World Journal of
Gastroenterology ®
Volume 17 Number 44
November 28, 2011

2008
中国精品科技期刊
OUTSTANDING S&T JOURNALS OF CHINA

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

ISSN 1007-9327
9 771007 932045 44>

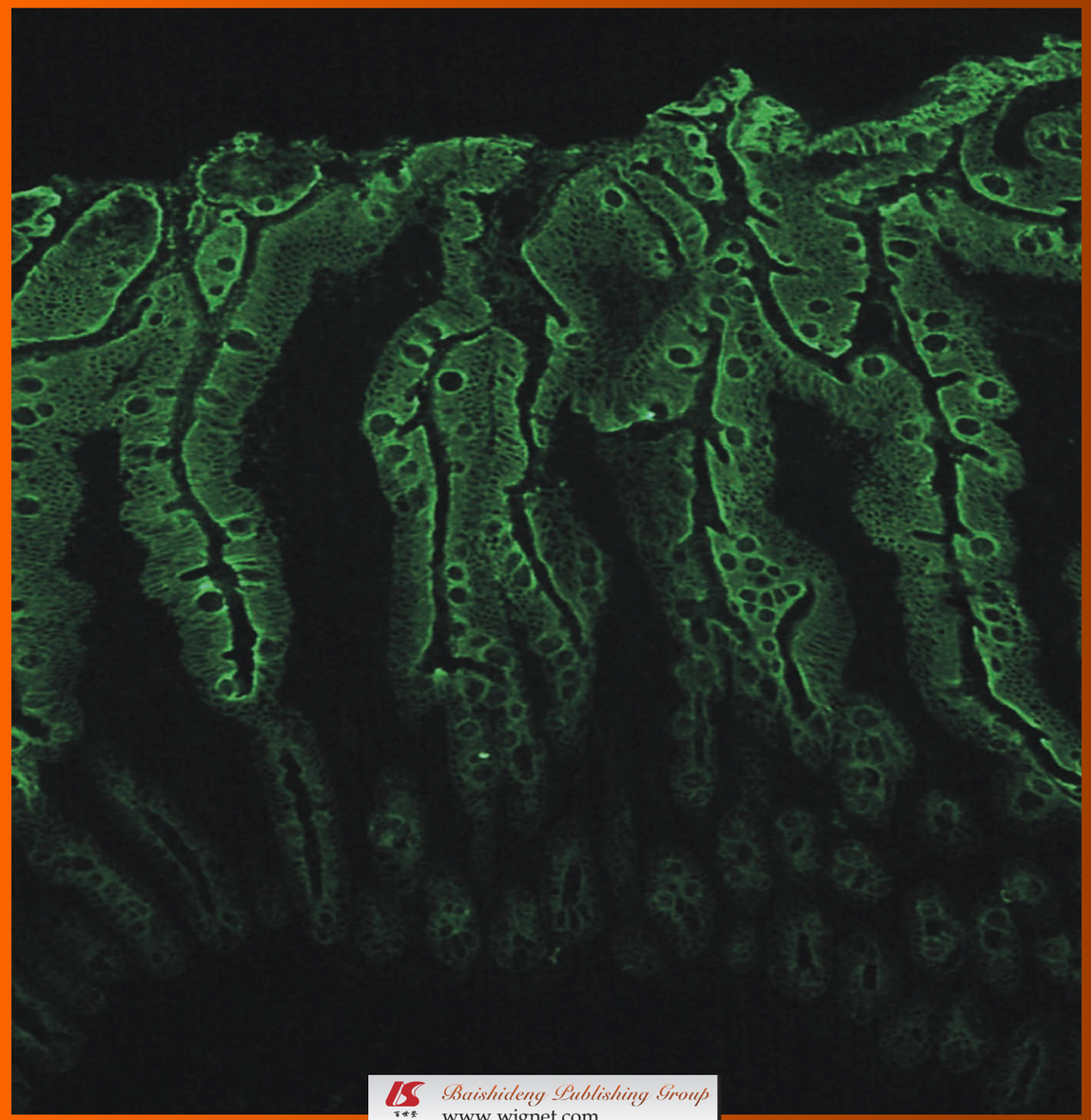
ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

World Journal of Gastroenterology
www.wjgnet.com
Volume 17 Number 44 Nov 28 2011

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

World Journal of *Gastroenterology*

World J Gastroenterol 2011 November 28; 17(44): 4839-4936



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WJG

World Journal of Gastroenterology

Contents

Weekly Volume 17 Number 44 November 28, 2011

World Journal of Gastroenterology
Volume 17 Number 44 November 28, 2011

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

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

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http://www.wjgnet.com

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

SUBSCRIPTION
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Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

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

PUBLICATION DATE
November 28, 2011

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

EDITORIAL OFFICE
Jian-Xia Cheng, Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4839

World J Gastroenterol 2011 November 28; 17(44): 4839-4844
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Juvenile polyposis syndrome

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Received: January 28, 2011 Revised: June 9, 2011
Accepted: June 16, 2011
Published online: November 28, 2011

Abstract

Juvenile polyposis syndrome is a rare autosomal dominant syndrome characterized by multiple distinct juvenile polyps in the gastrointestinal tract and an increased risk of colorectal cancer. The cumulative life-time risk of colorectal cancer is 39% and the relative risk is 34. Juvenile polyps have a distinctive histology characterized by an abundance of edematous lamina propria with inflammatory cells and cystically dilated glands lined by cuboidal to columnar epithelium with reactive changes. Clinically, juvenile polyposis syndrome is defined by the presence of 5 or more juvenile polyps in the colorectum, juvenile polyps throughout the gastrointestinal tract or any number of juvenile polyps and a positive family history of juvenile polyposis. In about 50%-60% of patients diagnosed with juvenile polyposis syndrome a germline mutation in the *SMAD4* or *BMPR1A* gene is found. Both genes play a role in the BMP/TGF-beta signalling pathway. It has been suggested that cancer in juvenile polyposis may develop through the so-called "landscaper mechanism" where an abnormal stromal environment leads to neoplastic transformation of the adjacent epithelium and in the end invasive carcinoma. Recognition of this rare disorder is important for patients and their families with regard to treatment, follow-up and screening of at risk individuals. Each clinician confronted with the diagnosis of a juvenile polyp should therefore consider the possibility of juvenile polyposis syndrome. In addition, juvenile polyposis syndrome provides a unique model to study colorectal cancer pathogenesis in general and gives insight in the molecular genetic basis of cancer. This review discusses clinical manifestations, genetics, pathogenesis and management of juvenile polyposis syndrome.

Key words: Juvenile polyposis syndrome; Hamartoma; Colorectal cancer; *SMAD4*; *BMPR1A*

Peer reviewer: Zoran V Krivokapic, Professor, First Surgical Clinic, Third Department, Clinical Center of Serbia, 6, Dr Koste Todorovica, Belgrade, 11000 Serbia, Republic of Ireland

Brosens LAA, Langeveld D, van Hattem WA, Giardiello FM, Offerhaus GJA. Juvenile polyposis syndrome. *World J Gastroenterol* 2011; 17(44): 4839-4844 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4839.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4839

INTRODUCTION

Juvenile polyposis syndrome (JPS) is a rare autosomal dominant hereditary disorder characterized by multiple distinct juvenile polyps in the gastrointestinal tract and an increased risk of colorectal cancer. Sporadic solitary colorectal juvenile polyps occur in approximately 2% of the paediatric population but these polyps are not associated with an increased risk of gastrointestinal cancer[1,2]. Juvenile polyposis syndrome is defined by the presence of five or more juvenile polyps in the colorectum, juvenile polyps throughout the gastrointestinal tract or any

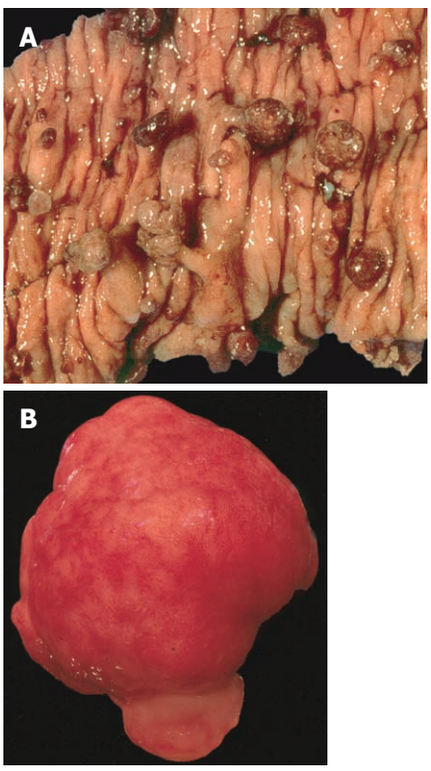

Figure 1 Macroscopic appearance of juvenile polyposis. A: Bowel resection of a patient with juvenile polyposis syndrome showing multiple spherical pedunculated polyps with a smooth surfaces; B: Gross appearance of a juvenile polyp from a patient with juvenile polyposis syndrome. Note the smooth surface, in contrast with a Peutz-Jeghers polyp.

number of juvenile polyps, and a positive family history of juvenile polyposis[1,3]. About 50%-60% of JPS patients have a germline mutation in the *SMAD4* or *BMPR1A* gene[4-6].

HISTOLOGY

The juvenile polyp is a histopathological entity first reported by Diamond[7] in 1939 and later described in more detail by Helwig[8]. Macroscopically, juvenile polyps vary in size from 5 mm to 50 mm, and typically have a spherical, lobulated and pedunculated appearance with surface erosion (Figure 1A and B). Microscopically, a juvenile polyp is characterized by an abundance of edematous lamina propria with inflammatory cells and cystically dilated glands lined by cuboidal to columnar epithelium with reactive changes (Figure 2A and B). The distinction between an inflammatory and a juvenile polyp is often difficult. In essence, juvenile polyps in juvenile polyposis syndrome appear similar to sporadic solitary juvenile polyps, although syndromic polyps often have a frond-like growth pattern with fewer stroma, fewer dilated glands and more proliferative smaller glands[9]. In addition, polyps in juvenile polyposis syndrome frequently show neoplastic changes to the epithelium not found in sporadic solitary juvenile polyps. Colorectal polyps from individuals with a *SMAD4* germline mutation often have a more proliferative epithelial phenotype and fewer stroma compared to those from patients with a *BMPR1A* germline mutation (Figure 2A and B)[10]. In addition, absence of the SMAD4 protein on immunohistochemistry of a juvenile polyp indicates that the patient carries a germline *SMAD4* mutation (Figure 2C)[11].

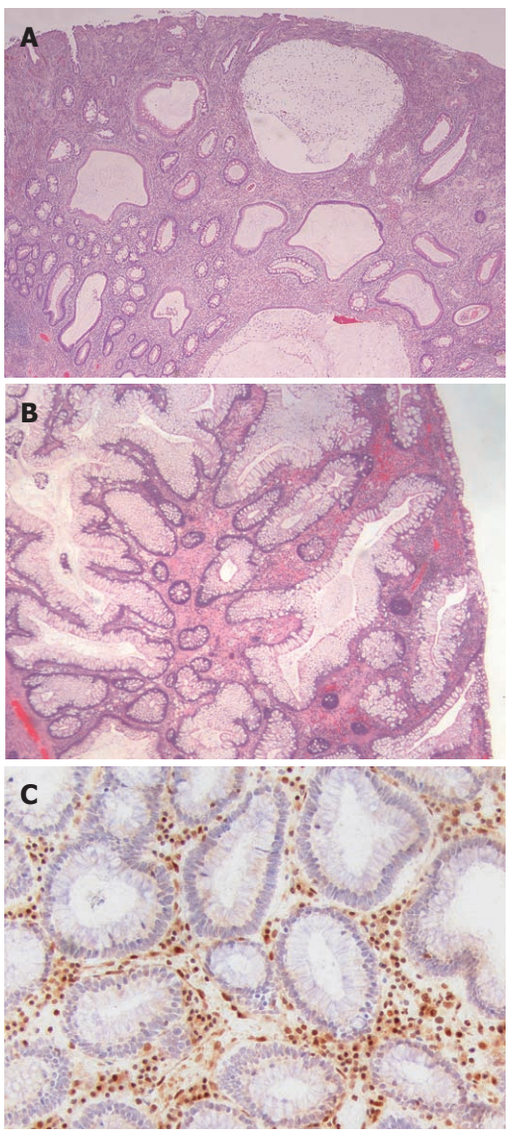

Figure 2 Histological appearance of juvenile polyposis. A: Histological section of a juvenile polyp from a juvenile polyposis patient with a germline mutation of *BMPR1A*. Typically, juvenile polyps are characterized by prominent lamina propria with edema and inflammatory cells, and cystically dilated glands lined by cuboidal to columnar epithelium with reactive changes; B: Histological section of a juvenile polyp from a juvenile polyposis patient with a germline mutation of *SMAD4*. This polyp shows relatively fewer stroma, fewer dilated glands and more proliferative smaller glands; C: SMAD4 immunohistochemistry on a juvenile polyp showing absent SMAD4 expression in the epithelium, indicating that this patient carries a germline *SMAD4* mutation.

Small intestinal polyps in JPS have been classified as juvenile[12,13], hyperplastic and/or inflammatory polyps[14-16], and as lymphoid hyperplasia[15,17]. The larger small intestinal polyps resemble juvenile polyps in the colon[17]. In addition, juvenile/hamartomatous polyps with dysplastic changes and adenomas have been found in the duodenum, jejunum, and ileum of patients with JPS[12,14,16]. Moreover,

we have seen a Brunner gland hamartoma in the duodenum of a juvenile polyposis patient with a *SMAD4* germline mutation. Most gastric polyps in JPS patients have been diagnosed as hyperplasic polyps[14] and are indistinguishable from gastric hyperplastic polyps[18].

GENETICS

A germline mutation in the *SMAD4* or *BMPR1A* gene is found in about 50%-60% of JPS patients[4-6]. Both genes are involved in the BMP/TGF-beta signalling pathway. Most germline defects are point mutations or small base pair deletions in the coding regions of *SMAD4* or *BMPR1A* that can be identified by conventional sequence analysis. About 15% of the germline genetic defects are deletions of one or more exons, or the entire *SMAD4* or *BMPR1A* coding sequence, which necessitates identification by techniques that analyze large genomic deletions, such as multiplex ligation-dependent probe amplification (MLPA)[4,6]. Recently, previously unknown mutations in the *BMPR1A* promoter region were found in about 10% of JPS patients[19].

About 30%-40% of JPS patients have no germline mutation; therefore, a number of candidate genes, mostly involved in the transforming growth factor β (TGF-β)/bone morphogenetic proteins (BMP) pathway, have been investigated for a role in JPS pathogenesis. Although not confirmed, and questioned by others, germline mutations of the TGB-β co-receptor Endoglin has been reported in two JPS patients[4,20]. In addition, *SMAD1*, *SMAD2*, *SMAD3*, *SMAD5*, *SMAD7*, *BMPR2*, *BMPR1B*, *ACVRL1, TGFBR II* and *CDX2* have been analyzed; however, no germline mutations have been found in these genes[20]. In addition, *PTEN*, the gene linked to Cowden (CS) and Bannayan-Riley-Ruvalcaba syndrome (BRRS), has been suggested as a *JPS* gene. However, *PTEN* mutations in patients with juvenile polyps likely represent CS or BRRS patients that have not (yet) developed extraintestinal clinical features specific to these conditions[21]. A recent study involving a large number of *PTEN* germline-mutation positive Cowden syndrome patients substantiated this notion by showing that both upper- and lower gastrointestinal polyps are a common manifestation of this syndrome[22]. Patients afflicted by Cowden syndrome may develop colorectal juvenile polyps indistinguishable from those in juvenile polyposis syndrome. Therefore, although the exact gastrointestinal manifestations of Cowden syndrome remain to be clarified, particularly with respect to the upper gastrointestinal tract, Cowden syndrome should be part of the differential diagnosis in a patient presenting with a juvenile polyp.

CLINICAL PRESENTATION

Clinically, juvenile polyposis can present in two forms. The first is called juvenile polyposis of infancy. This is a generalized form occurring in infants with polyps in the stomach, small bowel and colon. The polyps vary in size from 1 to 30 mm and may be sessile or pedunculated. These infants suffer from diarrhoea, haemorrhage, malnutrition and intussusception. Death usually occurs at an early age. In addition, many of these patients have congenital abnormalities, including macrocephaly and generalized hypotonia[23]. Some investigators suggest that this rare form of juvenile polyposis is caused by continuous deletion of *BMPR1A* and *PTEN* genes located on chromosome 10q23.2 and 10q23.3 respectively, although others disagree[24, 25].

In addition, generalized juvenile polyposis and juvenile polyposis coli (juvenile polyps restricted to the colorectum) have been defined[26]. However, these forms appear to be variable expressions of the same disease, because patients of both forms have been reported to segregate according to a dominant mode in the same family[24,27]. These forms may be sporadic, i.e., 'de novo', or inherited, and usually present later in childhood or in adult life. They are characterized by the presence of gastrointestinal juvenile polyposis and an increased risk of gastrointestinal cancer[28]. A variety of extra-intestinal manifestations have been reported in these patients[23]. In approximately 50% of juvenile polyposis coli or generalized JPS cases, a heterozygous germline mutation in the *SMAD4* or *BMPR1A* gene is identified[24]. Several differences in phenotypic expression between carriers of a *SMAD4* and *BMPR1A* mutations have been noted. *SMAD4* mutations are associated with a more aggressive gastrointestinal phenotype, involving higher incidence of colonic adenomas and carcinomas and more frequent upper gastrointestinal polyps and gastric cancer than patients with a *BMPR1A* mutation[6,29,30]. Also, the combined syndrome of JPS and hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome) is associated with germline mutations in *SMAD4*[31].

POLYP DISTRIBUTION

Polyps in JPS predominantly occur in the colorectum, varying in number from five to several hundred. In addition, polyps can be found in the stomach, duodenum, jejunum, and ileum, although the incidence of upper gastrointestinal tract polyps in JPS is less well studied. Rarely, profuse gastric juvenile polyposis is found in the absence colonic polyps[32]. As noted above, upper gastrointestinal polyposis and gastric cancer has been associated with *SMAD4* germline mutation[6,29,30].

Few studies systematically examined upper gastrointestinal tract involvement in juvenile polyposis[12,14,33]. One investigation found gastric polyps in 10 out of 12 (83%) patients, mostly located in the antrum, but throughout the stomach in six individuals. Rarely, profuse gastric juvenile polyposis is found in the absence of colonic polyps. Duodenal polyps were found in four out of 12 (33%) JPS patients, with two having multiple polyps ranging in size from 0.5 to 1.5 cm. and two others with minute polyps[14]. Using capsule endoscopy, small-bowel polyps beyond the range of standard gastroscopy were found

in 2 of 10 (20%) patients and duodenal polyps in 4 others (40%)[33]. Another study reported small bowel polyps in 8 of 56 JPS patients (14%)[12]. Moreover, a number of case reports of duodenal, jejunal, and ileal polyps in JPS patients exist[13,15-17,34], In addition, juvenile polyps are frequently found in the ileal pouch of juvenile polyposis patients who have undergone proctocolectomy[35,36].

CANCER RISK

Juvenile polyposis is associated with an increased risk of gastrointestinal cancer. A recent cancer risk analysis calculated a cumulative life-time risk for colorectal cancer in JPS of 39% and a relative risk of colorectal cancer of 34[37]. However, this may be a conservative estimate, because some patients in this study had already undergone prophylactic colectomy. Jass reported a 68% cumulative risk of colorectal cancer in patients from the St Mark's Registry, but details were not provided[38]. In addition, several cases of stomach, duodenal, and pancreatic cancer in JPS have been described in the literature, but no formal risk analysis for these malignancies exists[28]. One study found small bowel carcinoma in six out of 56 (11%) JPS patients, but four of these cancers occurred in one family[12]. Evaluation of literature reports suggests that gastric and small bowel carcinoma, together, occur at about one-fifth the frequency of colorectal cancers in this patient group[37].

CANCER PATHOGENESIS

Cancer pathogenesis in juvenile polyposis has not yet been unravelled and may develop through the so-called "landscaper mechanism". The landscaper model was proposed after the observation that the genetic alterations at chromosome 10q22 (*BMPR1A* locus) occurred predominantly in the stroma of juvenile polyps. This paradigm postulates that cancer develops as a result of an abnormal stromal environment, which leads to neoplastic transformation of the adjacent epithelium[39]. Support for a "landscaper" defect triggering juvenile polyposis came from a study in which disrupted BMP signalling, through expression of a natural pathway inhibitor, resulted in development a juvenile polyposis-like phenotype in mice[40]. BMP-4 expression is normally limited to the mesenchymal compartment of the murine intestine, suggesting that disruption of this mesenchymal signal is involved in mediating juvenile polyposis.

Notwithstanding the concept that faulty transmission or receipt of mesenchymal signals by the epithelium may trigger juvenile changes, others have found that homozygous *SMAD4* deletions are limited to the epithelium of juvenile polyps from JPS patients with germline *SMAD4* mutations and in *Smad4* knockout mice[11,41]. Although further studies are needed, this suggests that *SMAD4* may act as a "gatekeeper", instead of a "landscaper" in JPS pathogenesis, consistent with the role of *SMAD4* in other cancer types[42].

MANAGEMENT

Management of JPS is mainly based on expert opinion[23,43-45]. Patients at risk or with a high suspicion of JPS should have endoscopic screening of the colon and upper gastrointestinal tract at age 15 or at the time of first symptoms[44]. At diagnosis of JPS, the entire gastrointestinal tract should be examined for the presence of polyps[23]. Genetic testing can be useful for at-risk members from families, where germline mutations have been identified. If no germline mutation is found in at-risk persons, then they do not have JPS and can be followed according to the guidelines for screening programs for the general population[44].

Endoscopic examination of the colon and upper gastrointestinal tract is recommended every two to three years in patients with JPS. In patients with polyps, endoscopic screening should be performed yearly, until the patient is deemed polyp-free. Patients with mild polyposis can be managed by frequent endoscopic examinations and polypectomy[23,36,44]. Intraoperative enteroscopy to evaluate small intestinal polyps can be considered at the time of colorectal surgery[16]. Endoscopic treatment of gastric polyps can be difficult, and patients with symptomatic gastric polyposis (e.g., severe anaemia) may need subtotal or total gastrectomy.

Prophylactic surgery is considered in patients with colorectal polyposis unmanageable by endoscopy (> 50-100 polyps), those with severe gastrointestinal bleeding or diarrhoea, juvenile polyps with dysplasia, and patients with a strong family history of colorectal cancer[35-37]. Surgical options include subtotal colectomy with ileorectal anastomosis, or total proctocolectomy with pouch[35,36]. Analogous to familial adenomatous polyposis, surgical type may depend on the extent of rectal polyposis. Recurrence of rectal polyps in patients with subtotal colectomy is frequent, and about half of these individuals require subsequent proctectomy[35,36]. Therefore, total proctocolectomy has been advocated as the initial surgery for patients with massive juvenile polyposis, who are unable to be managed endoscopically[36]. Although the surgery of choice in JPS remains debatable, patients need frequent post-operative endoscopic surveillance because of the high recurrence rates of polyps in the remnant rectum and the pouch[35].

In JPS patients with a germline *SMAD4* mutation, screening should be considered for signs of hereditary hemorrhagic teleangiectasia, including chest radiography for arteriovenous malformations, magnetic resonance imaging of the brain, and liver sonography[31]. Digital clubbing and pulmonary osteoarthropathy are frequently described in combination with arteriovenous malformations[17].

COX-2 expression is higher in JPS polyps than in sporadic juvenile polyps and correlates with polyp size and dysplasia[46]. This observation suggests that chemoprevention using selective or non-selective *COX-2* inhibitors could be beneficial in JPS. Currently, nonsteroidal anti-in-

flammatory drugs (NSAID) chemoprevention in JPS has not been systematically studied; however, two JPS patients who had undergone proctocolectomy with pouch reconstruction and subsequent polypectomy from the pouch had no further polyp development in the pouch while on sulindac[35]. However, the value of NSAID chemoprevention in JPS requires further investigation.

CONCLUSION

JPS is a rare hamartomatous polyposis syndrome characterized by the presence of multiple distinct juvenile polyps in the gastrointestinal tract. The primary defect in JPS may be stromal rather than epithelial. This so-called 'landscaper' defect may ultimately lead to neoplastic transformation in the overlying epithelium, although the polyps are not neoplastic per se. On the contrary, juvenile polyps may be considered true hamartomas, i.e., anomalies in the developmental patterning of the gut. Juvenile polyposis syndrome is, therefore, a unique model for studying carcinogenesis in the gastrointestinal tract.

Although rare, recognition of this condition is important in view of the consequences for patients and their families. Each clinician confronted with the diagnosis of a juvenile polyp should consider the possibility of juvenile polyposis syndrome. The number of juvenile polyps should be documented, along with the family history of gastrointestinal polyps and cancer. If a patient fulfils the clinical criteria of JPS, further diagnostic evaluation is indicated.

Future studies on the molecular and clinical aspects of JPS will result in a better understanding of gastrointestinal carcinogenesis and improved management of patients afflicted by this disorder.

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S- Editor Wu X **L- Editor** Stewart GJ **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4845

World J Gastroenterol 2011 November 28; 17(44): 4845-4852
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Proteome-based biomarkers in pancreatic cancer

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Received: May 13, 2011 Revised: August 1, 2011
Accepted: August 8, 2011
Published online: November 28, 2011

Abstract

Pancreatic cancer, as a highly malignant cancer and the fourth cause of cancer-related death in world, is characterized by dismal prognosis, due to rapid disease progression, highly invasive tumour phenotype, and resistance to chemotherapy. Despite significant advances in treatment of the disease during the past decade, the survival rate is little improved. A contributory factor to the poor outcome is the lack of appropriate sensitive and specific biomarkers for early diagnosis. Furthermore, biomarkers for targeting, directing and assessing therapeutic intervention, as well as for detection of residual or recurrent cancer are also needed. Thus, the identification of adequate biomarkers in pancreatic cancer is of extreme importance. Recently, accompanying the development of proteomic technology and devices, more and more potential biomarkers have appeared and are being reported. In this review, we provide an overview of the role of proteome-based biomarkers in pancreatic cancer, including tissue, serum, juice, urine and cell lines. We also discuss the possible mechanism and prospects in the future. That information hopefully might be helpful for further research in the field.

Key words: Biomarkers; Mass spectrometry; Pancreatic cancer; Proteome; Screening

Peer reviewer: Tatjana Crnogorac-Jurcevic, MD, PhD, Cancer Research UK, Molecular Oncology Unit, Barts and The London School of Medicine and Dentistry, John Vane Science Centre, Charterhouse Square, London EC1M 6BQ, United Kingdom

Sun C, Rosendahl AH, Ansari D, Andersson R. Proteome-based biomarkers in pancreatic cancer. *World J Gastroenterol* 2011; 17(44): 4845-4852 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4845.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4845

INTRODUCTION

Pancreatic cancer is a highly lethal disease and despite continuous research efforts, results have only marginally improved patient outcome with minor overall changes in death rate over the last four decades. Pancreatic cancer is the fourth cause of cancer-related death and 36 800 pancreatic cancer-related deaths were reported in the United States in 2010, corresponding to 6.5% of all deaths from cancer[1]. Similar overall observations are reported from the other parts of the Western world[2-5].

Surgical resectable pancreatic cancer is associated with an improved outcome, especially if the diagnosis is obtained in an early phase. Regrettably, most symptoms, including e.g., profound weight loss, abdominal pain, new onset type 2 diabetes mellitus, jaundice and nausea, are usually vague and occur late during the course of disease. Only 20% of patients with pancreatic cancer are candidates for a potentially curative resection[6]. Efficient tumor markers for population screening are absent. Current markers used for pancreatic cancer, especially carcinoembryonic antigen and cancer antigen 19-9 (CA19-9), lack appropriate sensitivity and specificity. Biomarkers for therapeutic assessment, detection of residual or recurrent cancer and even for targeted therapy in pancreatic cancer in a more customized fashion are needed. The identification of biomarkers in pancreatic cancer is thus essential for improving outcome.

The development of proteomic techniques has increased the interest for clinical applications of biomarkers in pancreatic cancer. However, the identification of suitable biomarkers with good sensitivity and specificity for clinical use in pancreatic cancer has been sparse. In this review, we focus on potential proteome-based biomarkers to be used in pancreatic cancer (Table 1), hopefully indicative for further research within the field.

PROTEOMIC-BASED BIOMARKERS IN PANCREATIC CANCER TISSUE

Pancreatic cancer tissue is the most direct source of proteomic biomarkers for cancer detection, as it is likely to have the highest concentrations of cancer-specific markers. However, there are two major reasons that make it less available for cancer screening. Invasive biopsy material for screening is usually not readily available, and percutaneous biopsies might even result in seeding of cancer cells. Pathological evaluation renders the final diagnosis and is the best choice when pancreatic cancer tissue is available. Ongoing biomarker research obtained from pancreatic cancer tissue is not only done for diagnostic purposes, but also for the development of potential future targeted therapies.

