients with acute appendicitis before 1950, but it has become very rare due to major advances in antibiotic therapy and surgical treatment[4]. However, the reported mortality rate of pylephlebitis is 30%-50%, partly due to a delay in diagnosis from its atypical clinical findings and a low index of suspicion[1,5].

Reported cases of pylephlebitis are mainly young children, with a mortality rate of up to 50%[4,6-8]. Children are particularly at a great risk of appendiceal perforation because the diagnosis is delayed. As a result, young children are prone to develop pylephlebitis.

The clinical features of pylephlebitis are non-specific. High fever, chills, malaise, right upper quadrant pain, and tenderness are the initial clinical manifestations. Balthazar and Gollapudi[9] reported that only 30% of patients with pylephlebitis present with localizing clinical signs of a primary source of sepsis. Laboratory findings, such as leucocytosis and mild abnormalities of liver function tests, are usually non-specific, but jaundice is rare except in case of multiple liver abscesses[4,5,7,10]. Our patient was treated conservatively for epigastric pain, high fever, and diarrhea after appendectomy in a private clinic, as there was no suspicion of pylephlebitis.

Blood cultures revealed no microorganisms in our case. Baril *et al*[5] reported that bacteremia is present in less than one-half of patients, whereas Balthazar and Gollapudi[9] reported that up to 80% of patients have positive blood cultures, and *Escherichia coli*, *Bacteroides fragilis*, *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Enterobacter* spp. are the most common microorganisms isolated[4,7,9,10].

Modern diagnostic imaging techniques help the early

diagnosis of acute phase pylephlebitis. The sensitivity and specificity of CT scans for pylephlebitis are not known. However, CT scans could simultaneously detect the primary source of infection, extent of pylephlebitis, and intrahepatic abnormalities, such as liver abscesses. Thus, CT scan is the most reliable initial diagnostic choice[9-11]. Air bubbles or thrombi of the portal venous system are the critical CT findings of pylephlebitis[9]. Ultrasound scan with color flow Doppler is also a sensitive test for confirming partial patency of the portal vein and portal vein thrombosis[7].

Once a diagnosis of pylephlebitis is established, appropriate treatment should be initiated as soon as possible.

The principal of treatment for pylephlebitis is to remove the source of infection and eradicate the toxic microorganisms using appropriate antibiotics. Immediate surgical intervention is necessary in most cases, but Stitzenberg *et al*[8] reported that interval laparoscopic appendectomy can be performed 3 mo after treatment with antibiotics and anticoagulants. Regarding the treatment of portal thrombosis, Nishimori *et al*[11] reported that surgical thrombectomy can be performed through the ileocolic vein using a Fogarty catheter, but most reported cases are treated with systemic antibiotics and anticoagulants. A minimum of 4 wk of antibiotic therapy is usually recommended and patients presenting with a hepatic abscess should receive at least 6 wk of antibiotic therapy[7,10].

The effectiveness of anticoagulants in the treatment of pylephlebitis is still controversial. We administered low molecular heparin for 11 d initially and warfarin for 1.5 mo. Condat *et al*[12] recommended early anticoagulants therapy because the recanalization of the portal system was significantly higher in the anticoagulation group compared to the control group. Baril *et al*[5] insisted that anticoagulants should be considered carefully because complications could present in 20% of patients, and it is not necessary in patients with thrombus isolated to the portal vein, but could be used for prevention of intestinal ischemia in patients with involvement of the superior or inferior mesenteric vein. Lim *et al*[10] recommended anticoagulation for the prevention of septic pulmonary embolism from infected portal thrombi.

In summary, pylephlebitis is a rare, but fatal complication of acute appendicitis. Therefore, when pylephlebitis is suspected, immediate CT scan and antibiotic therapy, with or without surgical intervention, should be started to ensure the survival of patients.