When comparing pancreatic cancer tissue with normal pancreatic tissue by MALDI-TOF-MS, the levels of galectin-3 and calgizzarin (S100A11) protein were found to be 3-fold higher in cancer patients[7]. Galectin-3 is a member of a family of β-galactoside-binding animal lectins, and has been found helpful in diagnosing e.g., thyroid cancer[7], but is also up-regulated in liver, stomach, and tongue cancers. In the same family, galectin-1 has also been identified as a potential biomarker[8,9]. S100A11, a calcium-binding protein and a member of the S100 protein family is expressed in the nucleus and cytoplasm. S100 proteins regulate a number of cellular processes like cell growth, cell cycle, differentiation, transcription and secretion. S100 proteins have been reported overexpressed in different cancers, like breast and thyroid cancer[10]. Other studies have shown up-regulation of S100A6, S100A8, S100A9 and S100A10 in pancreatic cancer[11-13]. Using laser capture microdissection and 2-DE to analyze protein expression in stromal components of pancreatic cancer, it was demonstrated that high levels of S100A8 and S100A9 were present in the tumor-associated stroma but not in benign or malignant epithelium[12]. Immunohistochemistry confirmed high levels of both S100A8 and S100A9 in specific stromal cells, which were later identified as monocytes or immature macrophages ($CD14^+$/CD68). In a subset of these cells, S100A8 and S100A9 were co-expressed, and this relationship appeared to be influenced by the Smad4 status of the corresponding tumor cells. This study provides further evidence of the complex tumor-stroma interaction and demonstrates that stromal tissue can become a novel and highly promising source of biomarkers.

The differential diagnosis between pancreatic cancer and mass-forming chronic pancreatitis is clinically challenging. A large-scale immunoblotting analysis with more than 900 primary antibodies was performed on cancer tissue, chronic pancreatitis and normal pancreas[14]. A total of 30 proteins were found to be differentially expressed between chronic pancreatitis and normal pancreas, while 102 proteins were different between pancreatic cancer and normal pancreas. Several proteins, such as UHRF1, ATP7A and AOX1, differed in their expression between chronic pancreatitis and pancreatic cancer, suggesting their importance in pancreatic carcinogenesis. The combination of these proteins can become a useful diagnostic tool for endoscopic ultrasonography-guided fine needle aspiration specimens obtained before surgery or treatment.

Pancreatic cancer (PDAC) develops through several phases of pancreatic intraepithelial neoplasia (PanINs) lesions from benign to fully malignant. Pancreatic cancer pathology may be helpful for diagnosis and treatment by providing knowledge on which phases the patient is in. Despite research showing different genetic alternations during different phases, such as *K-ras* in the early PanIN-1A/B, *p16* in the intermediate PanIN-2 and *p53* in the late PanIN-3 phases[15], biological mechanisms still remain largely unclear. One reason is the difficulty in studying early molecular changes in pancreatic cancer, due to lack of suitable tissue specimens, as patients in the early phase often are without existing symptoms, and thus tissue samples are not available. Plectin-1 has been shown to be up-regulated in PanINs and in PDAC in genetic defect mouse models and early-stage pancreatic cancer cell lines[16]. Plectin-1 is a large 500 kDa protein associated with filamentous-actin, microtubules and intermediate filaments. Plectin-1 was found to be exclusively associated with mitochondria and may thus provide an important link of this organelle with the intermediate filament system[17]. Plectin-1 can also bind specific peptides, which may be helpful in detecting precursor lesions and PDAC, when conjugated to magnetofluorescent nanoparticles.

Proteome changes of pancreatic cancer tissue during different stages have been identified by 2-DE. Five candidate protein biomarkers were selected from a total of 31 identified nonredundant proteins, including 14-3-3 sigma, major vault protein (MVP), anterior gradient 2 (AGR2) and Annexin A4[13]. AGR2 is increased early on during tumor progression, and is also present in pancreatic juice[18]. MVP expression, associated with the PI3K pathway, and 14-3-3 sigma were found to be increased in PanIN-2 and -3[19,20]. On the other hand, annexin A4 was down-regulated. Annexin A4 is a Ca^{2+}- and phospholipid-binding protein like annexin A2, which previously has been reported in PDAC[21].

To decrease the complexity and large dynamic range of proteins found in pancreatic tissue samples, subcellular fractionation with mass spectrometric techniques has been used to identify potential biomarkers associated with pancreatic cancer. McKinney *et al*[22] and colleagues

Table 1 A selection of potential biomarkers for pancreatic cancer

Potential biomarkers for pancreatic cancer	Expression	Proteomic tools	Ref.
Tissue			
14-3-3 sigma	+	2-D SDS PAGE, MS	[13]
Annexin A4	+	2-D SDS PAGE, MS	[13]
Anterior gradient 2	+	2-D SDS PAGE, MS	[13]
AOX1	-	PowerBlot	[14]
ATP7A	+	PowerBlot	[14]
Biglycan	+	SDS-PAGE, LC-MS/MS	[22]
Galectin-1	+	2-D SDS PAGE, MS	[9]
Galectin-3	+	MALDI-TOF-MS	[7]
Gelsolin	-	Proteomic chip	[23]
Major vault protein	+	2-D SDS PAGE, MS	[13]
Pigment epithelium-derived factor	+	SDS-PAGE, LC-MS/MS	[22]
Plectin-1	+	Western blotting	[16]
S100A6	+	2-D SDS PAGE, MS	[11]
S100A8 (stroma)	+	2DE, LC-MS/MS	[12]
S100A9 (stroma)	+	2DE, MALDI-TOF-MS	[12]
S100A10	+	2-D SDS PAGE, MS	[13]
S100A11	+	MALDI-TOF-MS	[7]
Thrombospondin-2	+	SDS-PAGE, LC-MS/MS	[22]
βIGH3	+	SDS-PAGE, LC-MS/MS	[22]
UHRF1	+	PowerBlot	[14]
Serum/plasma			
α-1B-glycoprotein precursor	+	DIGE, MS/MS	[38]
Anterior gradient 2	+	iTRAQ, MS/MS	[18]
Apolipoprotein A-Ⅱ	-	SELDI-TOF, MS	[24]
Apolipoprotein C-Ⅰ	+	SELDI-TOF, MS	[24]
Caldecrin	-	ICAT, MS	[43]
CXCL 7	-	LC-MS	[29]
DJ-1	+	DIGE, MS/MS	[38]
Fibrinogen β chain	+	ICAT, MS	[43]
HSP27	+	Protein-chip technology	[33]
Pancreatic juice			
Lithostathine I α	-	2DE, MALDI-TOF-MS	[40]
Matrix metalloproteinase-9	+	DIGE, MS/MS	[38]
Neural cell adhesion molecule L1	+	ICAT, MS	[43]
p-Akt	+	Bio-Plex suspension array	[32]
p-ERK1/2	+	Bio-Plex suspension array	[32]
Phosphor-cAMP response element binding protein	+	Bio-Plex suspension array	[32]
Phosphor-p90 ribosomal S6 kinase	+	Bio-Plex suspension array	[32]
p-IκB-α	+	Bio-Plex suspension array	[32]
Plasminogen	+	ICAT, MS	[43]
Platelet factor 4	-	MALDI-TOF	[31]
p-MEK1	+	Bio-Plex suspension array	[32]
Transthyretin	+	2DE, MALDI-TOF-MS	[45]
Urine			
Annexin A2	-	2-D SDS PAGE	[46]
CD59	-	2-D SDS PAGE	[46]
Gelsolin	-	2-D SDS PAGE	[46]
Cell lines			
Apoprotein E	+	SILAC	[48]
Cadherin	Not in metastatic tumor cell	LC-MS/MS	[52]
Catenin	Not in metastatic tumor cell	LC-MS/MS	[52]
CD9	+	SILAC	[48]
Fibronectin receptor	+	SILAC	[48]
Galectin	Not in primary tumor cell	LC-MS/MS	[52]
Glucagon	+	Protein array	[53]
Integrin	Not in metastatic tumor cell	LC-MS/MS	[52]
Perlecan	+	LC-MS/MS	[48]
Prolactin	-	Protein array	[53]

SILAC: Stable isotope labelling with amino acids in cell culture; DIGE: Difference gel electrophoresis; MS: Mass spectrometry; iTRAQ: Isobaric tags for relative and absolute quantification; ICAT: Isotope-coded affinity tag; βIGH3: Ig-h3 precursor; A1BG: α-1B-glycoprotein precursor; +: Up-regulated; –: Down-regulated expression in pancreatic cancer as compared with controls.

identified 2393 unique proteins in normal and pancreatic tissue with cancer, and determined 104 proteins that were significantly changed in pancreatic cancer. Four secreted and up-regulated proteins have been validated as potential

biomarkers for diagnosing pancreatic cancer, biglycan, pigment epithelium-derived factor, thrombospondin-2 and transforming growth factor β induced protein Ig-h3 precursor, though data for sensitivity and specificity for these markers are not yet available.

Information on lymph node metastasis is very important for the surgical strategy-making and also for deciding other additional treatments (e.g., chemotherapy). Proteome comparison of pancreatic cancer tissue with corresponding non-cancerous normal tissue obtained from the same patients on antibody capture-based proteomic chips[23] identified gelsolin as a candidate biomarker for detection of lymph node metastasis in pancreatic cancer. Gelsolin is an important actin-binding protein that plays a major role in maintaining an organized actin cytoskeleton. The expression of gelsolin in pancreatic ductal adenocarcinomas with lymph node involvement (71.4%) was reported markedly increased as compared to lymph node negative pancreatic cancers (20%)[23].

BIOMARKERS IN BODY FLUIDS

Serum and plasma

Blood is the most frequently used source for biomarkers, being minimally invasive, easily accessible, generally inexpensive and reproducible to obtain and analyse. However, some highly abundant proteins, such as albumin or globulin, can affect the detection of less abundant, but for the diagnosis, valuable proteins.

One study aimed to identify biomarkers in a total of 319 serum samples from pancreatic cancer patients and controls. Using SELDI-TOF MS technology, 21 peaks were identified to be differentially expressed between pancreatic cancer and disease controls (DC), and 18 peaks between pancreatic cancer and healthy volunteers (HV)[24]. Apolipoprotein C-Ⅰ (ApoC-Ⅰ) and apolipoprotein A-Ⅱ (ApoA-Ⅱ) were significantly increased and decreased, respectively. ApoC-Ⅰ plays an important role in controlling plasma lipid metabolism, and is expressed in gastric, breast and pancreatic cancer[25]. ApoA-Ⅱ is present on the surface of lipid particles and may play a diagnostic role in prostate cancer[26]. The receiver operating characteristic area under the curve (AUC) of ApoA-Ⅱ, ApoC-Ⅰ and CA19-9 was greater than that of CA19-9 alone for pancreatic cancer *vs* DC (0.90 *vs* 0.84) and for pancreatic cancer *vs* HV (0.96 *vs* 0.90), results supported by others[27].

CXCL-7 is a chemokine member of the angiogenic ELRb CXC chemokine family, expressed within the megakaryocyte lineage[28]. Using a novel combination of hollow fiber membrane-based low-molecular-weight protein enrichment and LC-MS-based quantitative shotgun proteomics identified a peptide derived from CXCL-7 to be significantly reduced in pancreatic cancer patients[29]. These authors compared the plasma proteome in a small cohort (24 patients with pancreatic cancer and 21 healthy controls) to get 53 009 MS peaks. They then further validated their CXCL-7 finding in an independent blinded cohort (n = 237) using a high-density reverse-phase protein microarray. Combination with CXCL-7 significantly improved the AUC value of CA19-9 to 0.961. However, in this study, the precise molecular mechanisms explaining the CXCL-7 reduction in patients with pancreatic cancer remained unclear. Platelet factor 4 (PF4) is another member of the CXC chemokine family (CXCL-4), and is present in α-granules of all mammalian platelets, as well as in the granules of mast cells[30]. PF4 had been identified as a potential marker for pancreatic cancer by MALDI-TOF-MS-based clinical serum profiling in 80 samples[31]. Validation by ELISA techniques in 40 serum samples showed the AUCs of PF4 concentrations used for the discrimination between healthy controls and pancreatic cancer was 0.833. The discrimination between patients with pancreatic cancer and acute pancreatitis was 0.829.

Protein phosphorylation is one of the most common ways of modifying biological systems, including the carcinogenic progress. Several phosphoprotein levels were significantly increased in serum from pancreatic cancer patients as compared to controls. Six candidate phosphoproteins have been found in serum of pancreatic cancer patients by using a Bio-Plex suspension array; p-ERK1/2, p-MEK1, phospho-p90 ribosomal S6 kinase (p-p90RSK), phospho-cAMP response element binding protein (p-CREB), p-Akt and p-IκB-α[32]. These phosphoproteins are associated with the Ras/Raf/MEK/ERK signalling pathway, which is a dominating growth promoting pathway in pancreatic carcinomas. Further data from the same study showed a simultaneous increase in phospho- and total-ERK1/2 with a positive correlation to pancreatic cancer patients. In detecting pancreatic cancer, a combination of p-ERK1/2 and CA19-9 can potentially avoid false-negatives (87.2%) and improve the discriminatory power.

Heat shock protein 27 (HSP27) is a powerful molecular chaperone that can prevent the aggregation of nascent and stress-induced misfolded proteins[33]. HSP27 has been identified in serum of pancreatic cancer patients by ProteinChip technology and 2-DE. HSP27 was found to be up-regulated in pancreatic cancer as compared with normal tissue, with a sensitivity of 100% and a specificity of 84% in the detection of pancreatic cancer, and has further been suggested to play an important role in gemcitabine resistance[34,35].

Pancreatic juice

Pancreatic juice is rich in proteins directly secreted from pancreatic ductal cancer cells and should therefore constitute a perfect source for specific protein biomarkers for pancreatic cancer detection. However, pancreatic juice is not readily accessible and in addition, the endoscopic retrograde cholangiopancreatography procedure per se, in order to obtain pancreatic juice, may induce acute pancreatitis in 4%-7% of patients[36]. To date approximately 170 proteins have been identified in human pancreatic juice, one third of which are enzymes[37].

When comparing pancreatic juice from 9 PDAC patients and 9 healthy volunteers by using difference gel electrophoresis and tandem mass spectrometry (MS/MS), three potential biomarkers were identified: matrix metalloproteinase-9 (MMP-9), oncogene DJ1 (DJ-1) and α-1B-glycoprotein precursor (A1BG)[38]. DJ-1 is a mitogen-dependent protein involved in the Ras signalling pathway, reported to be increased in serum from pancreatic cancer patients[33,39]. A1BG, a secreted plasma protein from the immunoglobulin superfamily, was also increased in the cytoplasm of malignant epithelia in 86.3% of pancreatic cancer tissue specimens.

Obstruction of the main pancreatic ducts may alter the protein composition of pancreatic juice. Comparing the 2-DE profiles of pancreatic juice from a patient with pancreatic body cancer and a patient with benign pancreatic disease, it was found that blockade of juice secretion strongly affected protein composition[40]. A subsequent analysis of patients with comparable obstruction of the pancreatic ducts was performed. The isomeric form of lithostathine I α was identified as one of five protein spots that were consistently reduced in pancreatic cancer.

Quantitative proteomic analysis using stable isotope labelling (iTRAQ) and MS/MS were applied to identify proteins abnormalities, elevated in the pancreatic juice from PanIN-3 patients[18]. Anterior gradient-2 (AGR2) was significantly increased in PanIN-3 juice samples among 20 differently expressed proteins. AGR2 is a secreted protein and over-expressed in many cancers, including pancreatic cancer and influences pancreatic cancer cell proliferation and invasion. Further analyses showed that AGR2 had 67% sensitivity and 90% specificity in predicting PanIN-3 in pancreatic juice samples[41,42]. Proteomics can also be used to differentiate pancreatic cancer from pancreatitis. In a comparative study between pancreatic cancer and pancreatitis by Isotope-Coded Affinity Tag and MS, 72 variable proteins were identified in pancreatic juice[43]. Some of the identified proteins, including fibrinogen β-chain, plasminogen, neural cell adhesion molecule L1 and caldecrin, demonstrated at least a 2-fold change in abundance in pancreatic juice. In addition, 9 proteins (hemoglobin, fibrinogen, trypsin Ⅰ, trypsin Ⅱ, chymotrypsinogen b, Ig-α1 chain c region, Ig-μ chain c region, ribonuclease, and human serum albumin) were up-regulated both in the pancreatic juice of pancreatitis and pancreatic cancer patients.

Transthyretin (TTR) was identified as a potential protein biomarker in pancreatic juice for the detection of pancreatic cancer. Using 2-DE and MALDI-TOF-MS, it was shown that TTR in the pancreatic juice increased more than 2-fold in pancreatic cancer as compared with chronic pancreatitis and choledocholithiasis[44]. However, TTR was only present in islet cells and not expressed in pancreatic cancer cells, in line with what has been reported by others[45].

Urine

Urine is a potential source of biomarkers, as it is easily and noninvasively available. However, a limitation of urine is the dilution of the proteins of potential interest. Secondly, the urine is derived from the kidneys, only being "in contact" with the pancreas through blood, and most of the proteomic information exists in circulating blood.

Using proteomic techniques multiple deregulated proteins were detected in urine samples from patients with pancreatic cancer, implicating urine to potentially be a valuable source of biomarkers for pancreatic cancer[46]. Five potential protein biomarkers (including annexin A2, gelsolin, CD59 and S100A9) from a total of 127 statistically valid and differentially expressed protein spots were identified, most of which have been reported associated with pancreatic cancer in other studies.

BIOMARKERS IN CELL LINES

Cell lines are the most easily obtained proteomic source. This allows analysis of secreted proteins. The most relevant limitation when using data obtained from cell lines is that it may not be representative for primary tissue samples in the clinical setting. Thus, few studies have used cell lines for identifying biomarkers of relevance in pancreatic cancer[47].

By analysis of secreted proteins between Panc-1 pancreatic cancer cells and immortalized non-neoplastic HPDE cells, 145 differentially secreted proteins were identified. Several proteins were validated by immunohistochemistry, such as CD9, perlecan, apoprotein E (ApoE) and fibronectin receptor[48]. CD9 is a membrane protein expressed on the surface of human platelets. CD9 plays a role in many cellular functions, like adhesion, migration, signal transduction and differentiation[49]. Perlecan is involved in angiogenesis and growth, as a receptor for basic fibroblast growth factor[50]. ApoE is a protein component of lipoproteins that has anti-tumor activity in pancreatic cancer[51]. Fibronectin receptor is another member of the integrin family.

Development of metastases, as part of the progress of pancreatic cancer, evidently involves a number of important proteins. Proteomic research comparing primary and metastatic PDAC cell lines can reveal functional proteins, which are helpful for the prediction of metastasis and potential therapy against this process. One metastatic PDAC cell line, AsPC-1 and one primary PDAC cell line, BxPC-3, were studied for this purpose[52]. Using SDS-PAGE and LC-MS/MS, 221 and 208 proteins were identified from AsPC-1 and BxPC-3 cells, respectively, with 109 proteins present in both cell lines. Analysis of other proteins showed different levels in the two cell lines, including catenin, cadherins, integrins, galectins, annexins and collagens. Cadherins are a class of type-1 transmembrane proteins that depend on calcium ions and combined complexes with catenin to mediate cell adhesion. They were all found in primary tumor cells (BxPC-3), but not in metastatic tumor cells (AsPC-1), suggesting a defect in cellular adhesion in metastatic AsPC-1 cells. Integrins are glycoprotein members that form heterodimeric receptors.

Integrin α2 and α5, which represent major adhesion molecules, were only identified in BxPC-3 cells. Conversely, galectins, as carbohydrate-binding proteins on the cell surface and extracellular glycoproteins, were found only in AsPC-1. Most of these proteins play a role in tumor cell adhesion and motility.

Springbio Antibody Microarrays were used to detect different proteins between the pancreatic cancer cell lines SW1990 and SW1990HM, highly liver metastatic-related cell lines[53]. Increased glucagon and decreased prolactin were selected as potential biomarkers for cancer detection from 40 reproducible, altered proteins. Glucagon induces glucose production and regulates carbohydrate and protein metabolism. Prolactin is a hormone released by the pituitary gland with effects on female breast development and milk production. Both are localized at the plasma membrane, and can influence tumor cell adhesion.

FUTURE ASPECTS

Compared with other types of cancers, pancreatic cancer is probably one of the solid tumors with the highest levels of genetic alterations resulting in aberrant expression of a large number of proteins. A panel of proteomic biomarkers with the appropriate combination of high sensitivity and specificity will likely be better than a single biomarker. Many researchers have focused on proteomic profiling for pancreatic cancer detection using a combined biomarker approach and results so far have gained interest[54-56]. In addition, some studies have investigated differentially expressed proteins of pancreatic cancer stem cells, where increasing evidence indicates an important role in tumorigenesis, growth and formation of metastasis[57]. Targeting and eliminating pancreatic cancer stem cells may significantly improve the prognosis and avoid recurrence in pancreatic cancer patients after pancreatic resection[58].

CONCLUSION

Due to the characteristics of pancreatic cancer, with often vague symptoms, but associated tumor aggressiveness, resistance to standard therapy and a poor prognosis, identification of sensitive and specific biomarkers is essential. Such biomarkers would be of extreme value for disease detection during an earlier phase. Up to now, despite the development of novel techniques and potential markers reported, only a limited number may be of potential use in the clinical situation. Research on pancreatic cancer biomarkers is, however, intensive and the use of proteomic technology may provide a completely novel tool and possibility of potential improvement, achieving early diagnosis, targeted therapy, and discovery of recurrence in patients with pancreatic cancer.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4853

World J Gastroenterol 2011 November 28; 17(44): 4853-4857
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Hepatitis B virus infection and the risk of hepatocellular carcinoma

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Received: March 20, 2011 Revised: July 6, 2011
Accepted: July 13, 2011
Published online: November 28, 2011

Abstract

Epidemiological studies have provided overwhelming evidence for a causal role of chronic hepatitis B virus (HBV) infection in the development of hepatocellular carcinoma (HCC). However, the pathogenesis of HBV infection and carcinogenesis of HBV-associated HCC are still elusive. This review will summarize the current knowledge on the mechanisms involved in HBV-related liver carcinogenesis. The role of HBV in tumor formation appears to be complex, and may involve both direct and indirect mechanisms. Integration of HBV DNA into the host genome occurs at early steps of clonal tumor expansion, and it has been shown to enhance the host chromosomal instability, leading to large inverted duplications, deletions and chromosomal translocations. It has been shown that the rate of chromosomal alterations is increased significantly in HBV-related tumors. Prolonged expression of the viral regulatory HBV x protein may contribute to regulating cellular transcription, protein degradation, proliferation, and apoptotic signaling pathways, and it plays a critical role in the development of hepatocellular carcinoma.

Key words: Hepatocellular carcinoma; Hepatitis B virus infection; Hepatitis B virus genotypic variations; Hepatitis B virus x protein

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Tan YJ. Hepatitis B virus infection and the risk of hepatocellular carcinoma. *World J Gastroenterol* 2011; 17(44): 4853-4857 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4853.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4853

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, and the third most common cause of cancer mortality[1,2]. This tumor, which arises from hepatocytes, is often associated with liver cirrhosis resulting from chronic liver diseases. Among the environmental risk factors, the prevalence of chronic hepatitis B and C virus infections is linked directly to the incidence of HCC. There is now evidence for persistence within the tumor cells of a low level HBV multiplication potential. Hepatitis B virus (HBV) DNA replicative molecules and covalently closed circular DNA (cccDNA) are detectable by polymerase chain reaction (PCR). Moreover, the association between HCC and HBV recurrence after liver transplantation, and the detection of cccDNA in HCC cells point toward the possibility of HBV replication in tumor cells. The latter could act as potential reservoirs for HBV recurrence, especially in patients who present with a recurrence of HCC[3]. So far, chronic and persistent infection with hepatitis B virus is a major risk factor for the development of HCC.

Globally, it is estimated that 350 million people are chronically infected with the HBV[4]. Approximately 25% of

chronically HBV-infected individuals will develop HCC[5]. Chronic carriers of HBV have up to a 30-fold increased risk of HCC[6]. In areas of high HBV endemicity, persons with cirrhosis have an approximately 16-fold higher risk of HCC than the inactive carriers, and a 3-fold higher risk for HCC than those with chronic hepatitis but without cirrhosis[7]. Although the mechanisms of oncogenesis of HBV remain obscure, several factors have been identified to be associated with a high risk of developing HCC among chronic hepatitis B (CHB) patients. HBV exerts its oncogenic potential through a multi-factorial process, which includes both indirect and direct mechanisms that likely act synergistically[8].

HEPATITIS B VIRUS INFECTION AND LIVER CARCINOGENESIS

Hepatitis B virus DNA genome is able to integrate into the cellular chromosomal DNA, causing both viral and host genome rearrangements

HBV DNA is a small, circular DNA with a highly compact genetic organization and overlapping open reading frames. It shares with retroviruses the use of reverse transcription during its replication. It is well known that the HBV DNA genome is able to integrate into the cellular chromosomal DNA, causing both viral and host genome rearrangements. HBV DNA integration enhances the instability of the host chromosome, leading to large inverted duplications, deletions and chromosomal translocations. As a result of spontaneous errors in viral reverse transcription, variations in the HBV genome occur naturally. These mutations arise during the course of chronic infection with HBV. In fact, several HBV mutant strains, including those with mutations in the Pre-C/C, core promoter and deletion in the *Pre-S/S* genes, are involved in the pathogenesis of progressive liver disease and HCC development[9]. Several studies have shown that HBV DNA insertion into cellular genes was frequent, and could occur in genes encoding for proteins that were crucial for the control of cell signaling, proliferation and apoptosis[10,11].

HBV-related HCC can also arise in the absence of significant liver damage. Many of these chromosomal segments contain key players in liver carcinogenesis such as P53, PB Wnt/β-catenin, cyclins A and D1, transforming growth factor β (TGF-β) and Ras signaling[12]. In another study HBV DNA was integrated at random sites of human DNA; the *MLL4* gene was one of the targets for integration during hepatocarcinogenesis[13]. Furthermore, viral DNA integration into the cellular DNA is not necessary for viral replication, but allows for the persistence of the viral genome in the cell. Viral DNA insertion as well as cellular DNA replication occurs during liver cell proliferation, secondary to the necrosis/apoptosis of adjacent hepatocytes.

Viral genotype and the risk of hepatocellular carcinoma

The viral genotype is another factor that affects cancer risk. Genotype C has a higher risk of causing HCC than genotype B[14,15], and genotype D has a higher cancer risk than genotype A[16]. Compared to the Asian genotypes (B and C), the European genotypes (A and D) are less well established.

Hepatitis B virus genotypic variations and the risk of hepatocellular carcinoma

Specific genotypic variations in HBV have been associated with cirrhosis and HCC. These variations include, in particular, mutations in the pre-core region (Pre-C, A1896G inside the ε structure of the genome), in the basal Core promoter (A1762T/G1764A), and in ORFs encoding PreS1/PreS2/S and Pre-C/C. There is an overlap between Pre-C or basic core promoter (BCP) mutations and genotype, since these mutations appear to be more common in genotype C as compared to other genotypes[14]. The $1762^{T}/1764^{A}$ double mutations (1762 A-to-T and 1764 G-to-A) in the BCP region were commonly found to be borne by HCC patients in some high-risk populations, and were thus suggested as potential biomarkers for hepatocarcinogenesis[17,18]. Comparison of HBV isolates from different studies indicates that the mutation rate of A1762T/G1764A is 64% for genotype C, 40% for genotype B and 35% for other genotypes[19]. Kusakabe *et al*[20] investigated a population-based cohort consisting of 19 393 subjects (middle aged or older) with a follow-up of over 13 years in Japan. They found that HBV mono-infected subjects with the A1762T/G1764A double mutation could be at high risk for HCC development during the natural course of HBV infection[20]. In addition, the 1753^{V} mutations (1753-to-C/A/G) were also associated with the progression of liver disease[21]. Li *et al*[22] evaluated the roles of genetic variations of HBV in the development of HCC in Southern Guangxi China. Their study supported the hypothesis that both the 1762T/1764A double mutations and the 1753V/1752V mutations were associated with increased risk for HCC. Fan *et al*[23] found that patients with higher viral load and genotype C had a higher incidence of 1762/1764 double mutations, and that Enhancer Ⅱ and DR1 were significantly more in the HCC group than in the CHB group, which may play an important role in HCC development via nucleotide substitution. The BCP mutations could affect the core promoter that regulates the expression of both HBeAg and the core protein, and this may be related to the higher rate of replication of genotype C. Substitutions in the BCP may increase genotype virulence by deregulating the transcription of pcARN/pgARN, increasing the risk of HCC in patients infected with genotype C[24]. Thus, the BCP overlaps with the X region of the HBV genome, and mutations in the amino acid sequence at positions 130 and 131 in this region have been proposed as prognostic markers for the development of liver cancer[9].

Yang *et al*[14] found that the Pre-C mutation (A1896G) could prevent the production of HBeAg, by introducing a premature stop codon into the ORF Pre-C/C that abolished the production of HBeAg. However, HBV DNA synthesis persisted under these conditions; this may cause liver damage with progression to cirrhosis and cancer.