REFERENCES

1 **Plemmons RM**, Dooley DP, Longfield RN. Septic thrombophlebitis of the portal vein (pylephlebitis): diagnosis and management in the modern era. *Clin Infect Dis* 1995; **21**: 1114-1120

2 **Giuliano CT**, Zerykier A, Haller JO, Wood BP. Radiological case of the month. Pylephlebitis secondary to unsuspected appendiceal rupture. *Am J Dis Child* 1989; **143**: 1099-1100

3 **Baddley JW**, Singh D, Correa P, Persich NJ. Crohn's disease presenting as septic thrombophlebitis of the portal vein (pylephlebitis): case report and review of the literature. *Am J Gastroenterol* 1999; **94**: 847-849

4 **Chang TN**, Tang L, Keller K, Harrison MR, Farmer DL, Albanese CT. Pylephlebitis, portal-mesenteric thrombosis, and multiple liver abscesses owing to perforated appendicitis. *J Pediatr Surg* 2001; **36**: E19

5 **Baril N**, Wren S, Radin R, Ralls P, Stain S. The role of anticoagulation in pylephlebitis. *Am J Surg* 1996; **172**: 449-452; discussion 452-453

6 **Slovis TL**, Haller JO, Cohen HL, Berdon WE, Watts FB Jr. Complicated appendiceal inflammatory disease in children: pylephlebitis and liver abscess. *Radiology* 1989; **171**: 823-825

7 **Vanamo K**, Kiekara O. Pylephlebitis after appendicitis in a child. *J Pediatr Surg* 2001; **36**: 1574-1576

8 **Stitzenberg KB**, Piehl MD, Monahan PE, Phillips JD. Interval laparoscopic appendectomy for appendicitis complicated by pylephlebitis. *JSLS* 2006; **10**: 108-113

9 **Balthazar EJ**, Gollapudi P. Septic thrombophlebitis of the mesenteric and portal veins: CT imaging. *J Comput Assist Tomogr* 2000; **24**: 755-760

10 **Lim HE**, Cheong HJ, Woo HJ, Kim WJ, Kim MJ, Lee CH, Park SC. Pylephlebitis associated with appendicitis. *Korean J Intern Med* 1999; **14**: 73-76

11 **Nishimori H**, Ezoe E, Ura H, Imaizumi H, Meguro M, Furuhata T, Katsuramaki T, Hata F, Yasoshima T, Hirata K, Asai Y. Septic thrombophlebitis of the portal and superior mesenteric veins as a complication of appendicitis: report of a case. *Surg Today* 2004; **34**: 173-176

12 **Condat B**, Pessione F, Helene Denninger M, Hillaire S, Valla D. Recent portal or mesenteric venous thrombosis: increased recognition and frequent recanalization on anticoagulant therapy. *Hepatology* 2000; **32**: 466-470

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CASE REPORT

Direct invasion to the colon by hepatocellular carcinoma: Report of two cases

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Abstract

Although hepatocellular carcinoma (HCC) is a common tumor, direct invasion of the gastrointestinal tract by HCC is uncommon. Recently, we encountered two cases of HCC with direct invasion to the colon. The first patient was a 79-year-old man who underwent transarterial chemo-embolization (TACE) for HCC 1.5 years prior to admission to our hospital. Computed tomography (CT) showed a 7.5-cm liver tumor directly invading the transverse colon. Partial resection of the liver and transverse colon was performed. The patient survived 6 mo after surgery, but died of recurrent HCC. The second patient was a 69-year-old man who underwent TACE and ablation for HCC 2 years and 7 months prior to being admitted to our hospital for melena and abdominal distension. CT revealed a 6-cm liver tumor with direct invasion to the colon. The patient underwent partial resection of the liver and right hemicolectomy. The patient recovered from the surgery. But, unfortunately, he died of liver failure due to liver cirrhosis one month later. Although the prognosis of HCC that has invaded the colon is generally poor due to the advanced stage of the disease, surgical resection may be a favorable treatment option in patients with a good general condition.

Key words: Hepatocellular carcinoma; Colon; Hepatoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide[1]. Direct invasion to the gastrointestinal (GI) tract by HCC is uncommon, with a reported incidence of 0.5%-2% among clinical HCC cases[2,3]. GI bleeding or stenosis due to HCC invasion is very uncommon. In such cases, the best treatment remains controversial[4].

Due to improved instruments, techniques, and perioperative management, surgical resection is now safely performed in patients with advanced HCC. Therefore, it is also possible to resect HCC with direct invasion to the GI tract. Here, we present two cases of HCC with direct invasion to the colon that were treated by surgical resection.