Mutations in Pre-S have been reported in HCC cases compared to chronic or asymptomatic cases. These mutations, including deletions in Pre-S in the integrated HBV DNA, may impair the secretion of HBsAg, leading to increased endoplasmic reticulum and oxidative stress in hepatocytes[25]. Truncated forms of Pre-S2 have also been shown to interact with cyclin A, a critical regulator of the cell division cycle, an observation that supports a role for Pre-S2 in hepatocyte hyperplasia and a likely role in the process of HBV-related tumorigenesis[26]. Thus, deletions of Pre-S may contribute to hepatocarcinogenesis by several mechanisms.

Altogether, this combination of genomic mutations, and/or deletions, together with transcriptional and post-transcriptional regulations, will therefore allow the establishment of viral persistence, and the ongoing synthesis of HBV antigens.

DNA METHYLATION AND THE RISK OF HEPATOCELLULAR CARCINOMA

DNA methylation occurs in the early stage of cancer development, including HCC. Genomic hypomethylation increases chromosome instability while localized hypermethylation decreases tumor suppressor gene expression, thus increasing the risk of HCC development[27]. Aberrant methylation of *RASSF1A* (Ras association domain family member 1) is thought to be an early event in the development of HCC[28]. The process is catalyzed by DNA methyltransferases (DNMT). DNMT inhibitors directly repress tumor angiogenesis, indicating that epigenetic modifications mediated by DNMT are involved in the regulation of gene expression during tumor angiogenesis[29]. Another significant link has been suggested between HCC development and the silencing by DNA hypermethylation of several tumor suppressor genes (*TSGs*). A number of TSGs, including $p16^{INK4A}$, *SOCS-1*, *APC*, *GSTP1* and *E-cadherin*, are silenced by DNA methylation in a large proportion of liver tumors, and this process often starts at the preneoplastic stage[30]. In some reports, a higher rate of promoter methylation for specific genes, such as $p16^{INK4A}$ and *E-cadherin*, has been observed in HBV-related tumors compared to nonviral tumors[31].

HEPATITIS B VIRUS X PROTEIN AND THE RISK OF HEPATOCELLULAR CARCINOMA

The hepatitis B virus x protein (HBx) protein is a 154 amino acid polypeptide with a molecular mass of about 17 kDa. HBx appears to play a critical role in the development of HCC. HBx is important for HBV replication and can regulate cellular transcription, protein degradation, proliferation, and apoptotic signaling pathways (reviewed by Bouchard and Schneider[32]). HBx protein does not bind directly to DNA, but rather acts on cellular promoters by protein-protein interactions and by modulating cytoplasmic signaling pathways. The cell cycle inhibition effect of HBx was validated through a liver regeneration experiment reported by Sidorkiewicz *et al*[33]. Kuo *et al*[34] reported that HBx can downregulate Wnt/β-catenin expression and suppress cell growth by not only repressing cell proliferation, but also triggering cell apoptosis. Furthermore, Hsien *et al*[35] have found that HBx protein interacts with the tumor suppressor adenomatous polyposis coli to activate Wnt/β-catenin signaling. Wnt/β-catenin has been shown to up-regulate the epithelial cell adhesion molecule in HCC cells to promote tumor initiation and stemness[36]. Thus HBx activation of Wnt/β-catenin may promote directly the transformation of liver cells into cancer initiating cells[37]. A number of ways in which HBx protein may induce antiapoptotic effects have been described. The most important of these is the ability of HBx to inhibit p53-mediated apoptosis. Recent experiments have suggested that HBx protein may increase the expression of telomerase reverse transcriptase and telomerase activity, prolonging the lifespan of hepatocytes and contributing to malignant transformation. The protein also interferes with nucleotide excision repair through both p53-dependent and p53-independent mechanisms. Carboxyl-terminal truncated HBx protein loses its inhibitory effects on cell proliferation and pro-apoptotic properties, and it may enhance the protein's ability to transform oncogenes. Dysregulation of IGF-Ⅱ enhances the proliferation and anti-apoptotic effects of oncogenes, resulting in uncontrolled cell growth. Another possible explanation for the anti-apoptotic effect of HBx protein involves the accumulation of the anti-apoptotic protein, survivin[37]. Guo *et al*[37] found that Hep3B cells expressing HBx protein increased the levels of hepatoma upregulated protein (HURP) RNA and protein, and showed resistance to cisplatin-induced apoptosis. Knockdown of HURP in these cells reversed this effect. The anti-apoptotic effect of HBx protein was shown to require activation of the p38/mitogen activated protein kinase (MAPK) pathway. In addition, the expression of survivin was upregulated by HBx protein in an HURP-dependent manner. High levels of HURP favored the expression of the anti-apoptotic survivin in HBx-expressing cells. These results indicate that HBx protein activates the expression of HURP *via* the p38/MAPK pathway, culminating in the accumulation of survivin. In recent years, evidence has accumulated that HBx protein modulates the transcription of methyl transferases, causing regional hypermethylation of DNA that results in silencing of tumor suppressor genes, or global hypomethylation. This, in turn results in chromosomal instability, thereby playing a role in hepatocarcinogenesis.

The $p16^{INK4A}$ gene is known as an abnormal tumor suppressor gene and critical cancer-related gene in human hepatocarcinogenesis. Several studies have shown that hypermethylation of the $p16^{INK4A}$ promoter is an important early event in carcinogenesis[38]. Zhu *et al*[39] found that HBx upregulates *DNMT1* and *DNMT3A* expression at both the mRNA and protein levels, and that HBx represses $p16^{INK4A}$ expression by inducing hypermethylation of

the $p16^{INK4A}$ promoter. Moreover, HBx induces the hypermethylation of the $p16^{INK4A}$ promoter through *DNMT1* and *DNMT3A*. Regulation of *DNMT1* and *DNMT3A* by HBx promotes the hypermethylation of the $p16^{INK4A}$ promoter region[39].

Among the activities of HBx, its trans-activation function may play a crucial role in hepatocarcinogenesis, because it is involved in the activation of a large number of signaling pathways and cellular genes that are involved in oncogenesis, proliferation and inflammation. For example, HBx transactivates a number of cellular promoters and enhancers containing binding sites for nuclear factor-kappa-B, activator protein 1 (AP-1), AP-2, cellular promoters of genes associated with cell proliferation such as IL-8, TNF, TGF-β, and epidermal growth factor receptor, and cytosolic signal transduction pathways including Src kinases, Cjun N-terminal kinase, JaK1/STAT and protein kinase, which have overlapping effects on cell proliferation and viability[40,41].

CONCLUSION

The studies we have reviewed here illustrate that HBV constitutes a major environmental etiological factor for primary liver cancer in humans. It will therefore be important to analyze gene expression and proteomic changes in a large series of samples from CHB at different stages, to identify suitable prognostic markers and therapeutic targets. Furthermore, detection of the viral genomes using sensitive, PCR-based, assays is mandatory to enable an accurate appraisal of their prevalence. Genomic alterations and epigenetic factors like methylation-associated gene silencing may play an important role in the deregulation of cellular functions, leading to malignant transformation. A better understanding of the complex role of HBV in liver tumorigenesis will undoubtedly contribute to the improvement of the management of liver diseases induced by CHB.

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S- Editor Lv S **L- Editor** Kerr C **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4858

World J Gastroenterol 2011 November 28; 17(44): 4858-4866
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Erlotinib inhibits progression to dysplasia in a colitis-associated colon cancer model

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Supported by National Institutes of Health Grants, No. U56 CA126379 (to Isidro AA and Appleyard CB), No. CA118809 (to Wu J); and a National Institutes of Health Predoctoral Fellowship No. F31 GM078951 (to Pagán B)
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Received: June 8, 2011 Revised: August 21, 2011
Accepted: October 14, 2011
Published online: November 28, 2011

Abstract

AIM: To investigate the role of epidermal growth factor receptor (EGFR) in colitis-associated dysplasia using the EGFR tyrosine kinase inhibitor erlotinib.

METHODS: Sprague-Dawley rats received trinitrobenzene sulfonic acid (TNBS; 30 mg in 50% ethanol, ic), followed 6 wk later by reactivation with TNBS (5 mg/kg, iv) for 3 d. To induce colitis-associated dysplasia, rats then received TNBS (iv) twice a week for 10 wk. One group received erlotinib (10 mg/kg, ip) for 1 wk before the start of the reactivation of the colitis and 2 wk after (21 d); the rest received the vehicle. After rats were euthanized, the colons were removed and analyzed for damage and expression of the EGFR downstream effectors Erk1/2 and c-Myc.

RESULTS: Ninety percent of the vehicle-treated animals had dysplasia in any region of the colon. Erlotinib-treated animals had a significant decrease in the incidence of dysplasia compared to vehicle-treated animals in all regions of the colon (50.00% ± 11.47% *vs* 90.00% ± 10.00% in proximal, $P < 0.05$; 15.00% ± 8.19% *vs* 50.00% ± 16.67% in mid, $P < 0.05$; and 20.00% ± 9.17% *vs* 70.00% ± 15.28% in distal, $P < 0.01$). Erlotinib-treated animals also had reduced cell proliferation, reduced active Erk1/2, and reduced c-Myc in colon epithelium compared with the vehicle-treated animals. *In vitro*, erlotinib treatment was shown to markedly decrease c-Myc and pErk1/2 levels in rat epithelial cells. Proliferation of rat epithelial cells was stimulated by epidermal growth factor and inhibited by erlotinib ($P < 0.05$).

CONCLUSION: Erlotinib can decrease the development of colitis-associated dysplasia, suggesting a potential therapeutic use for erlotinib in patients with long-standing colitis.

Key words: Animal model; Epidermal growth factor receptor; Colitis; Dysplasia; Erlotinib

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Pagán B, Isidro AA, Cruz ML, Ren Y, Coppola D, Wu J, Appleyard CB. Erlotinib inhibits progression to dysplasia in a colitis-associated colon cancer model. *World J Gastroenterol* 2011;

17(44): 4858-4866 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4858.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4858

INTRODUCTION

Patients with inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, are at increased risk of developing colorectal cancer (CRC)[1,2]. The known association of IBD with CRC presents an identifiable population for preventative intervention. This preventive effort, however, relies on understanding the molecular events critical for progression from IBD to dysplasia and CRC and on the identification of suitable molecular targets to block the disease development.

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase. Aberrant EGFR activity has been linked to different types of carcinoma, including CRC[3]. EGFR activates several signaling pathways that include Ras/Raf/Mek/Erk1/2 and phosphoinositide 3-kinase/PDK1/Akt to control epithelial cell proliferation and survival[4]. Aberrant EGFR activity, resulting in proliferative effects and anti-apoptosis, can be targeted with small molecule tyrosine kinase inhibitors or with specific antibodies. A number of EGFR tyrosine kinase inhibitors, such as erlotinib, gefitinib and lapatinib, have been developed to treat cancer patients or are in clinical development to treat various types of human cancer[3]. Moreover, two monoclonal antibodies to EGFR, cetuximab and panitumumab, have been approved by the US Food and Drug Administration to treat CRC patients[3,5].

During inflammatory processes like IBD, EGFR and its ligands play a repair role in colonic mucosa[6]. EGFR expression is increased in inflamed tissues of the bowel in animal models and in patients with IBD and colon cancer[7-9]. For example, in a study by Malecka-Panas, it was found that EGFR is increased in colonic mucosa by 35.2% in patients with adenomatous polyps, by 40.6% in patients with ulcerative colitis, and by 123% in patients with colon cancer[10]. One of the complications of long-standing IBD is the development of cancer[11]. The risk of cancer in patients with colitis increases with longer duration of the disease[1]. While it is not understood completely how IBD leads to neoplastic transformation and progression to CRC, higher levels of EGFR and its ligands could cause hyper-activation of growth promoting signaling pathways and may contribute to development of dysplasia. In the rat model of colon carcinogenesis induced by the carcinogen azoxymethane, EGFR is involved in the development of dysplastic lesions and colon cancer[12].

Erlotinib (Tarceva®) is the first EGFR tyrosine kinase inhibitor approved by the US Food and Drug Administration. It is currently used in clinics to treat lung and pancreatic cancer. In this study, we tested the effects of erlotinib on the occurrence of colitis-associated dysplasia in a rat model developed recently by us[13]. We hypothesized that, by inhibiting EGFR, the progression from chronic inflammation to dysplasia will be halted, as a result of the blockade of EGFR activity. Our data show that erlotinib significantly inhibits the colitis-induced dysplasia in this animal model.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 200-220 g at the start of the initial treatment were maintained in restricted-access rooms with controlled temperature (23 ℃) and 12-h light-dark cycle. Standard laboratory chow (8640 Teklad Rodent Diet, Harlan Laboratories; Tampa, FL) and drinking water were provided ad libitum. One week before beginning the protocol animals were acclimatized to avoid additional stress. Animal protocols were approved by the Institutional Animal Care and Use Committee at Ponce School of Medicine.

Induction and reactivation of the colitis and development of dysplasia

Chronic colitis was induced by intracolonic administration of trinitrobenzene sulfonic acid (TNBS; 0.5 mL of 60 mg/mL; Sigma Aldrich; St. Louis, MO) in 50% ethanol followed by reactivation with systemically administered TNBS 6 wk later[14]. The induction was performed by using a rubber catheter, and TNBS was introduced rectally into the colon, approximately 8 cm proximal to the anus. The reactivation was performed 6 wk after the induction. Briefly, the rats were lightly anesthetized with ether, and TNBS (5 mg/kg in 0.9% saline) was administered intravenously *via* a tail vein every 24 h for three consecutive days[14]. Dysplasia was developed by continuing to administer the TNBS (5 mg/kg) twice a week intravenously for 10 wk[13]. The rats were weighed weekly until they were sacrificed at 10 wk with an overdose of pentobarbital (about 1.5 mL of 65 mg/kg for rats of > 500 g). The experiments reported herein were performed in accordance with the principles described in the "Guide for the Care and Use of Laboratory Animals," publication No. DHHS (NIH) 86-23.

Drug treatment

One group of animals was treated with the EGFR inhibitor erlotinib (a kind gift of OSI Pharmaceuticals, Farmingdale, NY). Erlotinib was administered at a dose of 10 mg/kg per day (i.p. dissolved in 0.5% methyl cellulose) from 1 wk before the start of the reactivation of the colitis until 2 wk after (21 d, Figure 1)[15]. Methyl cellulose alone was administered to a control group for the same amount of time.

Measurement of macroscopic damage

After animals were euthanized, a macroscopic analysis of the colon was performed based on the criteria of Appleyard and Wallace[14]. Four variables were examined: the presence of diarrhea (0 or 1 for absence or presence), adhesions between the colon and other organs (0, 1 or 2 for none, minor or major, respectively), the thickness of each

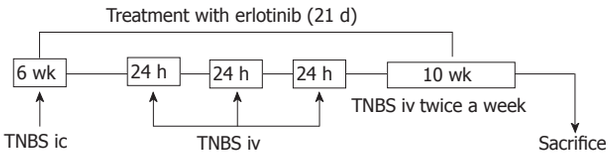

Figure 1 Experimental design for development of colitis-associated dysplasia in a rat model and timeline for administration of erlotinib (10 mg/kg, ip). TNBS: Trinitrobenzene sulfonic acid.

colon segment (in millimeters), and the degree of ulceration (0 for no damage; with increasing scores up to 10, depending on the extent of ulceration). These variables were added to give a total macroscopic damage score.

Sample collection

The colon length was measured in centimeters and cut in equal thirds representing the proximal, mid and distal parts of the colon. These segments were cut longitudinally; one half was weighed and stored at -80 ℃ for molecular analysis, and the other half was fixed in 10% buffered formalin for histological procedures. The Swiss-roll technique was used to evaluate each colon segment microscopically, allowing us to see the entire length of the intestine at once. Briefly, the tissue was rolled into a small piece with the help of forceps and then fixed and placed in a cassette for the remainder of the histological procedures[13].

Microscopic assessment

The tissues were scored microscopically for damage by a blinded observer, as previously described[13]. Criteria included loss of mucosal architecture (0-3: absent, mild, to severe), cellular infiltration (0, none; 1, in muscularis mucosae; 2, in lamina propria/villi; 3, in serosa), muscle thickening (0, muscle < 1/2 of mucosal thickness; 1, muscle = 1/2 to 3/4 of mucosal thickness; 2, muscle = mucosal thickness; 3 = all muscle), goblet cell depletion (0, absent; 1, present), and crypt abscess formation (0, absent; 1, present). The score of each variable was added to give a total microscopic damage score (maximum of 11).

Pathologic evaluation

Colonic sections (2-4 μm) stained with hematoxylin and eosin were analyzed by our pathologists in a blinded manner for dysplasia. Histologic analysis for dysplasia was scored based on previously published criteria[16,17]. Briefly, tissue sections were classified as either negative for dysplasia or positive for dysplasia or carcinoma. The tissues classified as negative for dysplasia adhered to one of the following: normal (small basally located nuclei and normal architecture), non-specific inflammation (cryptitis and glandular invasion by neutrophils), or active colitis (cryptitis, glandular invasion by neutrophils, crypt abscesses, microabscesses). A classification of positive dysplasia was characterized by low-grade dysplasia, which included hyperchromasia, loss of mucin, increased nuclear/cytoplasmic ratio, nuclear elongation and stratification, irregular nuclear outline, and increased number of normal mitoses. The criteria for high-grade dysplasia included the characteristics of low-grade dysplasia plus mucosal architectural distortion including fusion of glands (cribiform pattern) and presence of vesicular polygonal nuclei. For a diagnosis of carcinoma, the characteristics of high-grade dysplasia were included in addition to presence of atypical mitosis and/or of single tumor cells within the lamina propria[16].

Immunohistochemistry

Formalin-fixed 4 μm tissue sections were deparaffinized with xylene, 2 changes, 15 min each, and then hydrated through descending grades of ethanol to deionized water. Antigen retrieval was performed on a hot plate using a beaker with distilled water with the appropriate buffer (0.01 mol/L citrate-ethylene-diamine-tetra-acetic acid (EDTA) buffer, pH 6.0 - high to boiling or EDTA - high to boiling). Slides were cooled at room temperature for 20 min, rinsed with deionized water, and placed in phosphate-buffered saline (PBS) for 5 min. Endogenous peroxidase was blocked with 3% aqueous hydrogen peroxide. After slides were washed with PBS for 5 min, they were blocked with normal serum for 20 min, followed by incubation with the primary antibody. Antibodies were used as follows: phosphorylated epidermal growth factor receptor-pY1068 (Cell Signaling; Danvers, MA), 1:400, overnight; antigen retrieval-EDTA buffer; and 5-bromo-2' deoxyuridine (BrdU), mouse monoclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA), 1:100, overnight, antigen retrieval-citrate-EDTA buffer. The secondary antibody (Bio-Genex Kit; San Ramon, CA) was added to the sections for 20 min and washed again with PBS for 4 minutes. Using the Bio-Genex Kit, we incubated sections with streptavidin-LSab-Peroxidase for 20 min and washed them with PBS for 4 min. The development of the sections was performed using 3,3'-diaminobenzidine tetrahydrochloride (Bio Genex, San Ramon, CA). All samples were lightly counterstained with Mayer's hematoxylin for 15 s, dehydrated through graded alcohol, cleared with xylene, and mounted with resinous mounting medium.

Immunohistochemistry of c-Myc was performed using a Ventana Discovery XT automated slide staining instrument. The antigen retrieval method was Ventana Cell Conditioning-1. Immunohistochemical conditions for c-Myc (ab32072, Abcam) were as follows: 1:25 dilution (60 min), Ventana UltraMAP anti-rabbit (20 min).

The stains were semiquantitatively examined by two independent pathologists using the Allred 8-unit system with a combination of a proportion score from 0 to 5 and an intensity score on a scale from 0 to 3 (none, weak, moderate, strong). A total score of 2-3 was considered low, a score of 4-5 was considered intermediate, and a score of 6-8 was considered high[18].

Western and immunoblot analysis

Proteins were extracted using lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 25 mmol/L NaF, 5 mmol/L $Na_4P_2O_7$, 1% Triton X-100, 1 mmol/L Na_3VO_4, 20 mmol/L

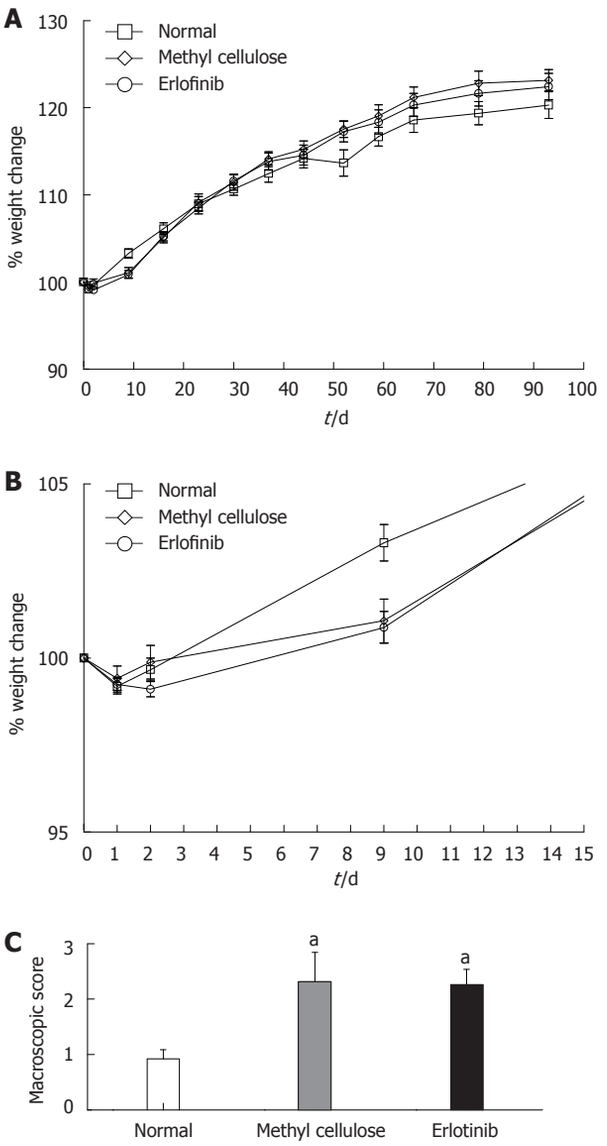

Figure 2 Erlotinib treatment did not worsen inflammation. A: Effect of erlotinib treatment on weight change in an animal model of colitis-associated dysplasia (n = 10-20 per group ± SE); B: Weight change following initial reactivation of colitis; C: Effect of erlotinib treatment on macroscopic damage score in an animal model of colitis-associated dysplasia. The average macroscopic score (including the presence of adhesions, thickness of the tissue, presence or absence of diarrhea, and grade of ulceration) was significantly higher in all trinitrobenzene sulfonic acid-treated animals whether treated with vehicle or drug ($^aP < 0.05$ *vs* normal animals, n = 12-20 ± SE).

p-nitrophenyl phosphate, 2 mg/mL leupeptin, 2 mg/mL aprotinin, and 1 mmol/L phenylmethyl-sulfonyl fluoride). Equal amounts (30 μg) of protein were separated on 12% sodium dodecyl sulfate gel and transferred to a polyvinylidene fluoride membrane (Bio Rad; Hercules, CA). Membranes were blocked with 5% non-fat dry milk in tris-buffered saline-tween (TBST) and incubated with one of the following primary antibodies: Erk1/2, pErk1/2, Akt, pAkt, Src, or Src-pY416 (Cell Signaling Technology; Danvers, MA). Membranes were washed with tris-buffered saline-tween and incubated with horseradish peroxidase-labeled secondary antibodies (Jackson ImmunoResearch Laboratories; West Grove, PA). The bands were detected using ECL-Plus reagent kit (GE Amersham; Piscataway, NJ). Western blotting bands were quantified by densitometry using ImageQuant 5.2 Software (Typhoon 9410; GE Amersham; Piscataway, NJ). pErk1/2, pAkt, and Src-pY416 bands were normalized for the corresponding total kinase.

Cell cultures

Rat intestinal epithelial-1 (RIE-1) cell line (American type culture collection CRL-1592) was cultured in RPMI 1640 containing 5% fetal bovine serum. For analysis of c-Myc, cells were treated with erlotinib (LC Laboratories) as indicated in the figure legends and epidermal growth factor (EGF, 10 ng/mL; Rocky Hill, NJ) for 24 h. For analysis of pErk1/2, erlotinib- or mock-treated RIE-1 cells were stimulated with EGF (10 ng/mL) for 5 min. Cell lysates (20 μg/each) were analyzed by immunoblotting.

Cell proliferation was assayed by plating cells in quadruplet in 96-well plates (1000 cells/well). Twenty-four hours after plating, EGF (10 ng/mL) or erlotinib (10 μmol/L) was added. Four days later, viable cells were measured using CellTiterGlo reagent (Promega) as reported previously[19].

Statistical analysis

Values are presented as means ± SEM where "*n*" represents one tissue from one animal used for a single replicate of an experiment. Statistical analyses were performed using GraphPad Instat V3.0 and Graph Pad Prism V4.0 (Graph Pad Software, San Diego, CA). Groups were analyzed using one-way analysis of variance with Turkey's post-test, and $P < 0.05$ was considered to represent a significant difference.

RESULTS

Erlotinib treatment did not worsen inflammation

During the study (10 wk), all of the rats increased their weight in comparison with their original starting weight, apart from the first 3 d of treatment, where all groups (normal, vehicle-treated, and erlotinib-treated) lost weight (Figure 2A and B). We have observed this phenomenon in prior studies and attribute it to a combination of the intravenous administration of TNBS and the stress initially associated with the procedure[13,16]. No differences in weight change were observed between the normal, vehicle-treated, and erlotinib-treated animals during the study, suggesting no major toxicity of the drug or vehicle.

After animals were euthanized, the colons were removed to score for ulceration, adhesions, diarrhea, and thickness and to measure colon length. As expected, animals treated with TNBS and receiving the vehicle methyl cellulose had significantly higher macroscopic damage scores than the normal animals (Figure 2C). Erlotinib treatment had no effect on the macroscopic score when compared with the vehicle-treated group, with damage scores still significantly higher than normal ($P < 0.05$; Figure 2C). The average length of the colon in TNBS/vehicle-treated animals was shorter that normal (10.55 ± 0.42 cm *vs* 12.17 ± 0.38 cm). This shortening was not at-

Table 1 Effect of erlotinib treatment on microscopic damage score (means ± SE, *n* = 10-20)

	Loss of mucosal architecture	Cell infiltration	Muscle thickness	Goblet cell depletion	Crypt abscess formation	Total microscopic score
Proximal						
Normal	0.67 ± 0.19	1.58 ± 0.23	0.83 ± 0.11	0.83 ± 0.11	0.33 ± 0.14	4.25 ± 0.55
Vehicle	1.40 ± 0.16[b]	2.50 ± 0.12[b]	1.60 ± 0.22[a]	1.00 ± 0.00	0.80 ± 0.13	7.50 ± 0.27[b]
Erlotinib	1.40 ± 0.11[b]	2.35 ± 0.11[b]	1.55 ± 0.17[a]	1.00 ± 0.00	0.60 ± 0.11	6.90 ± 0.27[b]
Mid						
Normal	0.75 ± 0.18	1.50 ± 0.67	0.92 ± 0.29	0.83 ± 0.39	0.45 ± 0.51	4.42 ± 0.51
Vehicle	1.50 ± 0.22[a]	2.70 ± 0.15[b]	2.00 ± 0.26[b]	1.00 ± 0.00	0.70 ± 0.15	7.90 ± 0.53[b]
Erlotinib	1.50 ± 0.14[b]	2.35 ± 0.49[b]	1.85 ± 0.59[b]	1.00 ± 0.00	0.45 ± 0.51	7.15 ± 0.31[b]
Distal						
Normal	1.08 ± 0.26	1.92 ± 0.23	1.50 ± 0.19	0.92 ± 0.08	0.25 ± 0.13	5.67 ± 0.66
Vehicle	1.80 ± 0.20	2.70 ± 0.15[a]	2.20 ± 0.20[b]	1.00 ± 0.00	0.50 ± 0.11	8.20 ± 0.49[b]
Erlotinib	2.05 ± 0.15[b]	2.50 ± 0.14[a]	2.45 ± 0.14[b]	1.00 ± 0.00	0.75 ± 0.10[a]	8.75 ± 0.32[b]

Vehicle is methyl cellulose. One-way analysis of variance between groups. [a]$P < 0.05$, [b]$P < 0.01$ *vs* normal animals within the same colonic region.

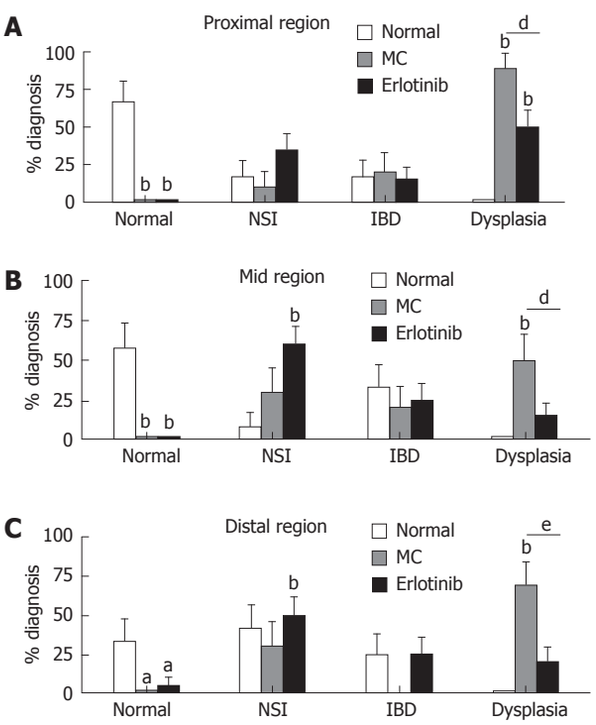

Figure 3 Effect of erlotinib treatment on pathological analysis in an animal model of colitis-associated dysplasia. The percentage of animals with the most severe diagnosis found in each region of the colon is shown ([a]$P < 0.05$, [b]$P < 0.01$ *vs* normal animals; [d]$P < 0.05$, [e]$P < 0.01$ *vs* vehicle-treated animals; *n* = 10-20 ± SE). MC: Methyl cellulose; NSI: Non-specific inflammation; IBD: Inflammatory bowel disease.

tenuated in erlotinib-treated animals (10.85 ± 0.45 cm).

Microscopic analysis of the colon revealed that total microscopic damage score was higher in all regions of the colon in animals receiving TNBS. The damage found was significantly higher in all regions of the colon from these animals than that shown in normal animals ($P < 0.01$, Table 1). Erlotinib had no effect on damage found.

Erlotinib treatment significantly decreased the occurrence of dysplasia

Pathological analysis identified areas of the colon as normal, showing inflammation (IBD and non-specific inflammation), and showing dysplasia. Ninety percent of the vehicle-treated animals had dysplasia in any region of the colon. This was decreased in the erlotinib-treated group such that only 55% of the animals had dysplasia in any area of the colon. When specific regions were analyzed, a decrease in dysplasia incidence was found in the proximal, mid, and distal regions with erlotinib treatment (50%, 15% and 20% in erlotinib *vs* 90%, 50% and 70% in vehicle). Moreover, in the erlotinib-treated group, a close to normal mucosal architecture was found, while in vehicle-treated animals a normal pathology was never observed (Figure 3). Erlotinib treatment increased non-specific inflammation in the mid-region when compared with both normal and vehicle-treated animals. No differences were observed in the identification of IBD in each region of the colon, suggesting that erlotinib may maintain animals in a milder stage of pathology, preventing progression to a more severe diagnosis (Figure 3). It was noted that some "normal" animals were found to have a finding of IBD in some areas; this may be explained by the fact that the normal animals were age-matched and, with increased age, inflammation in response to normal microflora begins to appear[20]. None of the normal animals developed dysplasia.

Erlotinib may decrease cell proliferation through Erk pathway

Proliferation of colon cells was examined by analyzing incorporation of BrdU. A significant increase in positively stained cells was found in animals treated with the vehicle compared with that shown in normal animals, suggesting that those animals have more cell proliferation (Figure 4). Erlotinib-treated animals had decreased cell proliferation compared to vehicle-treated animals, and this was not significantly different from that shown in the normal animals (Figure 4).

Attempts to examine EGFR Y1068 phosphorylation in rat tissue samples were not successful. The activation state of EGFR downstream signaling components Erk, Akt, and Src were then measured by Western blotting using phosphor-specific antibodies. Animals treated with erlotinib showed a tendency toward a decrease in the ratio of

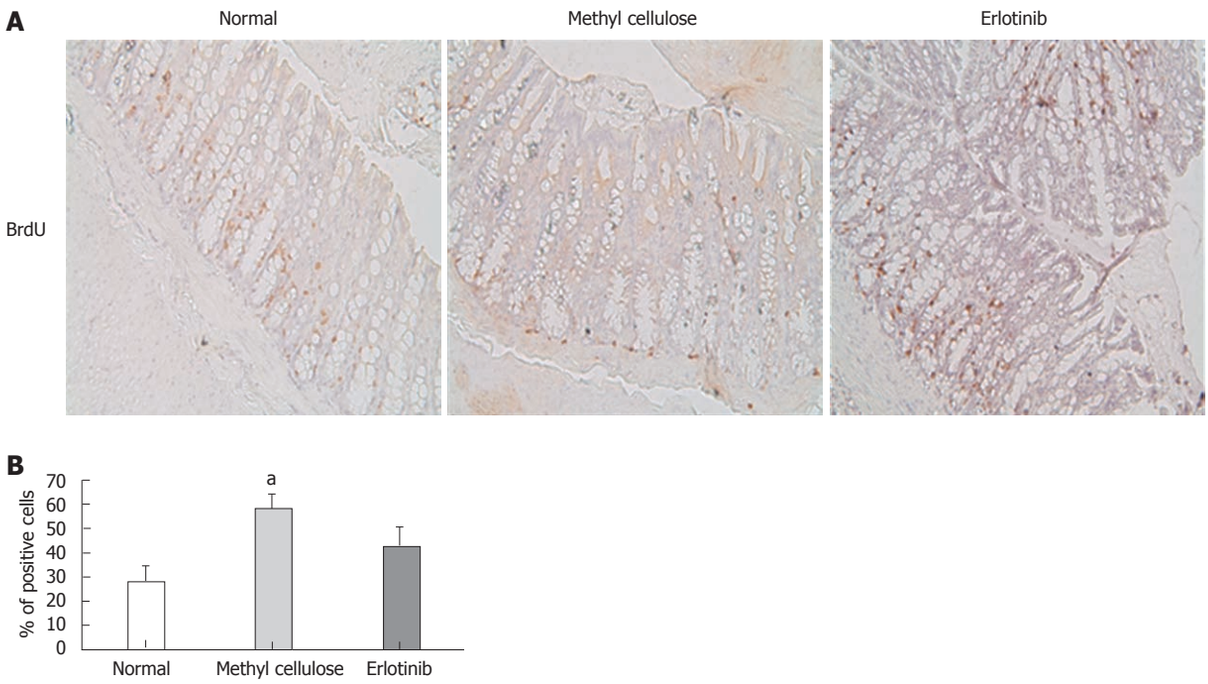

Figure 4 Erlotinib treatment inhibited cell proliferation. A: Representative immunohistochemistry for BrdU incorporation in normal, vehicle-treated and erlotinib-treated animals (200 x). B: Cell proliferation in erlotinib-treated animals was less than that shown in vehicle-treated animals and not significantly different from normal animals ($^aP < 0.05$; $n = 10 \pm SE$).

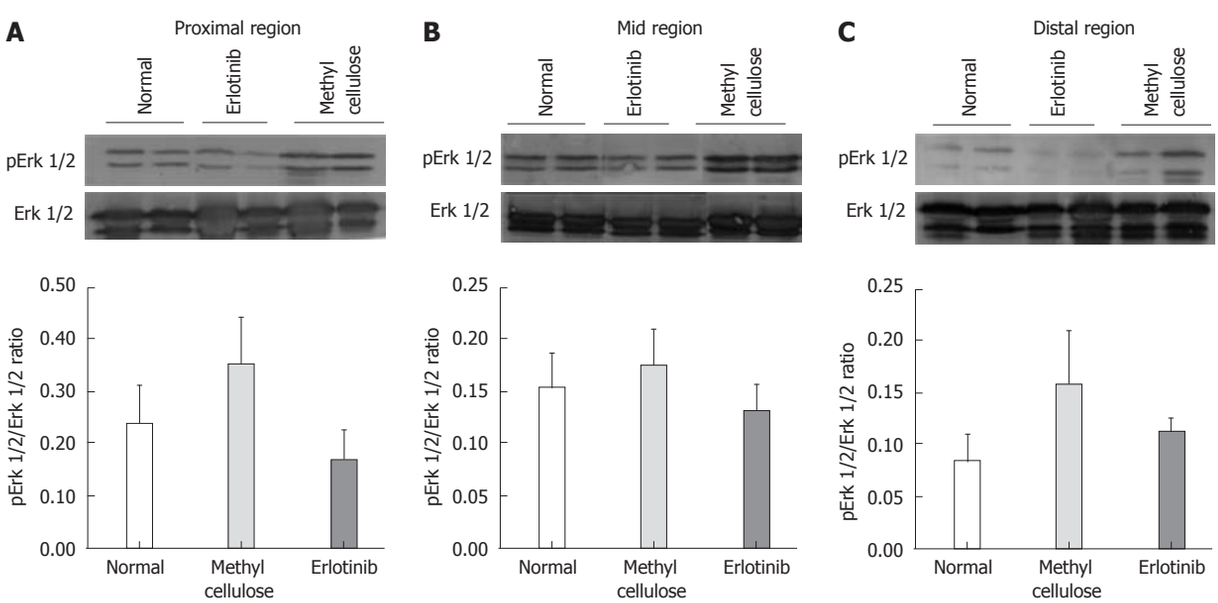

Figure 5 Effect of erlotinib in an animal model of colitis-associated dysplasia on mitogen-activated protein kinase components. Samples from (A) proximal, (B) mid, and (C) distal regions of normal rats and trinitrobenzene sulfonic acid (TNBS) + vehicle and TNBS + erlotinib treated animals were ground, and equal amounts of protein (30 μg) were separated by sodium dodecyl sulfate 12%-polyacrylamide gel electrophoresis before analysis by Western blotting. Antibodies against pErk1/2 and Erk1/2 were used. Lines are representative samples of 3 independent rats per group. Densitometry was performed by ImageQuant 5.2 Software (Typhoon 9410) (n = 10-20 ± SEM).

pErk1/2 when compared with the vehicle-treated group in the proximal, mid and distal regions, with an inhibition of the pErk1/2-to-Erk1/2 ratio of 63%, 24% and 31% in proximal, mid and distal regions, respectively, in animals treated with erlotinib compared to vehicle-treated animals (Figure 5). The ratios of pAkt/Akt and pSrc-pY416 /Src were unchanged in vehicle- and erlotinib-treated animals compared with normal animals (data not shown).

Erlotinib inhibits upregulation of c-Myc

c-Myc is frequently up-regulated in colon cancer and plays an important role in the tumorigenesis of colon cancer. We examined the presence of strong c-Myc staining in the nuclei of rat colon epithelial cells. Compared with the normal group, elevated c-Myc staining was observed in methyl cellulose-treated group, although the difference did not reach statistical significance. Importantly,

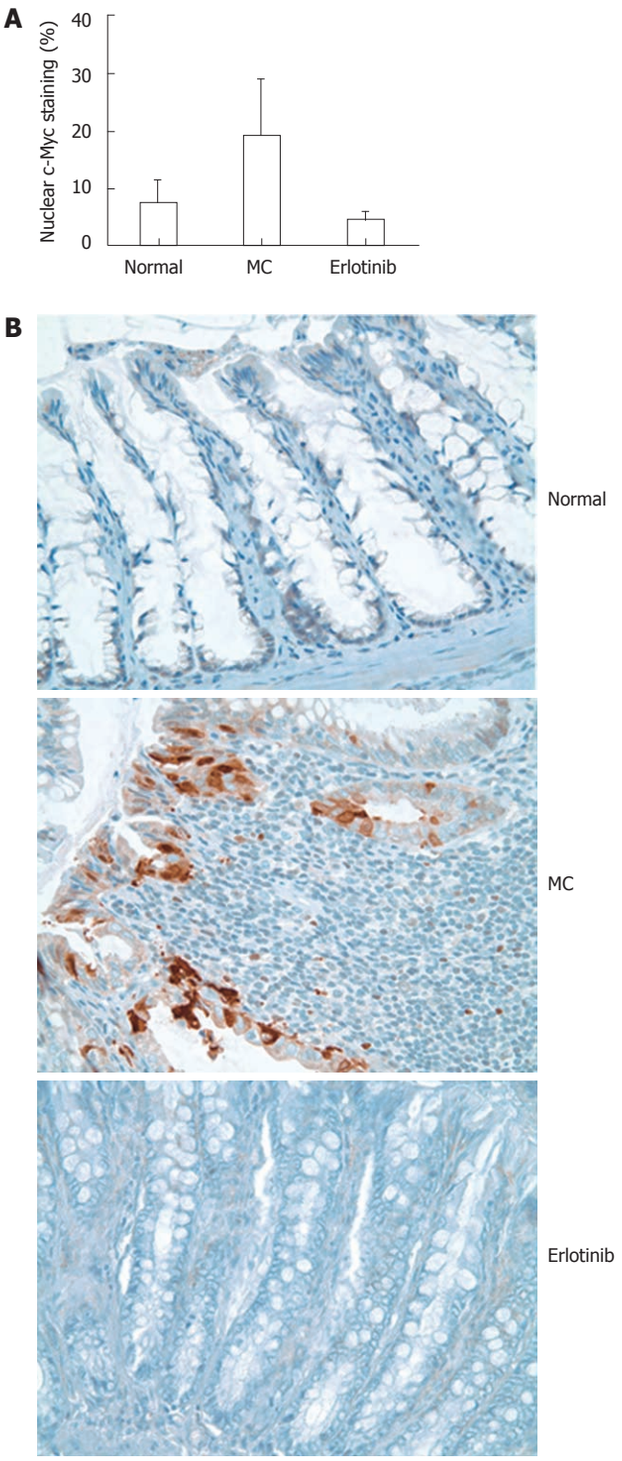

Figure 6 Immunohistochemistry analysis of c-Myc in rat colon tissue samples. A: Slides of formalin-fixed, paraffin-embedded tissues were stained with an anti-c-Myc antibody, and the percentage of strong nuclear c-Myc stain was enumerated. Samples (10, 8 and 11 samples, respectively) from normal, methyl cellulose-treated, and erlotinib-treated rat colon were analyzed; B: Representative immunohistochemistry staining of samples.

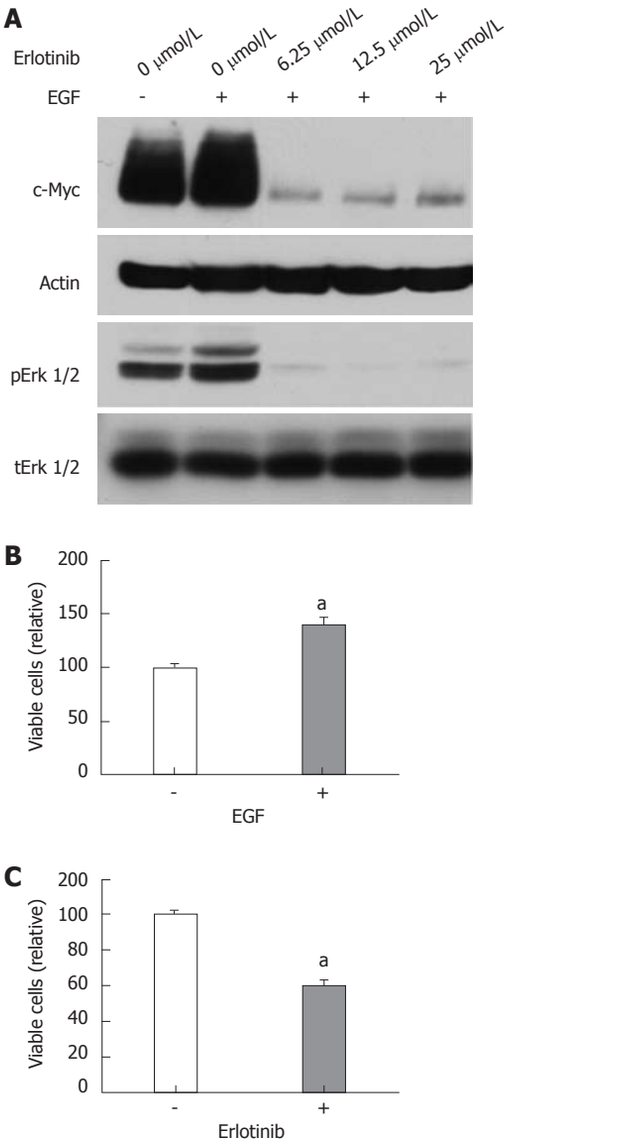

Figure 7 Effects of erlotinib on RIE-1 cells. A: Cells were incubated in RPMI 1640-5% FBS with or without EGF (10 ng/mL) and the indicated concentrations of erlotinib for 24 h. Cells were also pretreated with indicated concentration of erlotinib for 24 h and then stimulated with EGF (10 ng/mL) for 5 min. Cell lysates were analyzed by immunoblotting with indicated antibodies; B and C: Cell proliferation was assayed in the presence or absence of EGF (10 ng/mL) or erlotinib (10 μmol/L) as described in the Materials and Methods. EGF: Epidermal growth factor.

the c-Myc was reduced to a level similar to that found in the normal group in erlotinib-treated rats (Figure 6).

To further assess the effects of erlotinib on c-Myc expression and proliferation in rat epithelial cells, we examined the effects of erlotinib on c-Myc expression in RIE-1 cells. RIE-1 cells appeared very sensitive to serum starvation. In the presence of 5% fetal bovine serum, EGF slightly increased c-Myc and pErk1/2 levels in RIE-1 cells. Erlotinib treatment markedly reduced c-Myc and pErk1/2 levels (Figure 7A). RIE-1 cell proliferation was stimulated by EGF and inhibited by erlotinib (Figure 7B and C).

DISCUSSION

Although the underlying molecular mechanisms involved in colitis-associated cancer need to be further studied, EGFR has been implicated in the development of colonic dysplasia and CRC. The unraveling of molecules that play crucial roles in the transition from chronic inflammation to dysplasia and cancer is essential for iden-

tification of novel drug targets to develop for early intervention measures. Tumorigenesis and tumor promotion depend on cellular signaling pathways that control cell proliferation and survival, and many of these pathways are regulated by EGFR[4]. During chronic inflammation and tissue repair, EGFR activity is elevated. An overactive EGFR may promote the aberrant colonic epithelial cell proliferation and contribute to the development of dysplasia and CRC.

The investigation of the underlying events occurring in colitis-associated dysplasia is complicated by the fact that there are a limited number of animal models available to study the transition of inflammation to dysplasia. Our laboratory recently modified a well-established rat model of chronic colitis to develop an animal model that can be used to investigate ulcerative colitis-associated dysplasia[13,16]. The model uses a prolonged reactivation of inflammation with a proinflammatory drug (TNBS) to create an environment similar to that shown with long-standing colitis in humans, which progresses to cancer. This model shows a degree of dysplasia in 60%-70% of the rats[13,16], similar to what occurs in humans where not all patients develop cancer after a long period with colitis.

We show here that, in our TNBS-induced colitis-associated dysplasia model, animals that received an EGFR inhibitor (erlotinib) had significantly less dysplasia than vehicle-treated animals. This suggests that the EGFR inhibitor is effective in preventing the progression to dysplasia in this animal model. Importantly, we did not observe toxicity of erlotinib in the colon of animals treated with this EGFR inhibitor. It was reported previously that EGFR may have a protective role during acute and chronic inflammation in both the TNBS and dextran sulfate sodium animal models[6,21,22]. The signaling pathway proposed by those researchers involves substance P-NK-1R-EGFR, suggesting that the protective role of this pathway may be due to its effects on fibroblasts[6]. This cell type helps in the remodeling of damaged and/or dead cells or tissues; thus use of an EGFR inhibitor might have been expected to interfere with this process. However, our animals treated with erlotinib showed a milder expression of the disease than that shown in vehicle-treated animals (fewer animals treated with erlotinib progressed to IBD or dysplasia). Thus, erlotinib does not appear to worsen inflammation in our animal model of colitis-associated dysplasia. These data may also help to substantiate the idea of administering erlotinib in conjunction with an anti-inflammatory agent to treat the inflammation and prevent the risk of developing cancer.

The BrdU incorporation assay was used to measure proliferation activity of the colonocytes. Normal animals incorporated BrdU by 26%; this was more than doubled in our model of colitis-associated dysplasia, where vehicle-treated animals showed a 61% incorporation of BrdU, suggesting that these animals possess a higher proliferation rate. In contrast, erlotinib-treated animals showed less BrdU incorporation (37%). Consistently, higher levels of active Erk1/2 and c-Myc were observed in the colon mucosa of vehicle-treated animals but were reduced in erlotinib-treated animals. The Ras-Erk1/2 MAP kinase pathway is known to be activated by EGFR to control cell proliferation. c-Myc overexpression is commonly observed in colon cancer. In the adenomatous polyposis coli-mutant associated CRC, c-Myc is induced by β-catenin to promote colon tumorigenesis. Our data suggest that c-Myc is also up-regulated in colitis-induced dysplasia and erlotinib can inhibit such an increase. In support of this notion, we found that erlotinib is very effective in suppressing c-Myc expression in RIE-1 cells.

In summary, we found that erlotinib is effective in preventing colitis-associated dysplasia without causing unwanted side effects in our novel rat model. Several investigations are already underway to use erlotinib for the treatment of colorectal metastasis[23-25]. In contrast, there have been no investigations into its use as a possible treatment for colitis-associated cancer. Our results suggest that EGFR plays an important role in the progression from inflammation to dysplasia and that inhibition of EGFR, possibly in combination with an anti-inflammatory agent, is a potential approach for use in IBD patients to prevent the development of CRC.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Noel RJ and Dr. Santiago P for advice in this study and the technical assistance of Benitez A. We also thank Hamilton R (Moffitt Cancer Center) for editorial assistance.

COMMENTS

Background

Patients with ulcerative colitis are at increased risk of developing colorectal cancer. Epidermal growth factor receptor (EGFR) up-regulation is related to the development of some cancers including colorectal cancer. Erlotinib, a potent inhibitor of the EGFR tyrosine kinase, has been shown to inhibit the EGFR signaling pathway inside the cell and block tumor cell growth in pancreatic and non-small cell lung cancer; however, its role in the transition to dysplasia is unknown.

Research frontiers

The underlying mechanisms in ulcerative colitis-associated dysplasia are poorly understood. Understanding the mechanisms responsible for the transition from chronic inflammation to dysplasia and cancer might be helpful to diminish the risk that many patients have of developing colon cancer.

Innovations and breakthroughs

This is the first study to report that erlotinib is effective in preventing colitis-associated dysplasia without causing unwanted side effects in a rat model. Although erlotinib is currently under study for the treatment of colorectal metastasis, there have been no investigations into its use as a possible treatment for colitis-associated cancer. The results of this study demonstrated that erlotinib significantly inhibits the colitis-induced dysplasia in this animal model suggesting that EGFR plays an important role in the progression from inflammation to dysplasia.

Applications

Erlotinib may be an effective treatment for patients with long-standing colitis and with higher risk of developing cancer. In addition, it may be possible to combine an anti-inflammatory agent with erlotinib to reduce inflammation, and therefore the occurrence of dysplasia.

Terminology

EGFR: EGFR is a cell surface receptor involved in several downstream signaling pathways, and is also associated with many types of cancer including colorectal cancer. EGFR expression is increased in inflamed tissues of the

bowel in animal models and in patients with inflammatory bowel disease and colon cancer; Erlotinib: Erlotinib is a small molecule inhibitor which is already approved to treat non-small cell lung carcinoma and pancreatic cancer. Erlotinib exerts its biological action by reversible inhibition of tyrosine kinases on the intracellular domain.

Peer review

In the present paper, the authors used ertolinib, an EGFR tyrosine kinase inhibitor, and determined its effect on the occurrence of colitis-associated dysplasia in rat. They showed that this compound significantly inhibits colitis-induced dysplasia. This is an interesting and elegant study. The model of dysplasia, validated previously by the authors, is very original. These data have potential therapeutic implications in the domain of inflammatory bowel diseases.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4867

World J Gastroenterol 2011 November 28; 17(44): 4867-4874
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Vascular endothelial growth factor 165b expression in stromal cells and colorectal cancer

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Received: February 5, 2011 Revised: March 28, 2011
Accepted: April 5, 2011
Published online: November 28, 2011

Abstract

AIM: To characterize the implications of vascular endothelial growth factor (VEGF)-A in stromal cells and colorectal cancer and the expression of VEGF-A splice variants.

METHODS: VEGF-A expression in tumor and stromal cells from 165 consecutive patients with colorectal cancer was examined by immunohistochemistry. The association between VEGF-A expression status and clinicopathological factors was investigated. Twenty fresh-frozen samples were obtained for laser capture microdissection to analyze the splice variants of VEGF-A.

RESULTS: VEGF-A was expressed in 53.9% and 42.4% of tumor and stromal cells, respectively. VEGF-A expression in tumor cells (t-VEGF-A) was associated with advanced clinical stage (stage 0, 1/9; stage 1, 2/16; stage 2, 32/55; stage 3, 38/66; stage 4, 16/19, $P < 0.0001$). VEGF-A expression in stromal cells (s-VEGF-A) increased in the earlier clinical stage (stage 0, 7/9; stage 1, 6/16; stage 2, 33/55; stage 3, 22/66; stage 4, 5/19; $P = 0.004$). Multivariate analyses for risk factors of recurrence showed that only s-VEGF-A expression was an independent risk factor for recurrence (relative risk 0.309, 95% confidence interval 0.141-0.676, $P = 0.0033$). The five-year disease-free survival (DFS) rates of t-VEGF-A-positive and -negative cases were 51.4% and 62.9%, respectively. There was no significant difference in t-VEGF-A expression status. The five-year DFS rates of s-VEGF-A-positive and -negative cases were 73.8% and 39.9%, respectively. s-VEGF-A-positive cases had significantly better survival than s-VEGF-A-negative cases ($P = 0.0005$). Splice variant analysis revealed that t-VEGF-A was mainly composed of VEGF165 and that s-VEGF-A included both VEGF165 and VEGF165b. In cases with no venous invasion (v0), the level of VEGF165b mRNA was significantly higher (v0 204.5 ± 122.7, v1 32.5 ± 36.7, v2 2.1 ± 1.7, $P = 0.03$). The microvessel density tended to be lower in cases with higher VEGF165b mRNA levels.

CONCLUSION: s-VEGF-A appears be a good prognostic factor for colorectal cancer and includes VEGF165 and VEGF165b.

Key words: Colorectal cancer; Vascular endothelial growth factor-A; Vascular endothelial growth factor165; Microvascular density; Stromal cell

Peer reviewer: Josep M Pique, MD, Department of Gastroenterology, Hospital Clínic of Barcelona, Barcelona 08036, Spain

Tayama M, Furuhata T, Inafuku Y, Okita K, Nishidate T, Mizuguchi T, Kimura Y, Hirata K. Vascular endothelial growth factor 165b expression in stromal cells and colorectal cancer. *World J Gastroenterol* 2011; 17(44): 4867-4874 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4867.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4867

INTRODUCTION

The growth and metastasis of cancer depend on angiogenesis, and vascular endothelial growth factor (VEGF)-A. VEGF-A is known to be one of the most important angiogenic factors. VEGF-A protein was discovered by Ferrara in 1989 as a specific growth factor and a blood vascular permeability factor for endothelial cells[1-2]. As a result of alternative splicing, 6 VEGF isoforms of 121, 145, 165, 183, 189 and 206 amino acids are produced from a single gene[3-7]. Most studies suggest that VEGF165 is the most abundant and biologically active isoform[3,8]. The biological effects of VEGF165 are mediated by tyrosine kinase receptors, i.e., VEGF receptor (VEGFR) 1 (Flt-1), VEGFR2 (KDR/Flk-1), and VEGFR3 (Flt-4)[9-11]. In colorectal cancer, VEGF-A is highly expressed in the case of hematogenous metastasis; therefore, VEGF-A is assumed to have value as a prognostic factor. VEGF-A and its receptor system are deeply involved in tumor angiogenesis. Thus, they are important molecular targets in the therapeutic strategy against colorectal cancer. It has been reported that the combined chemotherapy and an anti-VEGF antibody improves the response ratio of the tumor and extends the length of survival[12-15]. Tumor cells are the predominant source of VEGF; however, stromal cells surrounding the tumor have also been shown to produce VEGF[16]. Researches on the invasive and metastatic mechanisms mainly focused on the characteristics of the cancer cell itself, and there are few reports concerning the stromal cells[17-19]. Over the past decade, the role of stromal cells has gradually become a matter of interest to many researchers. The median survival in stromal VEGF-A-positive patients was 9.7 years *vs* 4.3 years in stromal VEGF-A-negative patients with stage Ⅱ and Ⅲ colorectal cancers[20]. However, the reason why VEGF-A expression in stromal cells resulted in a better prognosis has not been clarified.

VEGF165b was recently isolated from kidney epithelial cells as an angiogenesis inhibitor[21]. This variant is identical to VEGF165 except for the last six amino acids encoded by alternative splicing. VEGF165b also binds to both the VEGF receptor 1 (VEGF-R1) and the VEGF receptor 2 (VEGF-R2) with a similar affinity to that of VEGF165. VEGF165b was shown to bind to VEGF-R2, but not to stimulate phosphorylation, and to inhibit VEGF165-mediated phosphorylation in human umbilical vein endothelial cells[22-25].

We examined the association between VEGF-A expression status and clinicopathological characteristics in order to determine how VEGF-A in stromal cells affects tumor progression. We also analyzed the expression of VEGF-165 and VEGF165b using fresh-frozen specimens.