CASE REPORT

Case 1

A 79-year-old man with chronic hepatitis C has been followed up since 1983. In August 1998, computed tomography (CT) revealed a 4-cm tumor in the caudate lobe of the liver, which was diagnosed as HCC. The lesion was treated by transarterial chemo-embolization (TACE). In February 2000, the patient suffered from epigastralgia, and was admitted to our hospital. CT revealed that the liver tumor increased to 7.5 cm in diameter and directly invaded the transverse colon (Figure 1). Liver function tests revealed no abnormalities. The serum α-fetoprotein (AFP) level was 331 ng/mL, and the protein induced by vitamin K absence or antagonist 2 (PIVKA-2) level was within normal range.

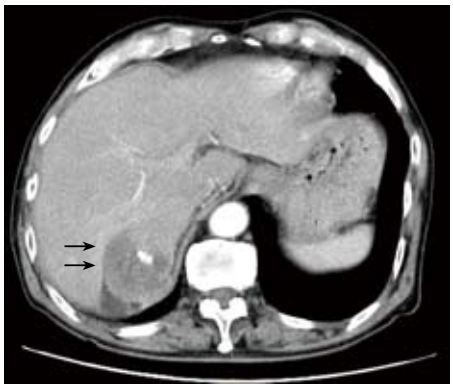

Figure 1 Computed tomography images showing a 7.5-cm liver tumor (arrows) arising from the caudate lobe in case 1(**A**), which appears to invade the transverse colon directly (arrows) (**B**).

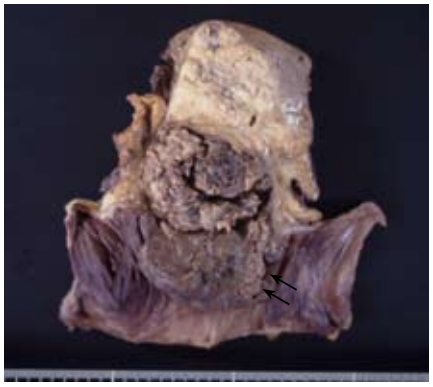

Figure 2 Macroscopic appearance of the surgical specimen in case 1. The liver tumor invades the colon (arrows).

HCC invading the transverse colon was diagnosed and partial resection of the liver and transverse colon was performed. In the resected specimen, a 96 mm × 58 mm liver tumor invading the transverse colon was found (Figure 2). Histopathologic examination of the specimen also showed poorly-differentiated HCC with direct invasion to the colon. The postoperative course was uneventful, and the patient was discharged on postoperative day 21. He survived symptom free for 6 mo and died of recurrent HCC.

Case 2

A 69-year-old man has been treated since 2000 for liver cirrhosis due to hepatitis C. In July 2004, CT revealed a 4-cm tumor in segment 6 of the liver, which was diagnosed as HCC. The lesion was treated by radiofrequency ablation (RFA) and TACE. In February 2007, the patient suffered from melena and abdominal distension and was admitted to our hospital. CT revealed that the tumor increased to 6 cm in diameter and directly invaded the diaphragm and the hepatic flexure of the colon (Figure 3). The ascending colon was dilated due to stenosis of the colon. Colonoscopic examination revealed a hemorrhagic and lobulated tumor in the hepatic flexure of the colon (Figure 4). Laboratory tests revealed 2.9 g/dL serum albumin, 1.9 mg/dL serum total bilirubin, 52.1% prothrombin activity, and 17.3% indocyanine green retention rate at 15 minutes,

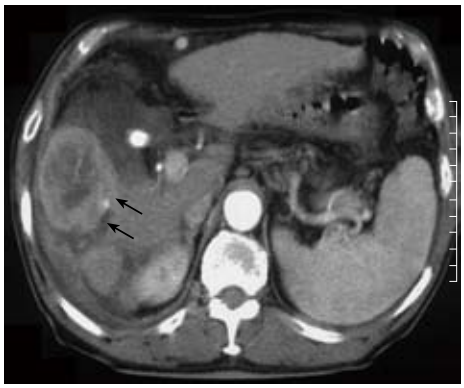

Figure 3 Computed tomography images showing a 6-cm liver tumor invades the colon and diaphragm (arrows) in case 2.

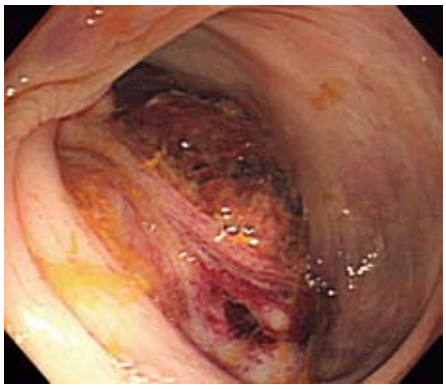

Figure 4 Colonoscopic view showing a hemorrhagic and lobulated tumor with a smooth surface is seen in the ascending colon in case 2.

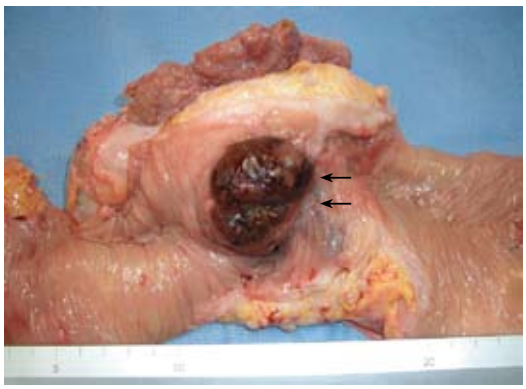

Figure 5 Macroscopic appearance of the surgical specimen in case 2. The liver tumor invades the colon (arrows).

15 ng/mL serum AFP, and 370 AU/mL PIVKA-2. HCC invading the hepatic flexure of the colon was diagnosed, and partial resection of the liver, right hemicolectomy and partial excision of the diaphragm were performed. In the resected specimen, a 65 mm × 47 mm liver tumor, which invaded the hepatic flexure of the colon, was found (Figure 5). Histopathologic examination of the specimen also showed moderately differentiated HCC with direct invasion to the colon and diaphragm. The patient recovered from the operation, and had no evidence of HCC recurrence, but unfortunately, he died of liver failure due to liver cirrhosis 1 mo later.

Table 1 Reported cases with invasion to the colon by HCC

Authors	Age (yr)/Sex	Viral infection	Symptom	Endoscopic shape	Tumor size (mm)	Previous treatment for HCC	Treatment	Prognosis
Hashimoto M[8] (1996)	72/F	HCV	Melena	Ulcerated	45	TAE (7 times)	Operation	4 mo alive
Chen CY[9] (1997)	71/M	Negative	Bloody stool	Lobulated	200	-	-	6 mo dead
Lin CP[2] (2000)	59/M	HCV	Bloody stool	Polypoid	80	TAE (3 times)	-	1.2 mo dead
Lin CP[2] (2000)	67/M	HBV	Stool OB (+)	Not observed	150	Operation TAE	-	1.5 mo dead
Lin CP[2] (2000)	69/M	HBV	Stool OB (+)	Not observed	200	-	-	1.2 mo dead
Lin CP[2] (2000)	63/M	Negative	Bloody stool	Not observed	200	-	-	4.0 mo dead
Strivastava DN[10] (2000)	32/M	HBV	Bloody stool	Not observed	n.d.	TAE	TAE	0.7 mo dead
Zech CJ[11] (2006)	57/M	HBV HCV	Abdominal pain	Not observed	n.d.	TACE (6 times)	Operation	ND
Our case	79/M	HCV	Epigastralgia	Not observed	75	TACE	Operation	6.0 mo dead
Our case	69/M	HCV	Melena	Lobulated	55	RFA TACE	Operation	1.0 mo dead

HBV: Hepatitis B virus; HCV: Hepatitis C virus; Stool OB (+): Stool positive for occult blood; TAE: Transarterial embolization; TACE: Transarterial chemoembolization; ND: Not described.