MATERIALS AND METHODS

Patients

Tumor specimens were obtained from 165 consecutive patients with colorectal cancer who underwent resection at the First Department of Surgery, Sapporo Medical University from 1997 through 2001. Of these 165 patients, 146 at stages 0-Ⅲ received curative resection. None of the patients received radiation or chemotherapy before surgery. The pathological stages, depth, histology, venous invasion, and lymphatic invasion of the primary tumor are shown in Table 1. Venous invasion and lymphatic invasion were both classified into four grades according to the Japanese Classification of Colorectal Carcinoma. v0 and ly0 represent no invasion, v1 and ly1, slight invasion, v2 and ly2, moderate invasion, and v3 and ly3, high invasion. immunohistochemical (IHC) analysis was performed in these 165 cases. We also obtained 20 fresh-frozen samples from patients with colorectal cancer in 2006-2007 to analyze the expression of VEGF165 and VEGF165b mRNAs.

Table 1 Characteristics of patients

	n	%
Gender		
Female	75	45.5
Male	90	54.5
Primary tumor location		
Ascending colon	29	17.6
Transverse colon	18	10.9
Descending colon	6	3.6
Sigmoid colon	30	18.2
Rectum	82	49.7
TNM stage		
0	9	5.5
Ⅰ	16	9.7
Ⅱ	55	33.3
Ⅲ	66	40.0
Ⅳ	19	11.5
T factor		
Tis	9	5.5
T1	7	4.2
T2	25	15.2
T3	111	67.3
T4	13	7.9
Histological differentiation		
Well	47	28.5
Moderate	95	57.6
Poor	8	4.8
Mucinous	10	6.1
Other	5	3.0
Venous invasion		
v0	46	27.9
v1	73	44.2
v2	32	19.4
v3	14	8.5
Lymphatic invasion		
ly0	53	32.1
ly1	79	47.9
ly2	28	17.0
ly3	5	3.0
Recurrence except stage IV cases		
No	95	65.1
Yes	51	34.9

Immunohistochemistry

For IHC staining, paraffin-embedded tissues were cut at 4 μm. Slides were deparaffinized in xylene for 3 min three times, 3 min in absolute alcohol, 3 min in 90% ethanol,

3 min in 70% ethanol, and finally, 3 min in phosphate-buffered saline (PBS) for three times. After being deparaffinized, sections were incubated in 3% H_2O_2-methanol for 20 min to inactivate endogenous peroxidase. Deparaffinized and rehydrated sections were heated in DAKO Target Retrieval Solution (DAKO Japan, Tokyo, Japan) for 15 min in an autoclave at 105 ℃. Nonspecific binding was blocked with 10% goat serum for 15 min at room temperature followed by incubation with the primary antibody in a moist chamber at 4 ℃ overnight. After rinsing in PBS for 3 min three times, the sections were incubated with a biotinylated secondary antibody, ENVISION + Mouse/HRP (Dako Japan, Tokyo, Japan), for 30 min. Sections were stained using aminoethylcarbazole (Dako Japan, Tokyo, Japan). Slides were mounted prior to observation under conventional light microscope.

Monoclonal antibodies

The primary antibodies were mouse monoclonal antibodies against VEGF-A, anti-human VEGF (N5) (IBL, Takasaki, Japan), CD34, anti-human CD34 (QBEnd10) and mouse monoclonal antibody Dako N1632 (Dako, Japan, Tokyo, Japan).

Evaluation of immunohistochemistry

VEGF-A expression was examined under light microscope, and both the tumor and the stromal cells were separately classified into stained cells and unstained cells. Three sections of tumor cells and stromal cells were counted respectively at × 400 magnification for marginal cancer tissue to determine whether the cells were positive for VEGF-A, and the percentage of stained cells was averaged. Specimens were regarded as VEGF negative if less than 5% of the cells were stained and as VEGF positive if more than 5% were stained. These criteria were used in many previous reports[26-27]. Microvessel density (MVD) was assessed using light microscopy in invasive tumors containing the highest number of capillaries and small venules per unit area. Any single endothelial cell or cell cluster stained with CD34 was counted as a single vessel at × 400 magnification for marginal cancer tissues[28]. Three sections were counted in one case, and the number of vessels was averaged.

Laser capture microdissection

Laser capture microdissection (LCM) is a method for obtaining pure populations of cells from heterogeneous samples. Using this technique, colorectal tumor tissues were separated into tumor and stromal tissues. The frozen tissues were sectioned at a thickness of 8 μm using a cryostat and mounted on nonadhesive glass slides. Tissue sections were rehydrated using 70% ethanol for 3 min and rinsed twice in distilled water (Invitrogen Corp., Carlsbad, CA). They were then stained using hematoxylin for 30 s and rinsed in distilled water, followed by dehydration with 95% and 100% ethanol for 10 s in each case. Counterstaining was performed three times with eosin. Dehydration with xylene was conducted twice for 1 min each time, followed by air drying for 20 min. The PixCell LM200 system (Arcturus Engineering, Mountain View, CA) was used to microdissect the tumor cells and the stromal cells from the colorectal tissue sections. Ten sections were used to obtain sufficient RNA for reverse transcription polymerase chain reaction (RT-PCR), and each section needed at least 10 000 pulses. Processing of the total RNA began immediately following LCM. Extraction and isolation were performed using a QIAGEN RNeasy Mini Kit (QIAGEN, Valencia, CA).

Real-time polymerase chain reaction

We constructed the following primers to amplify fragments of human VEGF165 and VEGF165b specifically. The forward primer was located in exon 7a (TGTTTG TACAAGATCCGCAGACGTG). One reverse primer complementary to exon 8 (TCACCGCCTCGG CTTGT-CACATCTGCAAGTACGTT) detected VEGF165 but not VEGF165b, and the other reverse primer complementary to exon 9 (GTTCTGTATCAGTCTTTCCTG-GTGAGAGATCTGCA) detected VEGF165b but not VEGF165. Denaturing was conducted at 96 ℃ for 30 s, with annealing at 55 ℃ for 30 s and extension at 72 ℃ for 60 s in reactions cycled 30 times. PCR products were run on 3% agarose gels containing 0.5 μg/mL ethidium bromide and visualized under a UV transilluminator. This reaction consistently resulted in amplicons of 121 bp consistent with VEGF165b and 119 bp consistent with VEGF165. To confirm the amplification of VEGF165 and VEGF165b, we performed sequence analysis of these PCR products.

Real time PCR was performed on a LightCycler (Roche, Basel, Switzerland) for the semi-quantitation of VEGF165 and VEGF165b mRNA levels. The primer sequences were the same as those of the primers used for RT-PCR. The calculated amounts of VEGF165 and VEGF165b mRNAs were normalized to the endogenous reference control gene, human glyceraldehyde-3-phosphate dehydrogenase (h-GAPDH). All data were presented as the ratio of the target gene/GAPDH expression.

Statistical analysis

The χ^2 test and Mann-Whitney *U* test were used to examine the association between the expression status of VEGF and clinicopathological characteristics. To analyze the risk factors for recurrence, logistic regression analysis was conducted. Survival curves were computed according to the Kaplan-Meier method. The log-rank test was used to compare the survival curves. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of VEGF-A in tumor and stromal cells

VEGF-A expression in tumor cells was positive in 53.9% (89/165) of the cases (Figure 1A). VEGF-A immunoreactivity was observed mainly in the cytoplasm of tumor cells. VEGF-A expression in stromal cells was observed in 42.4% (73/165) of the cases (Figure 1B).

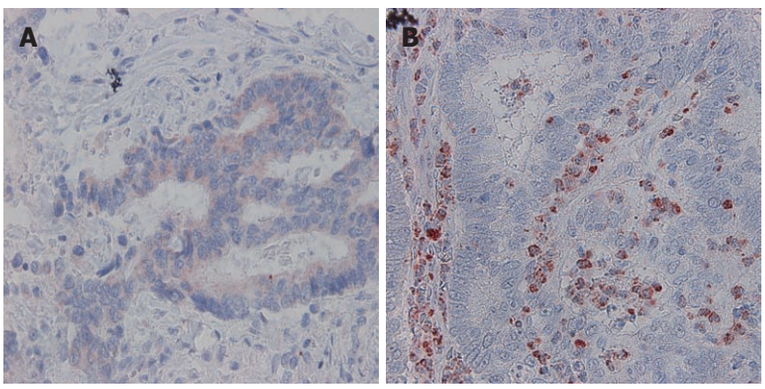

Figure 1 Immunohistochemical of colorectal cancer tissues used the anti-vascular endothelial growth factor-A antibody. A: Vascular endothelial growth factor (VEGF)-A was expressed in tumor cells but not in stromal cells; B: VEGF-A was expressed in stromal cells but not in tumor cells.

Table 2 Association between vascular endothelial growth factor-A expression and clinicopathological characteristics *n* (%)

	n	Tumor VEGF positive cases	Stromal VEGF positive cases
TNM stage			
0	9	1 (11.1)	7 (77.8)
I	16	2 (12.5)	6 (37.5)
II	55	32 (58.2)	33 (60.0)
III	66	38 (57.6)	22 (33.3)
IV	19	16 (84.2)	5 (26.3)
Total	165	89 (53.9)	73 (44.2)
		$P < 0.0001$	$P = 0.004$
T factor			
Tis	9	1 (11.1)	7 (77.8)
T1	7	0 (0.0)	6 (85.7)
T2	25	9 (36.0)	11 (44.0)
T3	111	70 (63.1)	45 (40.5)
T4	13	9 (69.2)	4 (30.8)
Total	165	89 (53.9)	73 (44.2)
		$P = 0.0002$	$P = 0.01$
Histological differentiation			
Well	47	15 (31.9)	23 (48.9)
Moderate	95	63 (66.3)	41 (43.2)
Poor	8	3 (37.5)	4 (50.0)
Mucinous	10	5 (50.0)	2 (20.0)
Other	5	1 (20.0)	3 (60.0)
		NS	NS
Venous invasion			
v0	46	14 (30.4)	27 (58.7)
v1	73	43 (58.9)	29 (39.7)
v2	32	23 (71.9)	15 (46.9)
v3	14	9 (64.3)	2 (14.3)
		$P = 0.001$	$P = 0.015$
Lymphatic invasion			
ly0	53	16 (30.1)	26 (49.1)
ly1	79	48 (60.8)	39 (49.4)
ly2	28	20 (71.4)	7 (25.0)
ly3	5	5 (100.0)	1 (20.0)
		$P < 0.0001$	$P = 0.04$

NS: Not significant; VEGF: Vascular endothelial growth factor.

Association between VEGF-A expression status and clinicopathological characteristics

A summary of the correlation between VEGF-A expression and clinicopathological characteristics is shown in Table 2. Tumor VEGF-A (t-VEGF-A) expression rates in tumors were 11.1% (1/9) in stage 0, 12.5% (2/16) in stage Ⅰ, 58.2% (32/55) in stage Ⅱ, 57.6% (38/66) in stage Ⅲ, and 84.2% (16/19) in stage IV. t-VEGF-A expression was associated with the clinical stage ($P < 0.0001$). VEGF-A (s-VEGF-A) expression rates in stromal cells were 77.8% (7/9) in stage 0, 37.5% (6/16) in stage Ⅰ, 60.0% (33/55) in stage Ⅱ, 33.3% (22/66) in stage Ⅲ, and 26.3% (5/19) in stage Ⅳ. The s-VEGF-A expression rate increased in the earlier clinical stage ($P = 0.004$). The t-VEGF-A expression rate increased with the depth of invasion ($P = 0.0002$). Conversely, the s-VEGF-A expression rate decreased with the depth of invasion ($P = 0.01$). There was no significant association between VEGF-A expression and the histological type. t-VEGF-A expression became significantly higher with the grade of venous and lymphatic invasion, while s-VEGF-A expression became significantly lower with the grade of venous and lymphatic invasion.

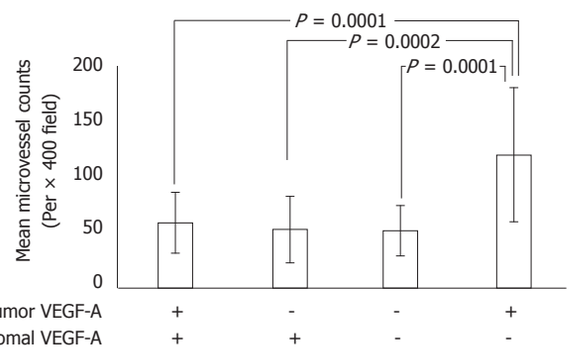

Figure 2 Microvessel density of vascular endothelial growth factor-A expression status. In s-vascular endothelial growth factor (VAEG)-A positive cases, microvessel density (MVD) was maintained at a low score regardless tumor VEGF-A (t-VEGF-A) expression. In s-VEGF-A negative cases, MVD was influenced by t-VEGF-A expression.

Microvessel density

MVD was calculated by counting CD34-positive vascular endothelial cells. The association between VEGF-A expression status and MVD is shown in Figure 2. The MVDs of t-VEGF-A and s-VEGF-A expression (+, +), (-, +), (-, -), and (+, -) were 58.5, 52.4, 51.2 and 119.0, respectively. In s-VEGF-A-positive cases, the low MVD score

Table 3 Logistic regression analysis for recurrence in colorectal carcinoma except for stage Ⅳ cases

Factor	*n* (Recurrence)	Univariate analysis			Multivariate analysis		
		Relative risk	95% CI	*P* value	Relative risk	95% CI	*P* value
Clinical stage							
0	9(1)	2.120	1.302-3.451	0.0250	1.718	0.980-3.010	0.0586
Ⅰ	16(3)						
Ⅱ	55(15)						
Ⅲ	66(32)						
Venous invasion							
v0	46(12)	1.500	1.050-2.143	0.0260	0.812	0.504-1.307	0.3907
v1	63(27)						
v2	27(6)						
v3	10(6)						
Lympathic invasion							
ly0	52(13)	2.094	1.354-3.238	0.0010	1.27	0.714-2.261	0.4155
ly1	68(24)						
ly2	23(12)						
ly3	3(2)						
s-VEGF-A positive	68(14)	0.269	0.135-0.535	0.0002	0.309	0.141-0.676	0.0033
t-VEGF-A positive	73(31)	2.340	1.218-4.495	0.0110	1.918	0.768-3.718	0.1918
Total	146(51)						

CI: Confidence interval.

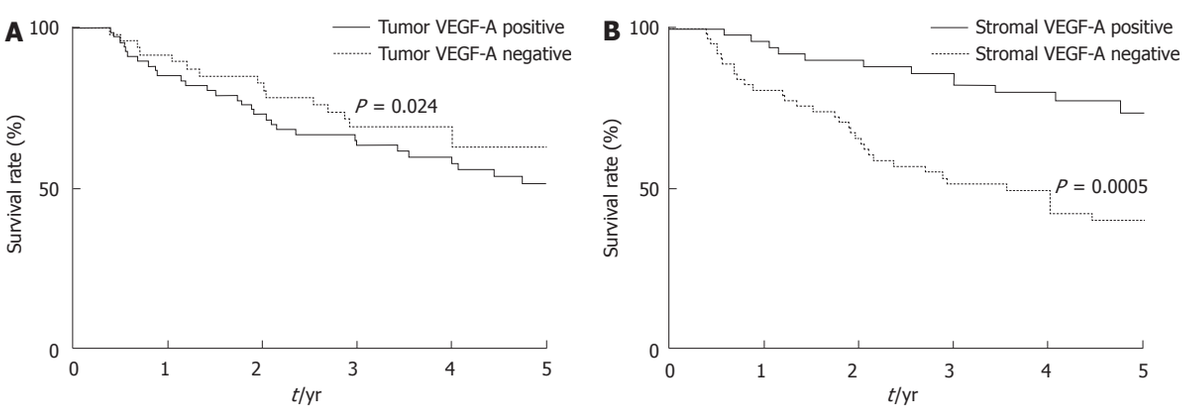

Figure 3 Disease-free survival of patients with stages Ⅱ and Ⅲ colorectal cancer. A: t-vascular endothelial growth factor (VAGE)-A positive *vs* negative. The log-rank test indicates *P*= 0.24 (not significant); B: s-VEGF-A positive *vs* negative. The log-rank test statistical analysis indicates a significant difference (*P* = 0.0005). VEGF: Vascular endothelial growth factor.

was almost the same regardless of t-VEGF-A expression. t-VEGF-A-positive and s-VEGF-A-negative cases had significantly higher MVD scores.

Recurrence

Risk factors for recurrence in the 146 cases excluding stage Ⅳ cases were examined using logistic regression analysis. In univariate analysis, clinical stage, venous invasion, lymphatic invasion, t-VEGF-A positivity and s-VEGF-A negativity were risk factors for recurrence (Table 3). Multivariate analyses of these risk factors were performed, which showed that only s-VEGF-A expression was an independent risk factor for recurrence ($P = 0.0033$) (Table 3).

Survival analysis

Survival analysis was performed for stage Ⅱ and Ⅲ patients ($n = 121$). The five-year disease-free survival (DFS) rates of t-VEGF-A-positive ($n = 70$) and -negative cases ($n = 51$) were 51.4% and 62.9%, respectively. There was no significant difference in t-VEGF-A expression status (Figure 3A). The five-year DFS rates of s-VEGF-A-positive ($n = 55$) and -negative ($n = 66$) cases were 73.8% and 39.9%, respectively. s-VEGF-A-positive cases had significantly better survival than negative cases ($P = 0.0005$) (Figure 3B).

Expression analysis of VEGF165 and VEGF165b

Expression analysis of VEGF165 and VEGF165b was performed using specimens of 20 cases obtained by LCM. RT-PCR was performed using specific primer sets (exon7/exon8 and exon7/exon9) to investigate the expression of VEGF165 and VEGF165b. Sequence analysis revealed that the PCR products were VEGA165 and VEGF165b (data not shown)[26]. IHC analysis was performed in the same 20 cases. Expression of s-VEGF-A and t-VEGF-A was positive in 40% (8/20) and 70% (14/20), respectively. mRNA levels of VEGF165 and VEGF165b were semi-quantified by real time PCR for each VEGF-A expres-

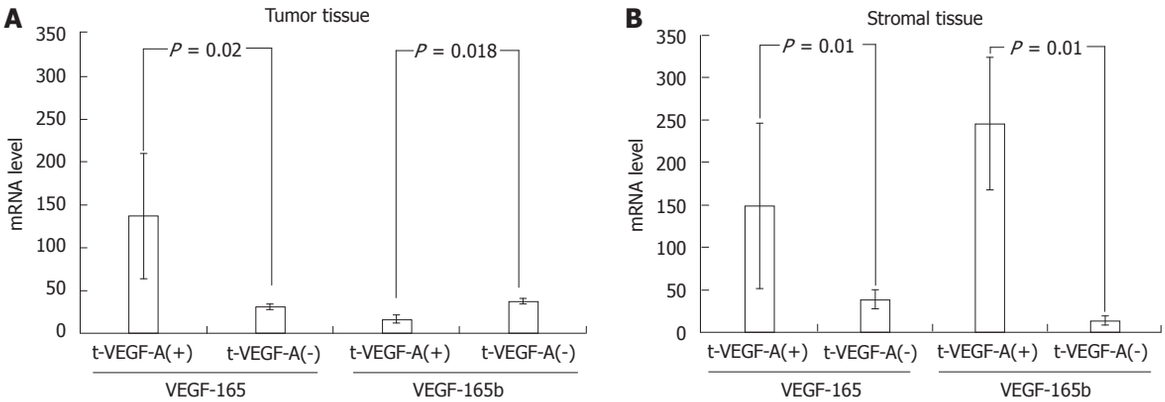

Figure 4 mRNA level of VEGF165 and VEGF165b semi-quantified by real-time polymerase chain reaction in tumor and stromal tissues. A: In tumor tissue, only vascular endothelial growth factor (VEGF) 165 expressed in t-VEGF-A positive cases; B: In stromal tissues, both VEGF165 and VEGF165b expressed in s-VEGF-A positive case.

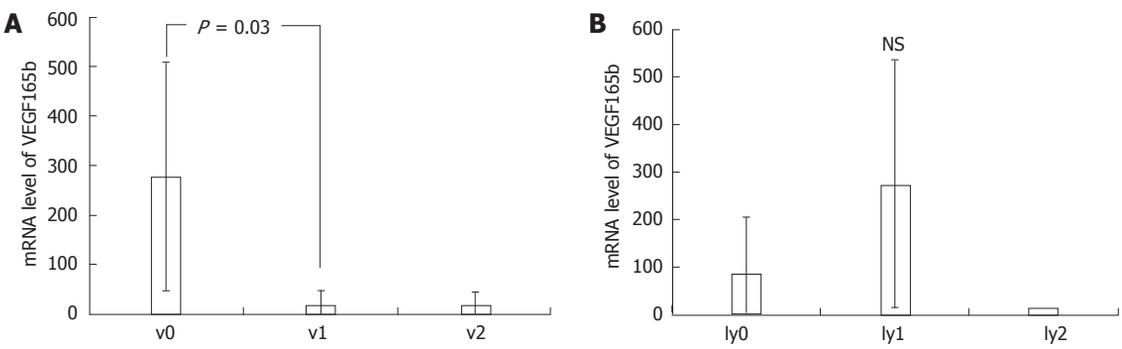

Figure 5 Correlations of vascular endothelial growth factor 165b expression in stromal tissue and tumors with venous and lymphatic invasion. A: Vascular endothelial growth factor (VEGF) 165b mRNA level in v0 cases was significantly higher than those in v1 cases; B: There were no significant differences of VEGF165bmRNA levels among degrees of the lymphatic invasion. NS: Not significant.

sion status determined by IHC. In tumor tissues, only VEGF165 was expressed in t-VEGF-A-positive cases (P = 0.02) (Figure 4A). In stromal tissues, both VEGF165 and VEGF165b were expressed in s-VEGF-A-positive cases (Figure 4B).

Correlation between VEGF165b expression in stromal tissues and venous invasion, VEGF165b expression in stromal tissues and lymphatic invasion

The VEGF165b mRNA level in v0 cases was significantly higher than in v1 cases (Figure 5A). There were no significant differences of VEGF165bmRNA levels among various degrees of lymphatic invasion (Figure 5B).

VEGF165 and VEGF165 mRNA levels and MVD in each case

In cases with lower VEGF165b mRNA levels (numbers 1-8), MVD depended on the VEGF165 mRNA level, while in cases with higher VEGF165b mRNA levels (numbers 14-20), MVD did not reach a high score regardless of the VEGF165 mRNA level (Figure 6).

DISCUSSION

Neoangiogenesis plays an important role in the progression and metastasis of colorectal cancer, and VEGF-A, among many molecules, is known to be of paramount importance because VEGF-A secreted from tumor cells chiefly binds to VEGFR-2 and induces angiogenesis. In colorectal cancer, it is well known that VEGF-A is highly expressed in cases with hematogenous metastasis[29,30]. Therefore, it is assumed that VEGF-A is one of the biomarkers for prognosis[31]. VEGF-A expression in tumor cells was examined to evaluate the degree of risk in many studies. However, there have been few reports focusing on stromal cells surrounding tumor cells. Concerning VEGF-A expression in stromal cells, stromal VEGF-A positivity generally results in a better prognosis than VEGF-A negativity[20].

In this report, IHC staining was performed in 165 consecutive patients with colorectal cancer to detect VEGF-A expression in tumor and stromal cells. Our results showed that s-VEGF-A expression might be a factor indicating a better prognosis. These results were consistent with a previous report[20] and implied that the functions of VEGF-A expressed in stromal cells might be different from those in tumor cells. Since VEGF has 6 splicing isoforms[2-6], we focused on one of them, VEGF165b, which was reported to inhibit neoangiogenesis. Our report demonstrated that s-VEGF-A, including VEGF165 and VEGF165b expressed in stromal cells, might inhibit

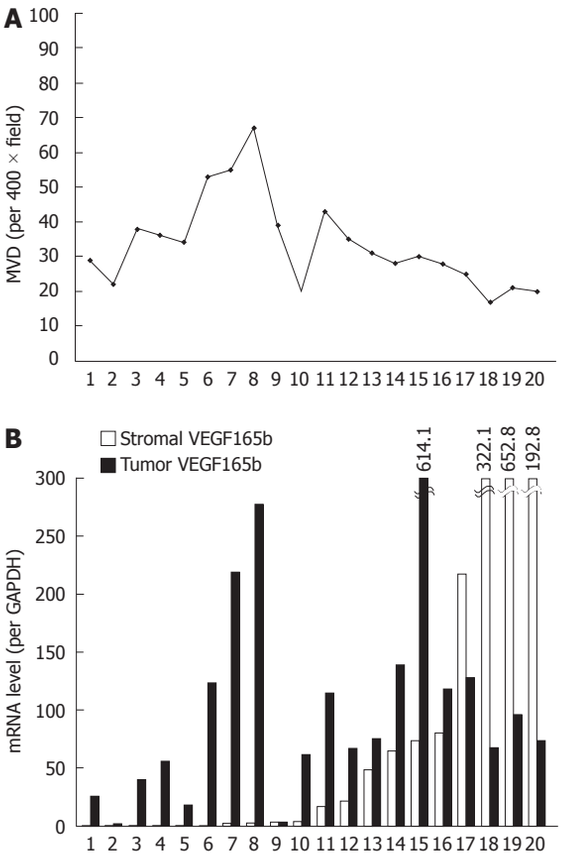

Figure 6 Relationship between the level of vascular endothelial growth factor 165 mRNA in tumor, vascular endothelial growth factor 165b mRNA in stromal tissues and microvessel density. Twenty cases are arrayed on the X axis in ascending order of the amount of vascular endothelial growth factor (VEGF)165b expression. A: The score of microvessel density (MVD); B: The mRNA level of VEGF165 and VEGF165b. MVD was maintained at a low level in the cases in which VEGF165b expressed in stromal tissues.

angiogenesis and reduce MVD. However, we could not conclude that VEGF165b expression improved the prognosis of colorectal cancer patients because the association between VEGF165b expression and the prognosis has not been investigated in a large series.

In this study, we clarified that s-VEGF-A, including VEGF165b, had a function to inhibit neoangiogenesis. However, it remains unexplained what kinds of cells secrete VEGF165b and what factors induce VEGF165b expression. A previous report showed that a subset of macrophages expressed VEGF-A resulting from CD68 (a macrophage-specific immunostain) macroIHC staining[32]. In our series, 76% of CD68-positive cases were s-VEGF-A positive and most of the s-VEGF-A(+) cells were identical to CD68(+) cells under light microscope (data not shown). CD68(+) stromal cells, and tumor-associated macrophages (TAMs) have been reported to have dual potential to improve and worsen the prognosis[33]. We speculate that CD68(+) stromal cells may secrete VEGF165b and inhibit the angiogenesis induced by VEGF165 from tumor cells to interfere with tumor progression. In the future, we will study TAMs in colorectal cancer, especially those expressing VEGF165b, which may be a key to developing a novel therapeutic strategy.

In summary, the s-VEGF-A appears be a good prognostic factor for colorectal cancer and includes VEGF165 and VEGF165b.

COMMENTS

Background

Neoangiogenesis plays an important role in the progression and metastasis of colorectal cancer and vascular endothelial growth factor (VEGF)-A, among many molecules, is known to be highly important because VEGF-A secreted from tumor cells chiefly binds to VEGFR-2 and induces angiogenesis. In colorectal cancer, it is well known that VEGF-A is highly expressed in cases with hematogenous metastasis. Therefore, VEGF-A is assumed to have value as a prognostic factor. VEGF-A and its receptor system are deeply involved in tumor angiogenesis. Thus, they are important molecular targets in the therapeutic strategy against colorectal cancer.

Research frontiers

It has been reported that combined chemotherapy and an anti-VEGF-A antibody improves the response ratio of the tumor and extends the length of survival. Tumor cells are the predominant source of VEGF-A; however, stromal cells surrounding the tumor have also been shown to produce VEGF-A. In many reports, VEGF-A expression in tumor cells was examined to evaluate the degree of risk. However, there have been few reports focusing on stromal cells surrounding tumor cells.

Innovations and breakthroughs

In this report, immunohistochemical staining was performed in 165 consecutive patients with colorectal cancer to detect VEGF-A expression in tumor and stromal cells. The results showed that s-VEGF-A expression might be a factor indicating a better prognosis. These results implied that the functions of VEGF-A expressed in stromal cells might be different from those in tumor cells. This report demonstrated that s-VEGF-A, including VEGF165 and VEGF165b, expressed in stromal cells, might inhibit angiogenesis and reduce microvessel density.

Applications

The authors clarified that s-VEGF-A, including VEGF165b, had a function to inhibit neoangiogenesis. However, it remains unexplained what kinds of cells secrete VEGF165b and what factors induce VEGF165b expression. Studies of TAMs in colorectal cancer, especially those expressing VEGF165b, may be a key to developing a novel therapeutic strategy.

Peer review

This is an excellent manuscript, with a well done methodological approach, and showing a correlation with stromal VGEF expression and colorectal cancer prognosis.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4875

World J Gastroenterol 2011 November 28; 17(44): 4875-4882
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Inhibition of tumor angiogenesis by TTF1 from extract of herbal medicine

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Received: August 11, 2011 Revised: October 12, 2011
Accepted: November 4, 2011
Published online: November 28, 2011

Abstract

AIM: To study the inhibition of tumor angiogenesis by 5,2,4′-trihydroxy-6,7,5′-trimethoxyflavone (TTF1) isolated from an extract of herbal medicine *Sorbaria sorbifolia*.

METHODS: Angiogenic activity was assayed using the chick embryo chorioallantoic membrane (CAM) method. Microvessel density (MVD) was determined by staining tissue sections immunohistochemically for CD34 using the Weidner capillary counting method. The mRNA and protein levels of vascular endothelial growth factor (VEGF), vascular endothelialgrowth factor receptor 2 (VEGFR2, Flk-1/KDR), basic fibroblast growth factor (bFGF), cyclo-oxygenase (COX)-2 and hypoxia-inducible factor (HIF)-1α were detected by quantitative real-time polymerase chain reaction and Western blotting analysis.

RESULTS: The TTF1 inhibition rates for CAM were 30.8%, 38.2% and 47.5% with treatment concentrations of 25, 50 and 100 μg/embryo × 5 d, respectively. The inhibitory rates for tumor size were 43.8%, 49.4% and 59.6% at TTF1 treatment concentrations of 5, 10, and 20 μmol/kg, respectively. The average MVD was 14.2, 11.2 and 8.5 at treatment concentrations of 5 μmol/kg, 10 μmol/kg and 20 μmol/kg TTF1, respectively. The mRNA and protein levels of VEGF, KDR, bFGF, COX-2 and HIF-1α in mice treated with TTF1 were significantly decreased.

CONCLUSION: TTF1 can inhibit tumor angiogenesis, and the mechanism may be associated with the down-regulation of VEGF, KDR, bFGF, HIF-1α and COX-2.

Key words: Chinese herbal medicine; *Sorbaria sorbifolia*; TTF1; Inhibition; Tumor angiogenesis

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Liu C, Li XW, Cui LM, Li LC, Chen LY, Zhang XW. Inhibition of tumor angiogenesis by TTF1 from extract of herbal medicine. *World J Gastroenterol* 2011; 17(44): 4875-4882 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4875.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4875

INTRODUCTION

Angiogenesis is the process by which a new blood-vascular system grows from the existing vascular bed through the interaction of cytokines, the cellular matrix and proteolylic enzymes. Tumor angiogenesis is closely associated with tumor growth, metastasis, recurrence and overall prognosis. For this reason, tumor angiogenesis is a desir-

able target for tumor treatment[1]. Anti-angiogenesis is an important strategy for tumor therapy[2]. Many studies have demonstrated that tumor angiogenesis can be inhibited by the flavones present in Chinese herbal medicines, including apigenin, silibinin, quercetin, wogonin, genistein and luteolin[3-9]. Previously, we have reported that acetic ether extracts of the medicinal plant *Sorbaria sorbifolia* (*S. sorbifolia*) inhibits the growth of HepG-2 cells[10] and mouse S180 sarcoma, down-regulates the levels of tumor necrosis factor (TNF)-α and interleukin (IL)-2, and reduced the cellular activity of natural killer cells[11]. In addition, extracts inhibit the placental glutathione S transferase formation of positive foci in hepatoma precancerous rats and down-regulated the expression of p53 and Bcl-2. They increase the activity of superoxide dismutase and glutathione peroxidase and decrease the nitrogen monoxide (NO) synthase activity and malondiadehyde and NO concentrations[12,13]. Six compounds have been identified in the *S. sorbifolia* acetic ether extracts, including 5,2′,4′-trihydroxy-6,7,5′-trimethoxyflavone (TTF1), 5,7- dihydroxy-8-methoxyflavone, rutin, quercetin, daucosterol, benzoate and p-hydroxybenzoic acid, and TTF1 was the first active flavonoid compound identified[11]. After testing the six compounds, we found that TTF1 inhibited vascular endothelial growth factor (VEGF) expression in HepG-2 cells and VEGF165-induced human umbilical vein endothelial cells proliferation and vascular endothelial growth factor receptor 2 (VEGFR2, Flk-1/KDR) protein expression[10]. This study focused on the effect of TTF1 specifically on the inhibition of tumor angiogenesis.

MATERIALS AND METHODS

Extraction of TTF1

TTF1 was separated using the water extraction and alcohol precipitation method from 10 kg *S. sorbifolia* (collected from Jilin Province) as previously described[11].

Cell culture

The HepG-2 cell line was purchased from KeyGEN Co., Ltd. (Nanjing, China). Cells were grown in RPMI1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin. Cells were cultured at 37 ℃ in a humidified incubator containing 5% CO_2. Cells in the logarithmic growth phase were used for tests.

Chick embryo chorioallantoic membrane assay

Angiogenic activity was assayed using a chick embryo chorioallantoic membrane (CAM) as described previously[14]. HepG-2 cell resuspensions (1×10^6) were inoculated into the chick embryo CAM. Using 4-d-old chick embryos in shells, 50 μL of different concentrations of TTF1, apigenin (KeyGEN), and normal saline were added to the chick chorioallantoic membrane once per day for 5 d. Each experimental group included five eggs, and experiments were repeated five times. Chorioallantoid membranes were collected for microscopy and photographic documentation. Five visual fields were randomly chosen for analyzing the angiogenesis inducing rate and inhibitory rate using the SmartScape microscope photography analysis system.

Inducing rate (%) = (vascular branchpoint number after inoculating tumor cells minus the vascular branchpoint number in non-inoculated tumor cells the vascular branchpoint number in non-inoculated tumor cells) × 100%

Inhibitory rate (%) = (vascular branchpoint number after inoculating tumor cells minus the vascular branchpoint number with drug treament/the vascular branchpoint number after inoculating tumor cells) × 100%

Nude mouse HepG-2 tumor model

BALB/c nude mice were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences (Jilin, China). All studies were in compliance with guidelines of the Institutional Animal Care and Use Committee. 0.1 mL HepG-2 cell resuspensions (1×10^6) were transplanted into the armpits of test mice subcutaneously as an experimental model. Ten days after HepG-2 cell transplantation, 40 mice bearing tumors were selected and divided into five groups, and orally administered 5, 10 or 20 μmol/kg of TTF1 or 10 μmol/kg of apigenin once a day for 10 d. The control group was treated with normal saline. Mice were sacrificed and the tumors were collected and weighed. The tumor inhibition rate was calculated as follows: inhibition rate (%) = (1- the tumor weight in treatment group/the tumor weight in control group) × 100%. Samples were fixed in a 10% formaldehyde solution to prepare the slides for hematoxylin and eosin staining and microscopy.

Immunohistochemistry

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Immunodetection of blood vessels in mouse tumor sections was performed with an anti-CD34 Ab (Boshide Biotechnology Company, Wuhan, China). Sections were incubated with a biotinylated anti-rat Ab (CD34) and then with peroxidase-conjugated streptavidin (Boshide Biotechnology Company, Wuhan, China). To quantify angiogenesis, microvessel density (MVD) was determined by staining tissue sections immunohistochemically for CD34 using the Weidner capillary counting method[15]. Entire sections were scanned under low magnification, and vascularization was subjectively graded. Three highly vascularized areas per tumor were then evaluated at low magnification (× 200). Any brown-staining CD34 distinct from adjacent microvessels, tumor cells, or other stromal cells was considered a single countable microvessel. The total number of microvessels was determined from five vessels in each area, and the average number was recorded for each tumor. To test TTF1 treatment effect on VEGF and basic fibroblast growth factor (bFGF) expression in tumor, the slides were prepared by following the protocol of S-P Kit. Using the double-blind method, the pictures from at least five representative high-power fields were observed in each slice, and no less than 100 cells in each field were counted for analysis.

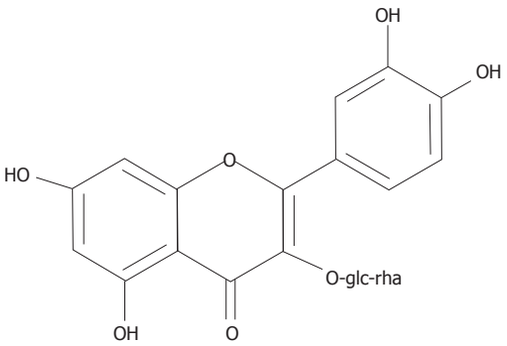

Figure 1 Chemical structure of TTF1.

Western blotting analysis

Tumors were lysed in lysis buffer (Pierce Roche, United States) and then centrifuged at 12 000 *g* for 15 min. Protein concentration was determined using the BCA kit (Pierce Rockford, United States) following the manufacturer's instructions. Seventy μg of protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane (Pall Corporation, Port Washington, NY, United States). After blocking for 1 h with 5% milk in tris-buffuered saline and tween 20, the primary antibody (anti-VEGF, KDR, bFGF, COX-2 or HIF-1α; 1:400) (Boshide Biotechnology Company) was added and incubated at 4 ℃ overnight. After incubation with secondary antibodies (1:5000), membranes were visualized by chemiluminescence. The intensity of protein bands was quantitatively determined using a ultraviolet crosslinkers (Bio-Rad, United States) and normalized with the intensity of Actin band in each gel.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from tumors using the RNeasy Plus Mini Kit (KeyGEN) following the manufacturer's instructions. cDNA was generated with the iScript Select cDNA Synthesis Kit (KeyGEN) and analyzed by quantitative real-time polymerase chain reaction (PCR) using SyberGreen qPCR primer assays (KeyGEN) and the iCycler iQ multicolor real time PCR detection system (KeyGEN). Relative expression levels were normalized against β-actin expression run concurrently as a reference control. The primers used were as follows: VEGF (forward, 5'-TACGTTGGTGCCCGCTGCTG-3'; reverse, 5'-GCCCTCCGGACCCAAAGTGC-3'; amplicon size of 400 bp), KDR (forward, 5'-AGCGTGTGGCACCCACGATC-3'; reverse, 5'-GGCAATCACCGCCGTGCCTA-3'; amplicon length of 338 bp); COX-2 (forward, 5'-TTGCCCGACTCCCTTGGGTGT-3'; reverse, 5'-CTCCTGCCCCACAGCAAACCG-3'; amplicon length of 397 bp); HIF1-α (forward, 5'-ACAGCAGCCAGACGATCATGCAG-3'; reverse, 5'-TGGCTACCACGTACTGCTGGCA-3'; amplicon length of 724 bp); β-actin (forward, 5'-GCTCGTCGTCGACAACGGCTC-3'; reverse, 5'-CAAACATGATCTGGGTCA TCTTCTC-3'; amplicon length of 353 bp).

Statistical analysis

Data in all experiments are shown as mean ± SD. Statistical difference was evaluated using a one-way ANOVA and independent *t* test of sample pairs with SPSS 13.0 software.

RESULTS

Effect of TTF1 on angiogenesis in chick embryo chorioallantoic membrane

The antiangiogenic activities of TTF1 (Figure 1) were tested using the CAM assay. HepG-2 cells induced CAM angiogenesis (Figure 2B). Capillary vessels were intensively spread in HepG-2 cell-inoculated regions, vessel branching significantly increased ($P < 0.05$) (Figure 2A, B and D), and the inhibitory rate was 53.9%. TTF1 inhibited angiogenesis: the number of capillary vessels significantly decreased ($P < 0.05$) in the TTF1 treatment group (Figure 2C and D), with inhibitory rates of 30.8%, 38.2% and 47.5% with TTF1 treatment concentrations of 25, 50 and 100 μg/embryo × 5 d, respectively (Figure 2D and E). The inhibitory effect on angiogenesis *in vivo* by TTF1 was dose-dependent. These results indicate that TTF1 inhibited angiogenesis induced by HepG-2 cells in CAM.

Changes in mouse tumor weight

To test whether TTF1 inhibited tumor growth, we measured tumor weight after TTF1 treatment. Compared to the control group, the tumor weights in the TTF1-treated group were significantly lower ($P < 0.01$), with inhibitory rates of 43.8%, 49.4% and 59.6% at treatment concentrations of 5, 10 and 20 μmoL/kg, respectively (Figure 3). These results suggest that TTF1 administration blocked the growth of HepG-2 cell-induced tumors in mice and that the inhibitory rate of TTF1 was dose-dependent.

Tumor pathology

Compared to the tumors in the control group, the tumors in the TTF1 and apigenin-treated groups were smaller in size with gray surfaces. Their texture was hard, and necrosis was present in the central area but few capillary hemorrhages were observed (data not shown). Microscopy of tumors from the TTF1 and apigenin-treated groups revealed that they had fewer tumor cells, increased cell gaps with clearly visible cell boundaries, and few capillaries in the central area (data not shown).

Changes in microvessel density in a mouse model

To quantify the HepG-2 cell-induced angiogenesis in mouse tumors, MVD was determined by staining tissue sections immunohistochemically for CD34. The positive staining of CD34 was brown and mainly located in the vascular endothelium of the cytomembrane and the cytoplast of capillary vessels, venules and arterioles (Figure 4A). The results showed that the number of capillary vessels greatly increased in tumor tissues in the control group, while they significantly decreased in the TTF1 treatment group (Figure 4B and C). The average MVD was 14.2, 11.2 and 8.5 at the treatment concentrations

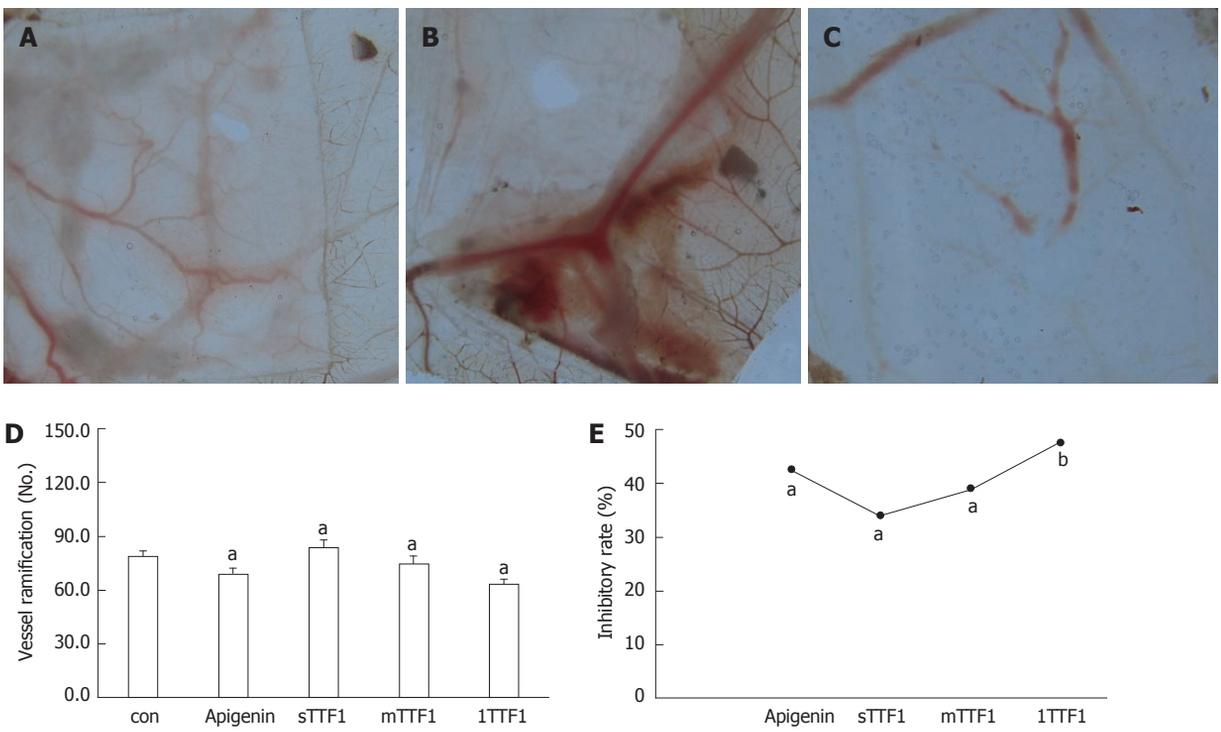

Figure 2 Inhibitory effect of TTF1 on HepG-2 cell-induced angiogenesis in chorioallantoic membrane. A: Control, inoculated with normal saline; B: Inoculated with HepG-2 cells; C: Inoculated with HepG-2 cells and treated with TTF1; D and E: Different doses of compound were used as treatments in the HepG-2 cell-induced angiogenesis in chorioallantoic membrane (CAM) as follows: sTTF1 (25 μg/embryo × 5 d); mTTF1 (50 μg/embryo × 5 d); lTTF1 (100 μg/embryo × 5 d); apigenin (100 μg/embryo × 5 d); and Con (control group treated with normal saline). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

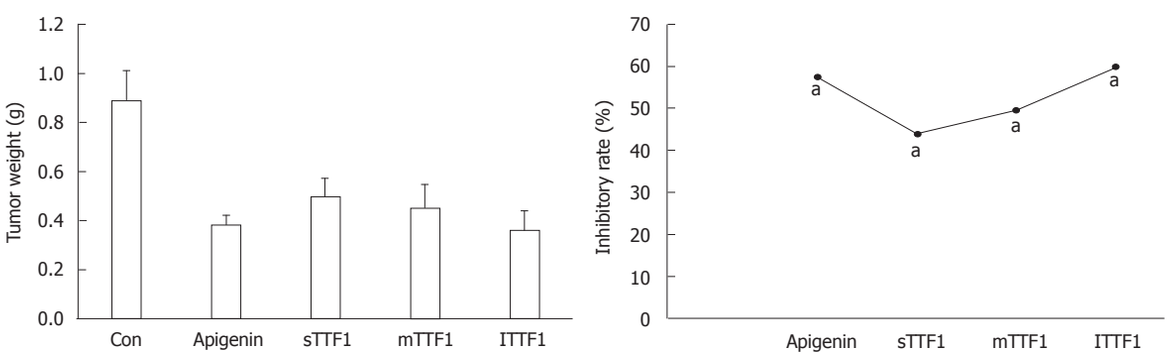

Figure 3 Effect of TTF1 on tumor weight in HepG-2-transplanted nude mice. Different doses of compound were used as treatments in the HepG-2-transplanted nude mice as follows: sTTF1 (5 μmol/kg); mTTF1 (10 μmol/kg); lTTF1 (20 μmol/kg); apigenin (40 μmol/kg); and Con (control group treated with normal saline). Compared to the control group. $^{a}P < 0.05$ *vs* control group.

of 5 μmol/kg, 10 μmol/kg and 20 μmol/kg TTF1, respectively, and it decreased in a dose-dependent manner (Figure 4C). These results indicated that TTF1 inhibited HepG-2 cell-induced angiogenesis in mouse tumors.

Effect of TTF1 on angiogenesis regulation factors

To test whether TTF1 affects the expression of the angiogenesis regulation factors including VEGF, KDR, bFGF, COX-2 and HIF-1α, we analyzed the protein levels of these factors in HepG-2 cell-induced tumors in mice. Immunohistochemistry results (as shown in Figures 5 and 6) showed the effect of TTF1 on the expression of VEGF and bFGF. In the control group, expression of VEGF and bFGF was demonstrated by brown staining of the cytoplasm and membrane of cancer cells, with a focal or diffuse distribution (Figures 5E and 6E). In the TTF1 treatment group, the brown-stained VEGF and bFGF cancer cells were significantly reduced, and most of the cells were stained blue (negative), as shown in Figures 5C and 6C. Combining these results showed that treatment with TTF1 resulted in significant down-regulation of VEGF and bFGF expression in tumors (Figure 7). Western blotting indicated that the protein levels of VEGF, KDR, bFGF, COX-2 and HIF-1α were lower in tumors that were treated with TTF1 than in control tumors (Figure 8A and B). We found that the decrease in protein levels occurred in a dose-dependent manner and showed significant differences at the 10 μmol/kg and 20 μmol/kg doses (as shown in Figure 8A and B) when compared to the controls. To explore whether TTF1

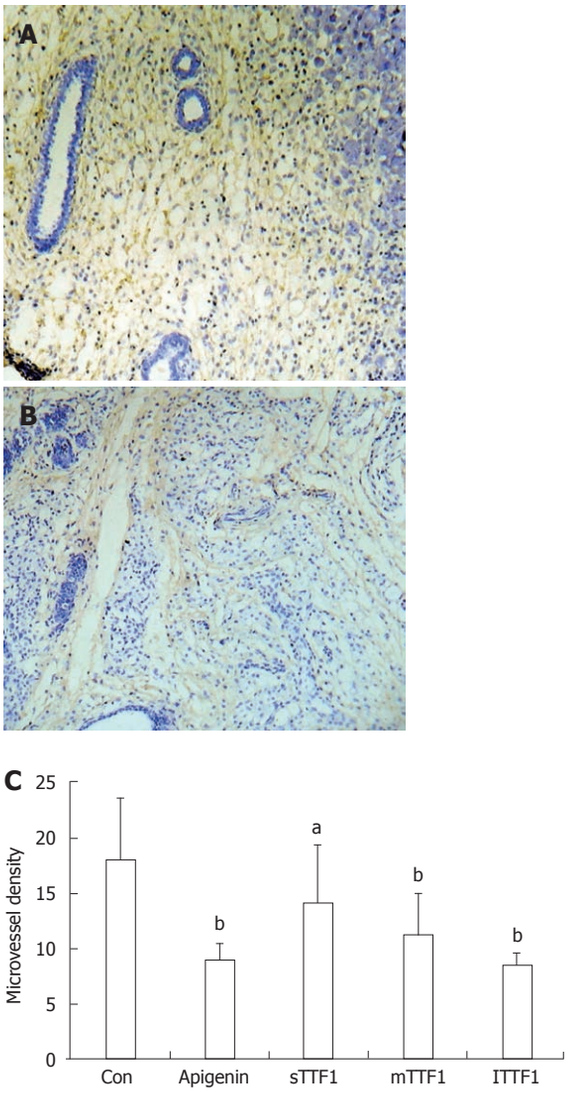

Figure 4 Microvessels in tumor tissue angiogenesis in HepG-2-transplanted nude mice. Brown staining indicates CD34 positive cells. A: Control, HepG-2-transplanted nude mouse treated with normal saline; B: TTF1, HepG-2-transplanted nude mouse treated with TTF1; C: Different doses of compound were used as treatments in the HepG-2-transplanted nude mice as follows: sTTF1 (5 μmol/kg); mTTF1 (10 μmol/kg); ITTF1 (20 μmol/kg); apigenin (40 μmol/kg); and Con (control group treated with normal saline). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

inhibits gene transcription to decrease the expression of these angiogenesis regulation factors, quantitative real-time PCR (qRT-PCR) was performed to determine the mRNA levels of VEGF, KDR, bFGF, COX-2 and HIF-1α in mice treated with TTF1. Representative qRT-PCR graphs for these genes is shown in Figure 8C-E. The effect of TTF1 on the mRNA levels of VEGF, KDR, bFGF, COX-2 and HIF-1α was consistent with the effect TTF1 on their protein levels. Our results indicate that TTF1 inhibits tumor angiogenesis by decreasing the RNA and protein levels of angiogenesis regulation factors (VEGF, KDR, bFGF, COX-2 and HIF-1α).

DISCUSSION

S. sorbifolia is a Chinese medicinal plant that grows on

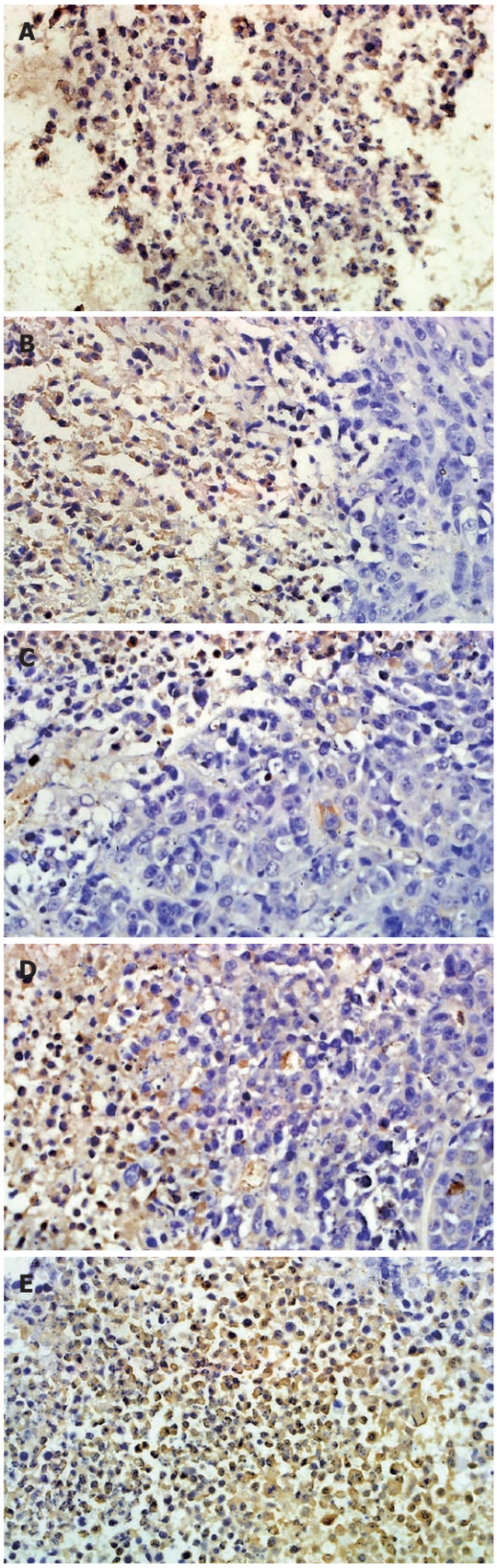

Figure 5 Down-regulation of expression of vascular endothelial growth factor by TTF1 (× 200). Brown staining indicates vascular endothelial growth factor positive. Different doses of compound were used as treatments in the HepG-2-transplanted nude mice as follows: A: sTTF1 (5 μmol/kg); B: TTF1 (10 μmol/kg); C: ITTF1 (20 μmol/kg); D: Apigenin (40 μmol/kg); E: Con (control group treated with normal saline).

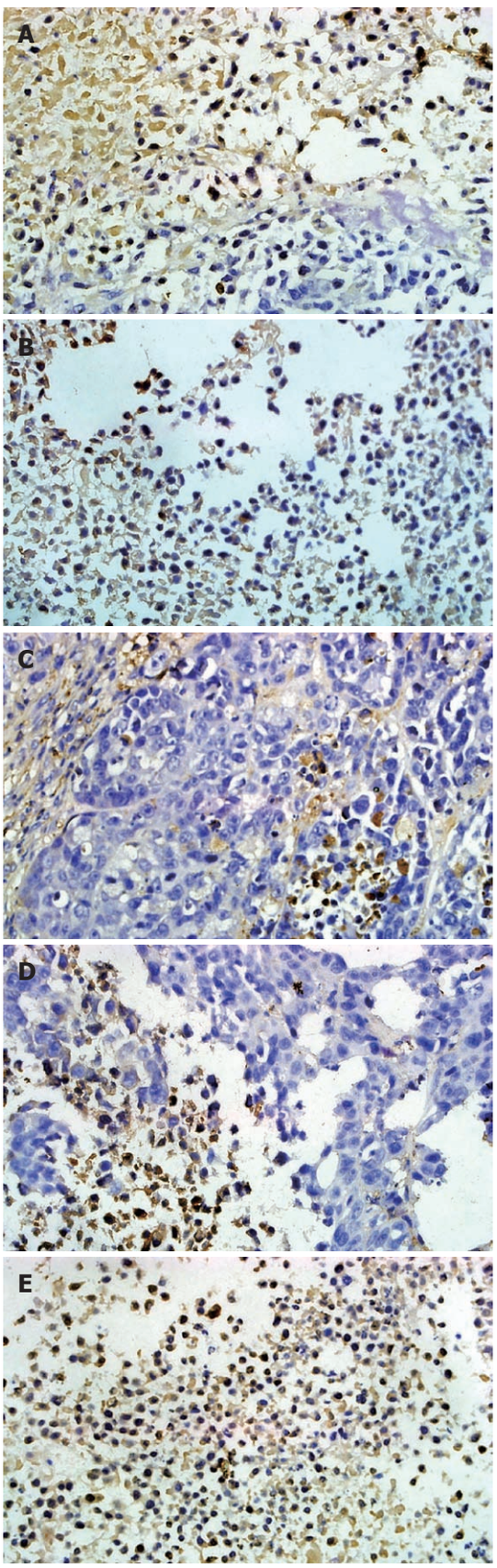

Figure 6 Down-regulation of expression of bFGF by TTF1 (× 200). Brown staining indicates bFGF-positive cells. Different doses of compound were used as treatments in the HepG-2-transplanted nude mice as follows: A: sTTF1 (5 μmol/kg); B: TTF1 (10 μmol/kg); C: lTTF1 (20 μmol/kg); D: Apigenin (40 μmol/kg); E: Con (control group treated with normal saline).