DISCUSSION

HCC, one of the most common malignant tumors worldwide, is responsible for more than 250000 deaths annually[1]. In some autopsy series, extrahepatic metastasis to the lung, lymphnodes, bone, heart, or adrenal glands has been found in 30%-75% of advanced HCC cases[5]. HCC with direct invasion to other organs can occur, with the most frequent sites being the diaphragm and gallbladder[6]. HCC only rarely invades the GI tract, the reported incidence is 0.5%-2% of clinical HCC cases and 4% of autopsy cases[2,3,7]. GI bleeding or stenosis due to HCC invasion is very uncommon[8]. The most frequently invaded GI tract sites are the duodenum and stomach[2] and invasion into the colon is very rare. To date, only eight cases of invasion to the colon by HCC have been reported in the English literature (Table 1)[2,3,9-11]. Among the 10 patients (including our two cases), the most frequent symptom was bloody stool (8 of 10 patients, 80%). Seven patients (70%) underwent transarterial embolization (TAE) or TACE for HCC prior to development of invasion. Surgical resection or supportive care was almost selected in almost all cases. However, the outcomes were very poor, and the median survival was only 2.5 mo.

GI tract invasion by HCC sometimes occurs after TAE or TACE[8]. TAE and TACE can induce exophytic growth of the HCC due to an inflammatory reaction and change in the extrahepatic blood supply. As a result, HCC may invade adjacent organs such as the diaphragm, stomach, duodenum, and colon.

TAE or TACE is not an effective treatment for GI invasion by HCC. RFA is difficult to perform in patients with GI tract invasion because of the risk of GI tract perforation. Fujii *et al*[12] reported that the median survival time of patients with GI tract invasion treated by surgical resection, nonsurgically, or by supportive therapies is 9.7 mo, 3.0 mo and 1.2 mo, respectively. Therefore, surgical resection may be the most effective treatment for GI tract invasion by HCC. In addition, surgical techniques including liver resection and perioperative management have recently improved. Surgical resection is probably the best treatment option for HCC invading the GI tract if the patient's general condition including liver function is good.

In conclusion, although the prognosis of colonic invasion of HCC is generally poor due to the advanced stage of the disease, surgical resection may be a favorable treatment option in patients with a good general condition.

REFERENCES

1 **Ramsey WH**, Wu GY. Hepatocellular carcinoma: update on diagnosis and treatment. *Dig Dis* 1995; **13**: 81-91
2 **Lin CP**, Cheng JS, Lai KH, Lo GH, Hsu PI, Chan HH, Hsu JH, Wang YY, Pan HB, Tseng HH. Gastrointestinal metastasis in hepatocellular carcinoma: radiological and endoscopic studies of 11 cases. *J Gastroenterol Hepatol* 2000; **15**: 536-541
3 **Chen LT**, Chen CY, Jan CM, Wang WM, Lan TS, Hsieh MY, Liu GC. Gastrointestinal tract involvement in hepatocellular carcinoma: clinical, radiological and endoscopic studies. *Endoscopy* 1990; **22**: 118-123
4 **Tung WY**, Chau GY, Loong CC, Wu JC, Tsay SH, King KL, Huang SM, Chiu JH, Wu CW, Lui WY. Surgical resection of primary hepatocellular carcinoma extending to adjacent organ(s). *Eur J Surg Oncol* 1996; **22**: 516-520
5 **Anthony PP**. Primary carcinoma of the liver: a study of 282 cases in Ugandan Africans. *J Pathol* 1973; **110**: 37-48
6 **Edmondson HA**, Steiner PE. Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. *Cancer* 1954; **7**: 462-503
7 **Nakashima T**, Okuda K, Kojiro M, Jimi A, Yamaguchi R, Sakamoto K, Ikari T. Pathology of hepatocellular carcinoma in Japan. 232 Consecutive cases autopsied in ten years. *Cancer* 1983; **51**: 863-877
8 **Hashimoto M**, Watanabe G, Matsuda M, Yamamoto T, Tsutsumi K, Tsurumaru M. Case report: gastrointestinal bleeding from a hepatocellular carcinoma invading the transverse colon. *J Gastroenterol Hepatol* 1996; **11**: 765-767
9 **Chen CY**, Lu CL, Pan CC, Chiang JH, Chang FY, Lee SD. Lower gastrointestinal bleeding from a hepatocellular carcinoma invading the colon. *J Clin Gastroenterol* 1997; **25**: 373-375
10 **Srivastava DN**, Gandhi D, Julka PK, Tandon RK. Gastrointestinal hemorrhage in hepatocellular carcinoma: management with transheptic arterioembolization. *Abdom Imaging* 2000; **25**: 380-384
11 **Zech CJ**, Bilzer M, Mueller-Lisse UG, Steitz HO, Haraida S, Reiser MF. Perforation of the colon: a rare complication of hepatocellular carcinoma. *Acta Radiol* 2006; **47**: 538-542
12 **Fujii K**, Nagino M, Kamiya J, Uesaka K, Sano T, Yuasa N, Oda K, Nimura Y. Complete resection of hepatocellular carcinoma with direct invasion to the stomach remnant. *J Hepatobiliary Pancreat Surg* 2004; **11**: 441-444

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LETTERS TO THE EDITOR

Ten mg dexrabeprazole daily is as effective as 20 mg dexrabeprazole daily

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Abstract

Ten mg dexrabeprazole daily has been shown to be more effective than 20 mg rabeprazole daily against gastroesophageal reflux disease (GERD). This report shows that the efficacy of 10 mg dexrabeprazole daily is equivalent to that of 20 mg dexrabeprazole daily against GERD. This implies that a dose of 10 mg dexrabeprazole is sufficient to block the maximum amount of proton pumps without any need to double the dose as suggested with rabeprazole.

Key words: Dexrabeprazole; Rabeprazole; Gastroesophageal reflux disease

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TO THE EDITOR

We read with interest the article by Pai *et al* on the efficacy of 10 mg dexrabeprazole[1]. However, the effects of rabeprazole on acid secretion are dose-dependent, and an increase in gastric pH, coupled with a reduction in oesophageal acid exposure, has been seen in gastroesophageal reflux disease (GERD) patients receiving 20 mg or 40 mg rabeprazole once daily[2]. Shimatani *et al*[3] showed that 20 mg rabeprazole, twice daily, may result in better acid suppression than 10 mg rabeprazole, twice daily, in GERD patients[3]. Hence, we wanted to find out whether 20 mg dexrabeprazole daily would provide a greater efficacy than 10 mg dexrabeprazole daily against GERD.

This was a randomized, double-blinded, comparative study in clinical setting, approved by the institutional review board and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Males and non-pregnant and non-lactating females between the age of 18-65 years, clinically diagnosed with GERD, were included after a written informed consent was obtained from each of them. Excluded from the study were those with abnormal laboratory tests at baseline (including liver enzymes greater than twice the upper limit of normal), those who were refractory to a 2-mo course of H_2-blocker or PPI therapy for GERD treatment, those who took PPI within 14 d of screening or a H_2-blocker or a prokinetic agent within 7 d of screening, those who required daily use of NSAIDs, oral steroids, aspirin or were unable to discontinue the use of anticholinergics, cholinergics, spasmolytics, opiates or sucralfate, and those with poorly controlled associated disease, such as heart disease, coagulation disorders, thyroid disorders. Patients having a history of infectious or inflammatory conditions of the intestine (including inflammatory bowel disease), malabsorption syndrome, obstruction, gastrointestinal malignancy, gastric or intestinal surgery including vagotomy, Barrett's esophagus, esophageal stricture, pyloric stenosis, scleroderma or a history of hypersensitivity to any of the PPIs, were also excluded from the study. Enrolled patients were randomized to receive 10 mg dexrabeprazole once daily (D10-OD), 10 mg dexrabeprazole twice daily (D10-BD) or 20 mg dexrabeprazole daily (D20-OD) for 28 d. Visual analog scale (VAS, 0-100) was used to assess the severity of GERD symptoms. A total of 136 patients were enrolled and all completed the study. No difference was found in the baseline demographics of the patients.