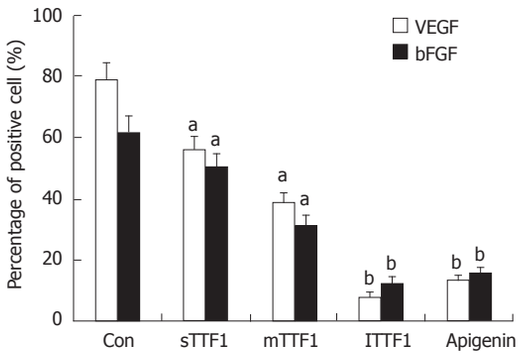

Figure 7 Quantify the effects of TTF1 on the expressions of vascular endothelial growth factor and basic fibroblast growth factor. Different doses of compound were used as treatments in the HepG-2-transplanted nude mice as follows: sTTF1 (5 μmol/kg), TTF1 (10 μmol/kg), lTTF1 (20 μmol/kg), apigenin (40 μmol/kg), and Con (control group treated with normal saline). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group. VEGF: Vascular endothelial growth factor; bFGF: Basic fibroblast growth factor.

Changbai Mountain. Our group began systematic research on its medicinal properties in 2002. An earlier study showed that an acetic ether extract of *S. sorbifolia* has anti-tumor, liver protective and anti-inflammatory effects. Six chemicals were identified in the acetic ether extract, and the novel monomeric compound TTF1 was separated for the first time.

Angiogenesis mainly occurs during embryo development as well as in some pathological conditions, such as damage repair, inflammation, and in particular, tumor growth and metastasis[16]. CAM is the ideal *in vivo* model to study angiogenesis and anti-vascular formation. Our research demonstrated that TTF1 inhibited HepG-2-induced CAM angiogenesis. We also found that TTF1 inhibited tumor growth in HepG-2-transplanted nude mice with an inhibition rate similar to that of apigenin, a flavone extracted from another Chinese medicinal plant that is currently in clinical use.

MVD is a marker to assess the level of tumor angiogenesis. An increase in MVD in tumor tissue suggests a fast-growing and potentially more metastatic tumor. After treatment with TTF1 on the transplanted tumors of nude mice, MVD decreased, suggesting that it inhibited tumor angiogenesis. VEGF is the most important inducing factor for angiogenesis, which specifically stimulates the proliferation of vascular endothelial cells and angiogenesis. VEGF proteins function in association with VEGF receptor (VEGFR) proteins. The five types of VEGFR include VEGFR-1 (Flt-1), VEGFR-2 (KDR), VEGFR-3 (Flt-4), NP-1 and NP-2. VEGF primarily functions through dimerization with KDR, and its intracellular tyrosine residues autophosphorylate after VEGF and KDR bind together. bFGF is another important inducing factor for angiogenesis. Tumor cells produce bFGF, and induce the vascular endothelial cells to produce bFGF, at the same time, increasing angiogenesis[17,18]. The expression levels of VEGF, VEGFR and bFGF were down-regulated after treatment with TTF1, suggesting that TTF1 may inhibit tumor growth through decreasing angiogenesis-inducing factors in HepG-2-transplanted nude mice.

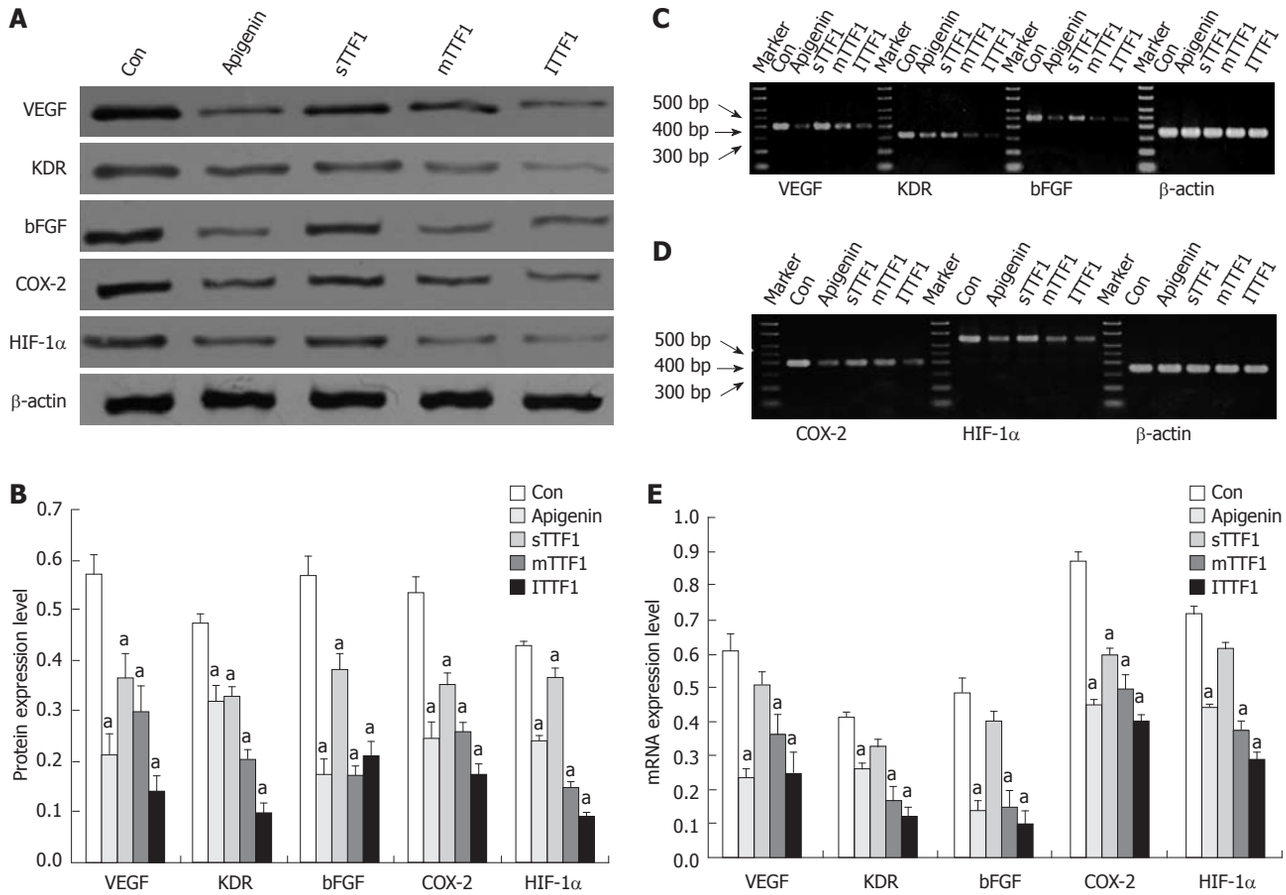

Figure 8 TTF1 decreases the gene and protein level of angiogenesis regulation factors. Western blotting analysis was used to determine the protein levels of VEGF, KDR, bFGF, COX-2 and HIF-1α. A: Tumor tissues were centrifuged at 12 000 *g* for 15 min and the supernatant (70 μg/lane) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Western blotting analysis was performed to detect the protein levels of VEGF, KDR, bFGF, COX-2 and HIF-1α. Different compounds and dosages were used as treatments in the HepG-2-transplanted nude mice as follows: sTTF1 (5 μmol/kg); mTTF1 (10 μmol/kg); ITTF1 (20 μmol/kg); apigenin (40 μmol/kg); and Con (control group treated with normal saline); B: The intensity of the VEGF, KDR, bFGF, COX-2 and HIF-1α protein bands were determined and normalized with β-actin's intensity by using the ultraviolet crosslinkers imager and plotted ($^aP < 0.05$ *vs* control group); C-E: Quantitative real-time PCR was performed to determine the mRNA expression levels of VEGF, KDR, bFGF, COX-2 and HIF-1α ($^aP < 0.05$ *vs* control group).

An insufficient blood supply in fast-growing tumor tissues may cause hypoxia. HIF-1α is the transcription factor that regulates gene transcription during tissue hypoxia. TTF1 may inhibit expression of HIF-1α by suppressing its association with the regulatory sequences of VEGF and bFGF, and therefore resulting in decreased transcription. The expression of VEGF and bFGF may further decrease the expression of KDR through negative feedback. Recent studies have shown that COX-2 is associated with tumor formation, development, and angiogenesis[19]. COX-2 was down-regulated after TTF1 treatment in tumor tissues, in accordance with the down-regulation of the other angiogenesis-inducing factors VEGF, bFGFand VEGFR.

Identification of the compounds responsible for the anti-tumor angiogenesis effects of Chinese herbal medicines is a research hotspot. Our study used the anti tumor drug apigenin as a positive control and comparison for TTF1 treament. Its mechanism of anti tumor activity includes inhibition of tumor angiogenesis, induction of tumor cell apoptosis, disturbing cellular signal pathways, and anti oxidation. Our experiments showed that the inhibitory effect of TTF1 on tumor angiogenesis surpassed that of apigenin.

S. sorbifolia is a rosaceous plant that grows extensively in Changbai Mountain, in Yunnan, Guizhou, Sichuan, Hubei, Gansu and Ningxia Provinces. It is traditionally used in activating blood, dissolving stasis, reducing swelling, easing pain, and healing fractures and injuries from falls[20]. It is a perennial herbaceous plant that has low toxicity and is liver-protective. Our study explored the inhibitory effect of TTF1 on tumor growth and angiogenesis. Further study needs to focus on the different regulatory factors and their interaction using molecular biological techniques after TTF1 inhibition of tumor angiogenesis. The relationship of the chemical structure of TTF1 to its activity should be studied, so that further structural modification may lead to new inhibitors of tumor angiogenesis with better curative effect and easier production. Moreover, further study is also needed to determine whether there are other pathways (such as inducing apoptosis, regulation of nuclear factor-κB or mitogen-activated protein kinase pathways) through which TTF1 inhibits tumor growth.

COMMENTS

Background

Anti-angiogenesis is an important strategy for tumor therapy. Many studies have demonstrated that tumor angiogenesis can be inhibited by the flavones present in Chinese herbal medicine. Previously, the authors reported that acetic ether extracts of the medicinal plant *Sorbaria sorbifolia* (*S. sorbifolia)* inhibites the growth of HepG-2 cells and mouse S180 sarcoma, down-regulates the levels of tumor necrosis factor-α and interleukin-2, and reduces the activity of natural killer cells. 5,2′,4′-trihydroxy-6,7,5′-trimethoxyflavone (TTF1) was the first active flavonoid compound identified in *S. sorbifolia*. This study focused on the effect of TTF1 specifically on the inhibition of tumor angiogenesis.

Research frontiers

Identification of the compounds responsible for the anti-tumor angiogenesis effects of Chinese herbal medicine is a research hotspot. The study used the anti-tumor drug apigenin, which is currently used clinically, as a positive control and comparison for TTF1 treatment. Its mechanism of anti-tumor activity includes inhibition of tumor angiogenesis, induction of tumor cell apoptosis, disturbing cellular signal pathways, and anti-oxidation. The experiments showed that TTF1 had an inhibitory effect on tumor angiogenesis, as did apigenin.

Innovations and breakthroughs

Six compounds were identified in acetic ether extracts of *S. sorbifolia*, including TTF1,5,7- dihydroxy-8-methoxyflavone, rutin, quercetin, daucosterol, benzoate, and p-hydroxybenzoic acid and TTF1 was the first active flavonoid compound identified in *S. sorbifolia*. After testing the six compounds, the authors found that TTF1 inhibited vascular endothelial growth factor (VEGF) expression in HepG-2 cells and VEGF165-induced human umbilical vein endothelial cell proliferation and vascular endothelial growth factor receptor 2 (VEGFR2, Flk-1/KDR) protein expression. The study explored the inhibitory effect of TTF1 on tumor growth and tumor angiogenesis.

Applications

The study results suggest that the TTF1 extracts of the medicinal plant *S. sorbifolia* is a potential therapeutic compound that could be used for tumor inhibition.

Terminology

Sorbaria sorbifolia (*S. sorbifolia)* is a Chinese medicinal plant that grows on Changbai Mountain. An earlier study has shown that an acetic ether extract of *S. sorbifolia* has anti-tumor, liver protective and anti-inflammatory effects. Chick embryo chorioallantoic membrane, is the ideal *in vivo* model for studying angiogenesis and anti-vascular formation. Microvessel density (MVD), is a marker to assess the level of tumor angiogenesis. An increase in MVD in tumor tissue suggests a fast-growing and potentially more metastatic tumor.

Peer review

The present paper examining the effects of extracts of the Chinese herb *S. sorbifolia* (TTF1) on tumor growth is work that extends and builds upon previously published work by this research group. The paper will gather a lot of interest amongst practicing gastroenterologists and oncologists.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4883

World J Gastroenterol 2011 November 28; 17(44): 4883-4889
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Human intestinal acyl-CoA synthetase 5 is sensitive to the inhibitor triacsin C

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Supported by Deutsche Forschungsgemeinschaft, No. GA785/6-1; Deutsche Krebshilfe, No. 109313; and the Rotationsprogramm of the Medical Faculty RWTH Aachen University (to Kaemmerer E)
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Received: July 1, 2011 Revised: August 25, 2011
Accepted: August 31, 2011
Published online: November 28, 2011

Abstract

AIM: To investigate whether human acyl-CoA synthetase 5 (ACSL5) is sensitive to the ACSL inhibitor triacsin C.

METHODS: The ACSL isoforms ACSL1 and ACSL5 from rat as well as human ACSL5 were cloned and recombinantly expressed as 6xHis-tagged enzymes. Ni^{2+}-affinity purified recombinant enzymes were assayed at pH 7.5 or pH 9.5 in the presence or absence of triacsin C. In addition, ACSL5 transfected CaCo2 cells and intestinal human mucosa were monitored. ACSL5 expression in cellular systems was verified using Western blot and immunofluorescence. The ACSL assay mix included TrisHCl (pH 7.4), ATP, CoA, EDTA, DTT, $MgCl_2$, [9,10-^{3}H] palmitic acid, and triton X-100. The 200 μL reaction was initiated with the addition of solubilized, purified recombinant proteins or cellular lysates. Reactions were terminated after 10, 30 or 60 min of incubation with Doles medium.

RESULTS: Expression of soluble recombinant ACSL proteins was found after incubation with isopropyl beta-D-1-thiogalactopyranoside and after ultracentrifugation these were further purified to near homogeneity with Ni^{2+}-affinity chromatography. Triacsin C selectively and strongly inhibited recombinant human ACSL5 protein at pH 7.5 and pH 9.5, as well as recombinant rat ACSL1 (sensitive control), but not recombinant rat ACSL5 (insensitive control). The IC50 for human ACSL5 was about 10 μmol/L. The inhibitory triacsin C effect was similar for different incubation times (10, 30 and 60 min) and was not modified by the N- or C-terminal location of the 6xHis-tag. In order to evaluate ACSL5 sensitivity to triacsin C in a cellular environment, stable human ACSL5 CaCo2 transfectants and mechanically dissected normal human intestinal mucosa with high physiological expression of ACSL5 were analyzed. In both models, ACSL5 peak activity was found at pH 7.5 and pH 9.5, corresponding to the properties of recombinant human ACSL5 protein. In the presence of triacsin C (25 μmol/L), total ACSL activity was dramatically diminished in human ACSL5 transfectants as well as in ACSL5-rich human intestinal mucosa.

CONCLUSION: The data strongly indicate that human ACSL5 is sensitive to triacsin C and does not compensate for other triacsin C-sensitive ACSL isoforms.

Key words: Acyl-CoA synthetase 5; Fatty acid metabolism; Mitochondria; Triacsin C

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Kaemmerer E, Peuscher A, Reinartz A, Liedtke C, Weiskirchen R, Kopitz J, Gassler N. Human intestinal acyl-CoA synthetase 5 is sensitive to the inhibitor triacsin C. *World J Gastroenterol* 2011; 17(44): 4883-4889 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4883.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4883

INTRODUCTION

Acyl-CoA derivatives play a fundamental role in the lipid metabolism of eukaryotic cells including enterocytes. Several biological processes are influenced by acyl-CoA thioesters (acyl-CoAs), ranging from intermediary and mitochondrial metabolism to nuclear gene transcription[1]. The formation of long-chain acyl-CoA derivatives is catalyzed by acyl-CoA synthetases (ACSLs; E.C. 6.2.1.3.), which convert long-chain fatty acids (FAs) into acyl-CoAs[2]. In humans and rodents, five ACSL isoforms have been identified so far, differing in their substrate preferences, enzyme kinetics, cellular and organelle locations, as well as their expression[3]. Human ACSL5 is strongly expressed by enterocytes of the small and large intestine, and is suggested as a modifier of enterocytic maturation and cell death[4-6]. Impaired ACSL5 expression and synthesis has been found in colorectal carcinogenesis[7]. The diversity of ACSL proteins is of functional interest because recent studies suggest that ACSL proteins may play a role in channelling fatty acids toward diverse and complex lipid functions with high relevance for cellular behaviour[8,9].

Triacsin C [1-hydroxy-3-(E,E,E-2',4',7'-undecatrienylidine) triazene], an alkenyl-N-hydroxytriazene fungal metabolite, has been reported to be a potent competitive inhibitor of acyl-CoA synthetase activity[10]. The inhibitory capacity of triacsin C depends on the N-hydroxytriazene moiety of the molecule. In different cellular systems, consequences of ACSL inhibition by triacsin C were found, including a dramatic reduction in cholesterol as well as triglyceride synthesis with non-transition of macrophages to foam cells or enhanced eicosanoid release in leucocytes[11,12]. In endothelial cells, arachidonoyl-CoA synthesis was considerably inhibited by triacsin C[13]. Interference of triacsin C with cellular proliferation *via* inhibition of hu-ACSLs has been found[14,15]. It has been speculated that the plethora of triacsin C effects results from differences in the triacsin C susceptibility of ACSL isoforms. In accordance with this hypothesis, it has been demonstrated that triacsin C inhibits recombinant rat ACSL1 (r-ACSL1), rat ACSL3 (r-ACSL3), and rat ACSL4 (r-ACSL4), but not ACSL5 (r-ACSL5) enzyme activity and may therefore, be useful for discriminating amongst ACSL functions[16,17]. The activity of recently described rat ACSL6 subtypes (r-ACSL6_v1 and r-ACSL6_v2) was not affected by triacsin C at concentrations as high as 50 μmol/L[17]. However, ACSL6 is not essentially expressed in intestinal tissues and enterocytes are widely negative for ACSL6 species.

The aim of the present study was to characterize the effect of triacsin C on human ACSL5 protein *in vitro* and in the intestinal cellular environment. Our findings show that human ACSL5 is, unlike rat ACSL5, sensitive to triacsin C.

MATERIALS AND METHODS

Materials

The rat anti-human ACSL5 antibody KD7 was prepared as previously described[5]. Additional antibodies and substances were anti-beta-actin (Sigma, Deisenhofen, Germany), anti-histidine antibodies (Roche, Mannheim, Germany), HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany; Dianova, Hamburg, Germany), enhanced chemiluminescence (PIERCE, Rockfort, United States), rainbow protein standard (Amersham Bioscience, United Kingdom), PVDF Immobilon-P membrane (Millipore, Bedford, United States), MitoTracker RedCMXRos (Molecular probes, Eugen, United States), and DAPI (Vysis Inc., Downer's Grove, United States). The alkenyl-N-hydroxytriazene fungal metabolite triacsin C (Biomol, Hamburg, Germany) and other materials were obtained from commercial sources. Standard cloning techniques were used for cloning hu-ACSL5 sequences (GeneBank accession no. AB033899) into the pENTRY vector of the GATEWAY cloning system (Invitrogen Life Technologies, Karlsruhe, Germany). Cytomegalovirus expression constructs were synthesized by recombination into the pcDNA_DEST40 vector. CaCo2 cell lines, stably transfected with hu-ACSL5 in pcDNA_DEST40 or empty pcDNA_DEST40, were cultured under appropriate culture conditions. In one approach, T5 promoter expression constructs with N-terminal fusion of hu-ACSL5 sequences to 6xHis-tag and factor Xa protease recognition sites were synthesized by recombination into the pQE-30Xa vector (Qiagen, Hilden, Germany). In a second approach, sequences of r-ACSL1 (EST clone IMAGp998O0814978Q in pExpress-1) and r-ACSL5 (I.M.A.G.E. full length cDNA clone IRBPp993B014D in pExpress-1), both delivered from RZPD Berlin, Germany, as well as the hu-ACSL5 sequence were cloned into pET22b(+) (Merck, Darmstadt, Germany) in *Escherichia coli (E. coli)* without a pelB leader sequence for cytosolic expression of C-terminal 6xHis-tagged proteins. Specimens of human normal intestinal mucosa (n = 10) were mechanically dissected from surgical resections and used fresh or immediately frozen in liquid nitrogen and stored at -80 ℃. The use of human tissues for study purposes was approved by each patient and by the local ethics committee at the University Hospital of the RWTH Aachen (EK019/06).

Cloning and recombinant expression of acyl-CoA synthetase species

For cloning of hu-ACSL5 into appropriate vectors, sequences were generated from human intestine by long distance RT-PCR using the following set of primers: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTACCATGCTTTTTTATCTTTAACTTTTTTGTTTTCCCCACTTCC-3' (sense) and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCATCCTGGATGTGCTCATACAGGCTGT-3' (antisense). Sequences of r-ACSL1 and r-ACSL5 were amplified for recombinant expression cloning. Correctness of all ACSL target sequences (accession nos.: AB033899, AM262166, NM_012820, NM_053607) were controlled by full length sequencing. Recombinant expression of different ACSL proteins in *E. coli* M15 or *E. coli* BL21 was induced with 1 mmol/L isopropyl beta-D-1-thiogalactopyranoside (IPTG) in LB medium when an OD_{600} of 0.6-0.9 was reached. In *E. coli* M15, formation of inclusion bodies was highly diminished by an incubation period of 45 min at 20 ℃. An incubation period of 24 h at 16 ℃ was used for r-ACSL1 and r-ACSL5 expression in *E. coli* BL21, whereas 6 h at 28 ℃ was optimal for expressing hu-ACSL5 in *E. coli* BL21. Expression of soluble ACSL proteins was verified by Western or Dot blotting of ultracentrifuged fractions and anti-ACSL5 or anti-His antibody probes. In control experiments, bacteria transformed with the empty vector were used.

Protein purification

Samples of IPTG-treated bacteria were sonicated in ice-cooled buffer (buffer A) containing 20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 1% triton X-100, and 0.1% sodium cholate, and further processed by ultracentrifugation. The resulting supernatants were applied to a Ni^{2+}-affinity column (5 mL HisTrap HP; Amersham Pharmacia GE Healthcare, Freiburg, Germany). Elution was performed at a flow rate of 1 mL/min at 20 ℃; a linear gradient from buffer A to A/B (50:50, buffer B: buffer A + 500 mmol/L imidazole); 10 min with A/B (50:50); a linear gradient from A/B (50:50) to solvent B; finally with buffer B. Purification of ACSL proteins was controlled by SDS gel electrophoresis with subsequent silver staining, Western blotting, and ACSL activity assays of the purified proteins. ACSL proteins were purified to near homogeneity, migrating as single bands. The Lowry procedure was used for measurement of total protein, and bacteria transformed with the empty vector was used as a negative control.

Acyl-CoA synthetase activity assay

ACSL activity assays were performed as previously described[6]. The standard ACSL assay mix contained 150 mmol/L TrisHCl (pH 7.4), 40 mmol/L ATP, 1.2 mmol/L CoA, 2 mmol/L EDTA, 2 mmol/L DTT, 0.1 mol/L $MgCl_2$, 0.5 μmol/L [9,10-^{3}H] palmitic acid, and 0.2% triton X-100. The 200 μL reaction was initiated by adding solubilized purified recombinant hu- or r-ACSL proteins (ca. 0.1-2.0 μg) or cellular lysates in 0.1% sodium deoxycholate and 1% triton X-100 in 20 mmol/L Tris (pH 7.4). Reactions were terminated after 10, 30 or 60 min incubation with Doles medium. After phase separation, the watery phase was washed twice with palmitic acid-enriched n-heptane. Radioactivity was measured using Ultima Gold cocktail in a Tri-Carb liquid scintillation (2900TR) counter equipped with QuantaSmart software (PerkinElmer, Rodgau, Germany). The studies were repeated three times, and virtually identical results were obtained from each experiment. Enzyme activity was always demonstrated in percent of the related peak activity. Error bars are SEM.

Western blotting analysis

Cellular proteins were separated by one-dimensional SDS-PAGE and transferred to a PVDF Immobilon-P membrane. Immunodetection was performed with primary antibodies [mAB KD7, specific for ACSL5 (undiluted); anti-beta-actin (1:1000)], probed with HRP-conjugated secondary antibodies (1:10 000) and developed with enhanced chemiluminescence as suggested by the provider. Rainbow protein standard was used for molecular weight estimation.

Immunofluorescence

Cells were incubated with anti-histidine antibodies, specific for His-tagged proteins, followed by Cy2-labeled anti-mouse antibodies. For negative controls, the primary antibody was replaced by normal serum. MitoTracker RedCMXRos was used as a mitochondrial marker following the manufacturer's recommendations. Cells were incubated with 25 nmol/L MitoTracker for 15 min, washed, fixed (methanol at -20 ℃ for 5 min, followed by acetone at 4 ℃ for 2 min), and permeabilized with 0.2% Triton X-100. After incubation of primary and secondary antibodies, DAPI was applied for nuclear staining. Images were visualized using a confocal laser microscope (Nicon, Düsseldorf, Germany).

RESULTS

Expression and purification of recombinant hu-acyl-CoA synthetase 5 proteins

Expression of soluble recombinant hu-ACSL5 protein in *E. coli* M15 was found after incubation with IPTG for 45 min at 20 ℃. Soluble activity in supernatants after ultracentrifugation (about 10% of total ACSL activity) was further purified to near homogeneity using Ni^{2+}-affinity chromatography. The specific activity of recombinant hu-ACSL5 proteins from different preparations were in the range of 1.08-2.31 nmol/min per mg and independent of the epitope and its location at the N- or C-terminus (Figure 1).

Triacsin C strongly inhibits recombinant hu-acyl-CoA synthetase 5, but not r-acyl-CoA synthetase 5 activity

Since hu-ACSL5 displayed activity peaks at pH 7.5[6,16] and pH 9.5[6], conditions favouring maximal enzyme activity were chosen when the inhibitory effects of triacsin

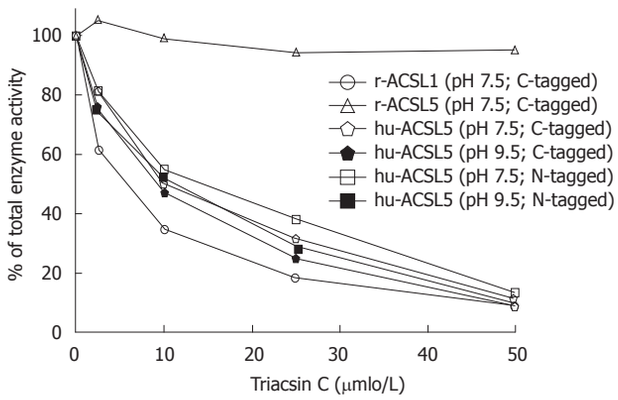

Figure 1 Sensitivity of recombinant acyl-CoA synthetase 5 proteins to triacsin C. Enzyme activity of Ni2+-affinity purified recombinant r-ACSL1, r-ACSL5, hu-ACSL5 C-terminal 6xHis-tagged enzyme, and hu-ACSL5 N-terminal 6xHis-tagged enzyme were assayed at pH 7.5 (white symbols) and pH 9.5 (black symbols) in the presence and absence of triacsin C as described in the methods section. ACSL5: Acyl-CoA synthetase 5.

C to ACSL proteins were tested. To test the effects of triacsin C on purified rat and human ACSL proteins in simultaneous reactions, the triacsin C substance dissolved in DMSO (2.5% of final assay reaction) was directly added to the reaction mixture (0-50 μmol/L) following the procedures by Kim *et al*[16]. Triacsin C selectively and strongly inhibited recombinant human ACSL5 protein (pH 7.5 and pH 9.5), as well as recombinant r-ACSL1 (sensitive control), but not recombinant r-ACSL5 (insensitive control) in a dose dependent manner (Figure 1). The IC50 for human ACSL5 proteins was about 10 μmol/L, and the inhibitory triacsin C effect was similar for different incubation times (10, 30 and 60 min) and was not modified by the N- or C-terminal location of the 6xHis-tag.

Triacsin C inhibits human acyl-CoA synthetase 5 activity in human intestine cellular systems

In order to further characterize the inhibitory potency of triacsin C on hu-ACSL5 protein in a cellular environment, stable hu-ACSL5 transfectants in CaCo2 cells (ATCC No. HTB-37) were used as previously published[6]. Transgenic and endogenous hu-ACSL5 protein expressions were controlled with Western blotting and indirect immunofluorescence (Figure 2). The shift between both ACSL5 proteins in Western blotting is due to the 6xHis-tag and a linker sequence, which result in a higher molecular weight of the transgenic ACSL5 protein (Figure 2A). N-terminal 6xHis-tagged hu-ACSL5 proteins are strongly co-localized with mitochondria. Some extramitochondrial signalling is found, including ER/ribosomes (Figure 2B). Monitoring of ACSL-activity in stable hu-ACSL5 N-terminal 6xHis-tagged transfectants and controls (wild type CaCo2 cells and CaCo2 transfectants with the empty vector) was performed between pH 5 and pH 10 (Figure 2C). In stable hu-ACSL5 CaCo2 transfectants, peak activity was found at pH 7.5 and pH 9.5, corresponding to the properties of recombinant hu-ACSL5 protein. This bimodal curve of ACSL activity was not observed in control cells, where the main ACSL activity was detectable at pH 7. The data further indicate that the bimodal distribution of ACSL activity was mainly due to hu-ACSL5 activity in the CaCo2 cellular environment.