A significant reduction ($P < 0.001$, Tukey-Kramer multiple comparison test) from baseline (day 0, before therapy) VAS scores of heartburn and regurgitation was

Table 1 Improvement in Visual Analog Scale (VAS) scores of symptoms (values expressed as mean ± SD)

	Day 0			Day 14			Day 28		
	D10-OD (A, *n* = 74)	D10-BD (B, *n* = 34)	D20-OD (C, *n* = 28)	D10-OD (A, *n* = 74)	D10-BD (B, *n* = 34)	D20-OD (C, *n* = 28)	D10-OD (A, *n* = 74)	D10-BD (B, *n* = 34)	D20-OD (C, *n* = 28)
Heartburn	48.5 ± 22.2	59.7 ± 12.4	58.2 ± 13.6	25.1 ± 16.2[b]	38.2 ± 13.1[b]	32.1 ± 15.5[b]	7.6 ± 15.2[b]	13.8 ± 10.7[b]	12.9 ± 14.4[b]
Between group	0.007[1]	0.007	0.033	0.0001[1]	0.0001	0.052	0.034[1]	0.034	0.114
P values	0.033[2]	0.652[3]	0.652	0.052[2]	0.078[3]	0.078	0.114[2]	0.779[3]	0.779
Regurgitation	45.9 ± 20.4	57.6 ± 12.6	56.4 ±14. 5	21.9 ± 16.2[b]	35.9 ± 12.1[b]	30.7 ± 16.8[b]	6.2 ± 14.2[b]	11.8 ± 10.3[b]	10 ± 13.6[b]
Between group	0.003[1]	0.003	0.014	< 0.0001[1]	< 0.0001	0.017	< 0.0001[1]	< 0.0001	0.225
P values	0.014[2]	0.729[3]	0.729	0.017[2]	0.162[3]	0.162	0.225[2]	0.556[3]	0.556

[b]$P < 0.001$ *vs* baseline values (Tukey-Krammer Multiple comparison test). Between-group difference (*T*-test): [1]A *vs* B; [2]A *vs* C; [3]B *vs* C.

seen in all the treatment groups on day 14 with a further reduction on continuing the therapy until 28 d. Improvement in the VAS scores in the D10-OD group was significantly better than in the D10-BD and D20-OD groups on day 14 with no significant difference between the D10-BD and D20-OD groups. On day 28, the D10-OD group showed significantly higher improvement than the D10-BD group with no significant differences between the D10-OD & D20-OD and the D10-BD & D20-OD groups (Table 1). Percentage of patients with ⩾ 50% relief in symptoms of heartburn and regurgitation on day 28 was 86.5% and 91.9% in the D10-OD group, 91.2% and 97.1% in the D10-BD group, 89.3% and 92.9% in the D20-OD group, respectively. No between-group difference in proportion of patients with ⩾ 50% relief was observed ($P > 0.05$, chi-square test). None of the patients reported any adverse drug reaction and no differences were seen in baseline laboratory parameters after therapy, indicating that dexrabeprazole at different doses was well-tolerated.

The results of this study demonstrates that the efficacy of 10 mg dexrabeprazole daily is equivalent to that of 20 mg dexrabeprazole daily in relieving symptoms of GERD. This implies that 10 mg dexrabeprazole daily is potent and sufficient enough to block the maximum amount of proton pumps, thus precluding the need to use higher doses as has been suggested with rabeprazole.

REFERENCES

1 **Pai V**, Pai N. Randomized, double-blind, comparative study of dexrabeprazole 10 mg versus rabeprazole 20 mg in the treatment of gastroesophageal reflux disease. *World J Gastroenterol* 2007; **13**: 4100-4102

2 **Langtry HD**, Markham A. Rabeprazole: a review of its use in acid-related gastrointestinal disorders. *Drugs* 1999; **58**: 725-742

3 **Shimatani T**, Moriwaki M, Xu J, Tazuma S, Inoue M. Acid-suppressive effects of rabeprazole: comparing 10mg and 20mg twice daily in Japanese Helicobacter pylori-negative and -positive CYP2C19 extensive metabolisers. *Dig Liver Dis* 2006; **38**: 802-808

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
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June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
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June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
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June 26-28, Bratislava, Slovakia
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www.ilts.org

September 10-13, Budapest, Hungary
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E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
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APDW 2008
September 13-16, New Delhi, Indian
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III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
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European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
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16th United European Gastroenterology Week
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E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
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November 6-9, Lucerne, Switzerland
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E-mail: ngm2008@mci-group.com
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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

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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]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

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

Author(s) and editor(s)

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

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

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

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