In the presence of triacsin C (25 μmol/L), total ACSL activity was dramatically diminished in hu-ACSL5 transfectants as well as in controls. Importantly, the bimodal curve of ACSL activity with peak values at pH 7.5 and pH 9.5, due to hu-ACSL5 transgenic over-expression, was essentially smoothed in the presence of triacsin C (Figure 2C).

Next, mechanically dissected normal human intestinal mucosa with high ACSL5 expression (Figure 3A) was monitored for ACSL activity in the pH range of 5 to 10 (Figure 3B). Strong ACSL activity was found at pH 7, pH 8, and pH 9.5, partly reflecting the characteristic bimodal activity of human ACSL5 proteins. In the presence of triacsin C (25 μmol/L), ACSL activity was dramatically inhibited; the pH 9.5 peak was especially diminished (Figure 3B). The resulting smooth curve paralleled the findings with triacsin C treated purified hu-ACSL5 recombinant proteins and stable hu-ACSL5 transfected CaCo2 cells.

DISCUSSION

A considerable amount of data indicates that long chain fatty acids are essential in intestinal physiology and pathophysiology. In the modifier concept of intestinal carcinogenesis, the activity of intestinal long chain fatty acids is suggested as an important cell cycle modifier[18]. The function of ACSL mediated metabolic channelling of fatty acids in the regulation of intestinal cell behaviour includes the lipidation of proteins and translation of long chain fatty acid modifiers in several signalling cascades and receptor structures[19]. Specific inhibitors of enzyme activity are well established and powerful tools for determining enzymatic functions in cellular and non-cellular systems. Both sensitivity and specificity of inhibitors are essential prerequisites for a stringent functional analysis of target enzymes. Several molecular mechanisms in enzyme inhibition have been characterized so far, including covalent and non-covalent binding of substrate-like or non-substrate compounds. Competitive binding with the substitution of a characteristic substrate frequently underlies non-covalent enzyme inhibition. Competitive inhibition is the mechanism behind the triacsin C-mediated biochemical effects on ACSL isoforms[10,16,20].

The overwhelming number of studies concerning triacsin C mediated effects on ACSL molecules have been performed in rat models. There is convincing experimental data demonstrating the triacsin C sensitivity of rat ACSL isoforms 1, 3, 4 and 6, but insensitivity of the rat isoform 5[16,17]. However, many ACSL isoforms have splice variants, most of which have not been tested and characterized for triacsin C sensitivity. In addition, species-related differences of ACSL protein sensitivity to triacsin C have not been systematically examined up-

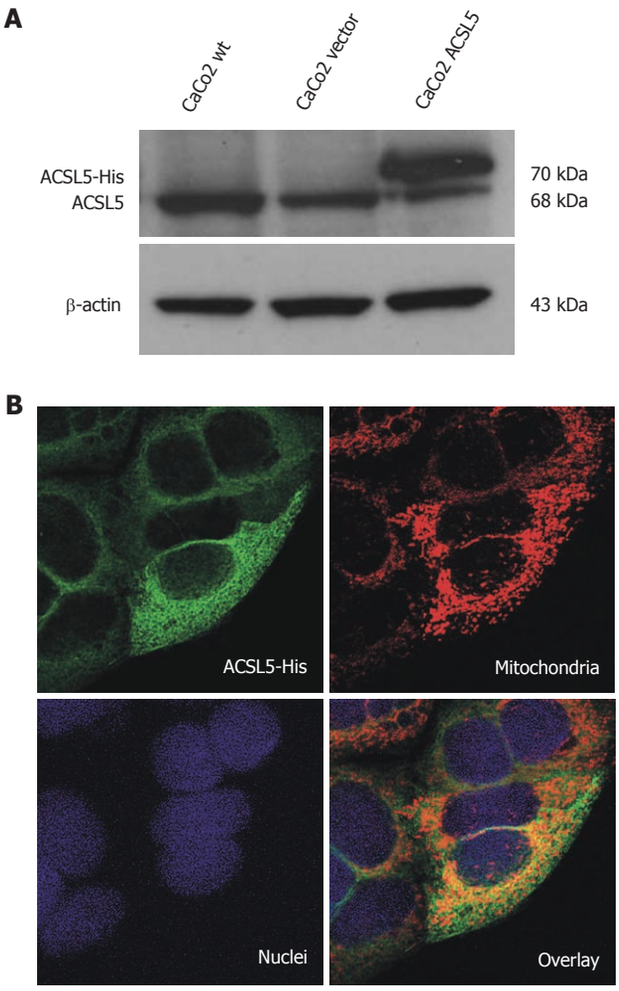

Figure 2 Human acyl-CoA synthetase 5 is triacsin C sensitive in CaCo2 cells. A: CaCo2 cells were stably transfected with the full-length ACSL5 expressing pcDNA_DEST40 plasmid or empty control vector. Expression of N-terminal 6xHis-tagged hu-ACSL5 protein in CaCo2 was analyzed by Western blot using anti-ACSL5 and anti-His antibodies; B: Indirect immunofluorescence of N-terminal 6xHis-tagged hu-ACSL5 proteins, mitochondria, and nuclei in CaCo2 cells shows the mitochondrial localization of recombinant ACSL5 proteins. Original magnification × 400; C: Analysis of ACSL activity (pH 5-10) in hu-ACSL5 CaCo2 transfectants and controls (pcDNA_DEST40 CaCo2 transfectants and CaCo2 wild type) in the presence (white symbols) or absence (black symbols) of 25 μmol/L triacsin C. ACSL5: Acyl-CoA synthetase 5.

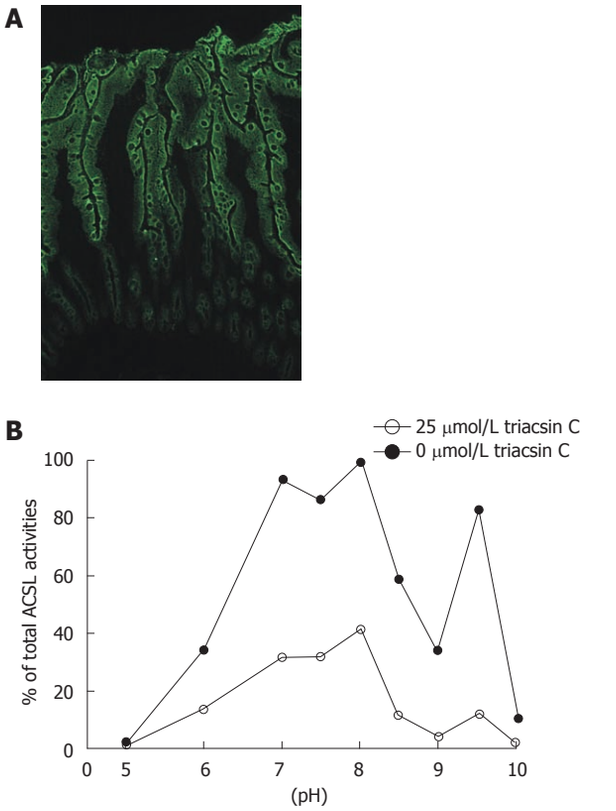

Figure 3 Acyl-CoA synthetase 5 related acyl-CoA synthetase activity is sensitive to triacsin C in human small intestinal mucosa. A: Human small intestinal mucosa after indirect immunofluorescence with anti-ACSL5 antibodies; B: Analysis of ACSL activity in human intestinal mucosa in the presence or absence of 25 μmol/L triacsin C. Triacsin C strongly inhibits ACSL activity in human intestinal mucosa. ACSL5: Acyl-CoA synthetase 5.

to-now. The present study was, therefore, designed to systematically analyze triacsin C effects on human ACSL5 proteins, which are predominantly found in enterocytes of the human small intestinal mucosa.

In order to analyze the biochemical behaviour of hu-ACSL5, we cloned the respective sequences from an intestinal or plasmid cDNA resource, expressed the 6xHis-tagged recombinant proteins, and further purified the enzymes to near homogeneity using Ni^{2+}-affinity chromatography. As demonstrated by digestion experiments and the subsequent analysis of ACSL-activity, the N- or C-terminal 6xHis-tag did not alter the enzymatic activity of ACSL5 at 30 ℃ or 37 ℃ in a broad range of pH values (pH 5-10). Monitoring ACSL-activity of recombinant hu-ACSL5 protein revealed triacsin C-sensitivity with an IC50 about 10 μmol/L. In this experimental setting, insensitivity of recombinant r-ACSL5 and sensitivity of recombinant r-ACSL1 was found, identical to previously described data by Kim *et al*[16].

Additional experiments ruled out the possibility that triacsin C-sensitivity of purified recombinant hu-ACSL5 protein could be a result of the absence of a cellular environment. Stable hu-ACSL5 transfectants and stable controls were established from CaCo2 cells, and in another set of experiments, human intestinal mucosa was investigated. The ACSL activity of cellular systems and tissues

was monitored at different pH values in the presence or absence of triacsin C (25 μmol/L), and similar results to those with recombinant proteins were seen. Especially at pH 9.5, a pH value highly characteristic for hu-ACSL5 activity[6], ACSL activity was significantly decreased in cultured cells as well as intestinal mucosa. The background ACSL activity was probably due to enzymes other than ACSL5, including triacsin C insensitive ACSL splice forms, ACSL isoforms, and fatty acid binding proteins. In conclusion, we demonstrate experimental evidence that hu-ACSL5 is triacsin C sensitive as a purified recombinant protein, in hu-ACSL5 over-expressing epithelial cell lines, and in human small intestinal mucosa, a tissue with high ACSL5 expression levels. These findings imply that human ACSL5 is not able to compensate for triacsin C-inhibited ACSL isoforms, and triacsin C cannot be used to differentiate functions of different ACSL enzymes in human cells or tissues.

The insensitivity of human ACSL5 to triacsin C has been postulated by the observation that recombinant rat ACSL5 was not inhibited by this fungal metabolite[16]. This has been addressed in several studies[15,21-24]. In all of these studies, triacsin C was preferentially used to incubate cultured cells in a concentration clearly below the $IC50^{hu\text{-}ACSL5}$ of approximately 10 μmol/L, and the substance was not directly added to the acyl-CoA activity assay mixture. In our study triacsin C was always used as a competitive inhibitor. Identical to the experimental approach of Kim *et al*[16], triacsin C was directly added to the acyl-CoA activity assay mixture.

We hypothesize that the divergent triacsin C effects on ACSL activity are species-related and determined by human and rat ACSL5 protein sequences. A sequence analysis of the proteins revealed that these were only 81% identical, and that discrepancies existed in exon 20 splicing as well as in the organization and length of functional domains, like the ATP-binding domain or the FA activation domain (NCBI data base: http//www.nlm.nih.gov/nlmhome.html). Species-related differences in ACSL5 activity are further suggested by expression experiments. Over-expression of r-ACSL5 in a rat cellular environment increases fatty acid incorporation into diacylglycerol and triacylglycerol but does not affect fatty acids used for beta-oxidation[25], whereas r-ACSL5 in a human cellular environment increases palmitate oxidation[26].

In the present study, sensitivity of human ACSL5 protein to triacsin C was demonstrated using purified recombinant protein, CaCo2 cells, and human intestinal mucosa. The divergent inhibitory effect of triacsin C with sensitivity of hu-ACSL5 and insensitivity of rat-ACSL5 is most likely species-related. Our findings indicate that human ACSL5 does not compensate for other triacsin C sensitive ACSL isoforms.

ACKNOWLEDGMENTS

The authors are grateful to Petra Akens for typing and proofreading the manuscript.

COMMENTS

Background

Strong expression of acyl-CoA synthetase 5 (ACSL5) is found in surface lining epithelia of the large and small intestine. ACSL5 enzyme activity is probably related to enterocytic maturation and intestinal carcinogenesis. Triacsin C is an inhibitor of several ACSL isoforms and is used to differentiate amongst ACSL functions. In rat, ACSL5 is insensitive to triacsin C.

Research frontiers

Analysis of human ACSL5 *in vitro* as well as in a cellular environment revealed sensitivity to the inhibitor triacsin C, which is in contrast to rat ACSL5. The data indicate that a species-related difference in triacsin C inhibition of ACSL5 exists, and human ACSL5 does not compensate for other triacsin C-sensitive human ACSL isoforms.

Innovations and breakthroughs

In previous triacsin C related studies of ACSL activity, cultured cells were preferentially incubated with triacsin C in a concentration clearly below the $IC50^{hu\text{-}ACSL5}$ of approximately 10 μmol/L. Moreover, triacsin C was not used as a competitive inhibitor. In particular, species-related differences of ACSL protein sensitivity to triacsin C have not been systematically addressed up to now.

Applications

The recent finding that human ACSL5 is sensitive to the inhibitor triacsin C should be considered in related experiments. In human tissues, the differentiation of ACSL activities and the characterization of ACSL5 function with triacsin C are limited. The current finding suggests that human ACSL5 does not compensate for other triacsin C-sensitive ACSL isoforms.

Terminology

Acyl-CoA synthetase 5 is an enzyme that catalyzes formation of long-chain acyl-CoA derivatives and belongs to the family of acyl-CoA synthetases (ACSLs; E.C. 6.2.1.3.). Five ACSL isoforms differing in their enzyme kinetics, substrate preferences, and cellular expression have been identified so far in humans and rodents. Triacsin C [1-hydroxy-3-(E,E,E-2',4',7'-undecatrienylidine) triazene], an alkenyl-N-hydroxytriazene fungal metabolite, has been reported to be a potent competitive inhibitor of acyl-CoA synthetase activity.

Peer review

Kaemmerer *et al* showed that human intestinal ACSL5 is sensitive to triacsin C using purified recombinant protein, CaCo2 cells, and human intestinal mucosa. The experimental design is good and interpretation of results was conducted appropriately.

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S- Editor Lv S **L- Editor** O'Neill M **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4890

World J Gastroenterol 2011 November 28; 17(44): 4890-4898
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Serum manganese superoxide dismutase and thioredoxin are potential prognostic markers for hepatitis C virus-related hepatocellular carcinoma

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Received: April 26, 2011 Revised: August 17, 2011
Accepted: October 14, 2011
Published online: November 28, 2011

Abstract

AIM: To evaluate the clinical significance of oxidative stress markers in patients with hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC).

METHODS: Sixty-four consecutive patients who were admitted to Kagoshima University Medical and Dental Hospital were enrolled in this retrospective study. All patients had chronic liver disease (CLD) due to infection with HCV. Thirty patients with HCV-related HCC, 34 with HCV-related CLD without HCC (non-HCC), and 20 healthy volunteers (HVs) were enrolled. Possible associations between serum manganese superoxide dismutase (MnSOD) and thioredoxin (TRX) levels and clinical parameters or patient prognosis were analyzed over a mean follow-up period of 31.7 mo.

RESULTS: The serum MnSOD levels were significantly higher in patients with HCV-related HCC than in patients without HCC ($P = 0.03$) or HVs ($P < 0.001$). Similarly, serum TRX levels were also significantly higher in patients with HCV-related HCC than in patients without HCC ($P = 0.04$) or HVs ($P < 0.01$). However, serum levels of MnSOD and TRX were not correlated in patients with HCC. Among patients with HCC, the overall survival rate (OSR) was lower in patients with MnSOD levels $\geqslant$ 110 ng/mL than in patients with levels < 110 ng/mL ($P = 0.01$), and the OSR tended to be lower in patients with TRX levels < 80 ng/mL ($P = 0.05$). In addition, patient prognosis with HCC was poorest with serum MnSOD levels $\geqslant$ 110 ng/mL and serum TRX levels < 80 ng/mL. Furthermore, a multivariate analysis using a Cox proportional hazard model and serum levels of five factors (MnSOD, prothrombin time, serum albumin, serum α-fetoprotein (AFP), and serum des-γ-carboxy prothrombin) revealed that MnSOD levels $\geqslant$ 110 ng/mL (risk ratio: 4.12, 95% confidential interval: 1.22-13.88, $P = 0.02$) and AFP levels $\geqslant$ 40 ng/mL (risk ratio: 6.75; 95% confidential interval: 1.70-26.85, $P < 0.01$) were independent risk factors associated with a poor patient prognosis.

CONCLUSION: Serum MnSOD and TRX levels are potential clinical biomarkers that predict patient prognosis in HCV-related HCC.

Key words: Oxidative stress; Manganese superoxide dismutase; Thioredoxin; Hepatitis C virus; Hepatocellular carcinoma

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INTRODUCTION

As a significant cause of global cancer morbidity and mortality, hepatocellular carcinoma (HCC) is the fifth- and seventh-most frequently diagnosed cancer worldwide in men and women, respectively, and is the second- and sixth-most frequent cause of cancer deaths in men and women, respectively[1]. HCC is most frequently caused by persistent infection with hepatitis C or B virus. Early HCC diagnosis and better treatments have helped to improve the prognosis for patients with HCC. Also, interferon (IFN)-based treatments not only eliminate hepatitis C virus (HCV) infection, but also prevent HCC in patients with chronic hepatitis C (CHC)[2]. However, IFN-based therapies do not always effectively eliminate HCV infection or prevent HCC. Thus, biomarkers that are indicative of HCC pathological condition would have many clinical benefits, including aiding in the selection of the most appropriate treatment for a patient's disease.

Oxidative stress results from an imbalance in the production of reactive oxygen species (ROS) and the antioxidative defenses that maintain a cellular redox state. ROS include superoxide anions, hydrogen peroxide, hydroxyl radicals and nitric oxide, all of which are indispensable elements in many biochemical processes[3]. ROS are mainly derived from Kupffer and inflammatory cells in the liver[4], and upon exposure to other cells are thought to induce apoptosis, necrosis, inflammation, immune responses, fibrosis and tissue regeneration[5]. In liver disease, there is an overproduction of ROS from endogenous sources such as the mitochondria, peroxisomes, and activated inflammatory cells. In particular, ROS of mitochondrial origin were recently reported to be elevated in patients with alcoholic liver disease, non-alcoholic steatohepatitis (NASH)[6,7] and HCV-related chronic liver disease (CLD)[8]. Conversely, cells are protected from oxidative stress by intracellular antioxidants such as glutathione (GSH) and thioredoxin (TRX) and by various antioxidant enzymes such as superoxide dismutase (SOD), GSH peroxidase, catalase, and heme oxygenase-1[9-11]. Collectively, the relative expression levels of these molecules may serve as biomarkers for various liver diseases, including HCV-related HCC.

Manganese SOD (MnSOD) is an antioxidant enzyme that catalyzes the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2. We have previously demonstrated that MnSOD expression was induced in primary cultured hepatocytes that were loaded with hydrogen peroxide *in vitro* and that serum MnSOD levels can be used to distinguish between NASH and simple steatosis in patients with nonalcoholic fatty liver disease[7]. However, the clinical significance of serum MnSOD levels in HCV-related CLD has not been fully investigated.

TRX was originally discovered in *Escherichia coli* as a proton donor for ribonucleotide reductase[12]. Subsequently, the human *TRX* gene was cloned as an adult T-cell leukemia-derived factor and was originally described as an interleukin-2 receptor inducer present in the cell culture supernatant of human T-lymphotropic virus type-1 -transformed cells[13]. TRX expression is induced by various oxidative stressors in patients with acquired immunodeficiency syndrome[14], Sjögren's syndrome[15], rheumatoid arthritis[16], and malignant neoplasms[17,18]. Previous studies have reported that serum TRX is an oxidative stress marker and that serum TRX levels increase in patients with HCV-related CLD during liver fibrosis progression[19]. In addition, serum TRX levels are reported to be elevated in patients with NASH compared to patients with simple steatosis[20]. However, the clinical significance of elevated TRX levels among patients infected with HCV in relation to HCC diagnosis and prognosis has not been elucidated.

In this study, we aimed to clarify the clinical significance of serum levels of MnSOD and TRX in patients with HCV-related CLD, and in particular among patients with HCC.

MATERIALS AND METHODS

Patients

Sixty-four consecutive patients who were admitted to Kagoshima University Medical and Dental Hospital between December 2006 and November 2008 were enrolled in this retrospective study. All patients had CLD due to an HCV infection and were diagnosed with HCC (30 patients; HCC group) or without HCC (34 patients; non-HCC group). Twenty healthy volunteers (HVs) were also enrolled in this study.

In this study, HCC was diagnosed based on findings from abdominal ultrasound, abdominal computed tomography, and serum levels of α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP, also known as PIVKA-Ⅱ). Patients were excluded from this study if they were positive for hepatitis B surface antigen; other types of hepatitis, including autoimmune hepatitis and alcoholic liver disease; or other malignancies.

The study endpoint was patient death, the available follow-up date, or December 31, 2010. Patient follow-up

periods ranged from 5.1 to 44.6 mo, with a mean observation time of 31.7 mo. Informed consent was obtained from all study patients and healthy controls. This study was approved by the ethical committees of Kagoshima University Graduate School of Medical and Dental Sciences and Kagoshima University Medical and Dental Hospital.

Laboratory markers

The clinical laboratory parameters assessed included platelet count (Plt), prothrombin time (PT), albumin (Alb), total bilirubin (T-Bil), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), AFP and DCP. The serologically defined HCV genotype (HCV serotype) was determined using a serological genotyping assay kit (Immunocheck F-HCV Grouping; International Reagents Co., Tokyo, Japan). If the HCV serotype could not be determined, the HCV genotype was evaluated using the HCV Core Genotype assay (SRL, Tokyo, Japan). HCV genotype 1b was included with serotype Ⅰ, while genotypes 2a and 2b were included with serotype Ⅱ. No other HCV genotype was detected in this study population. HCV RNA titers were quantified using either quantitative RT-PCR (Amplicor monitor version 2, Roche, Tokyo, Japan) or the Cobas TaqMan PCR assay (Roche, Tokyo, Japan). Patients were categorized as having a high viral load if their values were 100 KIU/mL or greater based on quantitative RT-PCR analysis, or 5 log IU/mL or more based on the Cobas TaqMan PCR assay.

Evaluation of clinical stage

Hepatic function was assessed in the HCC group using Child-Pugh staging based on both clinical (ascites and encephalopathy) and laboratory (Alb, T-Bil, and PT) parameters. HCC clinical stage was assessed based on a patient's Cancer of the Liver Italian Program (CLIP) score, which was calculated by adding points for the following four variables: Child-Pugh stage, tumor morphology, AFP value, and portal venous invasion[21,22]. The Japan Integrated Staging (JIS) system[23,24], developed by the Liver Cancer Study Group of Japan and based on a combination of Child-Pugh stage and HCC TNM classification, was used to clinically stage HCC.

Serum MnSOD and TRX levels

Serum was obtained from peripheral blood samples by centrifugation at 4000 *g* for 5 min at room temperature. Serum samples were frozen at -80 ℃ until further use. Serum MnSOD or TRX levels were measured using the Human Superoxide Dismutase 2 (AbFRONTIER, Seoul, Korea) and human thioredoxin (Redox Bio Science, Kyoto, Japan) ELISAs, respectively.

Statistical analysis

Results are expressed as the mean and standard deviation. *P* values less than 0.05 were regarded as statistically significant. Statistical analyses were performed using the Fischer's exact test or the Mann-Whitney *U* test, as appropriate. The area under the curve (AUC) was calculated for the receiver operating characteristic (ROC) curve in order to measure the overall accuracy of the test. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of diagnostic test were additionally determined according to the protocol described previously[25]. Differences among the three groups were evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparison tests. Correlation coefficients were calculated using Spearman's rank correlation analysis. The Kaplan-Meier method was used to estimate death for each parameter that had been identified at enrollment, and the death distribution curves were compared using the log-rank test. Univariate and multivariate analyses of patient outcome risk ratios were performed using Cox's proportional hazards regression analyses. All statistical analyses were conducted using PASW Statistics v. 18 (SPSS Inc., Chicago, IL).

Table 1 Patient clinical characteristics

Characteristics	Non-HCC group (*n* = 34)	HCC group (*n* = 30)	*P* value[1]
Age (yr)	62.3 ± 11.0	72.2 ± 7.5	< 0.001
Sex (male/female)	10/24	21/9	< 0.01
Plt (× 10^4/μL)	17.0 ± 5.5	10.3 ± 5.2	< 0.001
PT (%)	99.7 ± 13.3	77.6 ± 11.8	< 0.001
Alb (g/dL)	4.3 ± 0.4	3.6 ± 0.6	< 0.001
T-Bil (mg/dL)	0.8 ± 0.3	1.5 ± 0.8	< 0.001
ALT (IU/L)	44.8 ± 30.2	52.0 ± 28.2	0.12
γ-GTP (IU/L)	31.3 ± 16.1	56.2 ± 44.3	< 0.01
AFP (ng/mL)	7.2 ± 22.8	85.9 ± 197.6	< 0.001
DCP (mAU/mL)	22.8 ± 14.7	485.5 ± 1982.6	0.001
HCV serotype group (1/2)	18/10 (*n* = 28)	21/3 (*n* = 24)	0.06
HCV RNA level (high/low)	28/5 (*n* = 33)	21/4 (*n* = 25)	0.99

Data are shown as the mean ± SD. *n*: Number of patients or the number of samples analyzed. [1]Differences between mean values were evaluated using either the Fischer's exact test or the Mann-Whitney *U* test, as appropriate. Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: alpha-fetoprotein; DCP: des-γ-carboxy prothrombin; HCV: Hepatitis C virus; RNA: Ribonucleic acid.

RESULTS

Patient characteristics and classification according to the presence of hepatocellular carcinoma

Table 1 summarizes the baseline clinical characteristics of the 64 patients who were classified based on the presence or absence of HCC. Age, sex, and clinical laboratory parameters, including Plt, PT, Alb, T-Bil, γ-GTP, AFP and DCP, were significantly different between these two groups.

Serum MnSOD and TRX levels in hepatocellular carcinoma patients

Serum MnSOD levels were significantly higher in patients with HCC compared to patients without HCC ($P = 0.03$) and HVs ($P < 0.001$) (Figure 1A). The serum TRX levels were also significantly higher in the HCC group compared to the non-HCC group ($P = 0.04$) and HV group ($P < 0.01$) (Figure 1B). However, there was no correlation between these two markers in the HCC group ($P = 0.28$, $r = 0.20$) (Figure 1C).

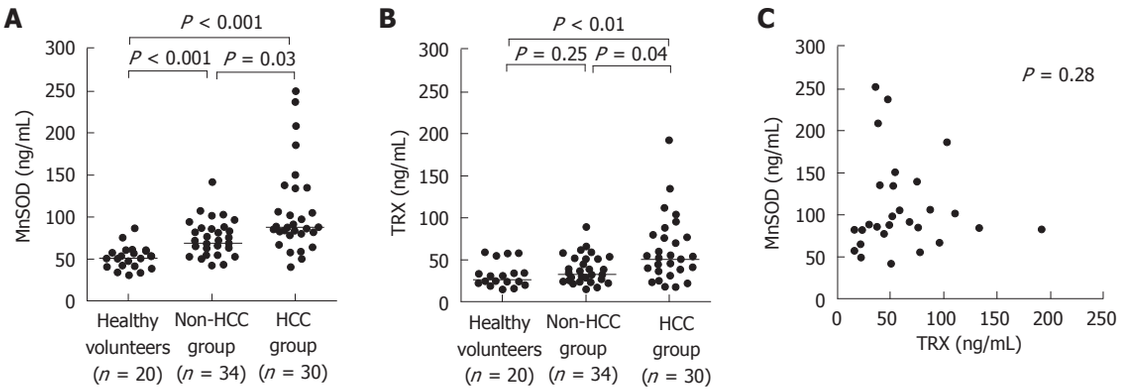

Figure 1 Serum levels of manganese superoxide dismutase and thioredoxin in the hepatocellular carcinoma, non-hepatocellular carcinoma and healthy volunteer groups. A: Serum manganese superoxide dismutase (MnSOD) levels were significantly higher in the hepatocellular carcinoma (HCC) group than in either the non-HCC group ($P = 0.03$) or the healthy volunteers (HV) group ($P < 0.001$); B: Serum thioredoxin (TRX) levels were also significantly higher in the HCC group than in either the non-HCC group ($P = 0.04$) or the HV group ($P < 0.01$); C: No significant correlation was detected between serum MnSOD and TRX levels in the HCC group.

Table 2 Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of manganese superoxide dismutase and α-fetoprotein serum levels for diagnosis of hepatocellular carcinoma in all patients (%)

Factors	Sensitivity	Specificity	PPV	NPV	Accuracy
MnSOD (≥ 110 ng/mL)	26.7	97.1	88.9	60.0	64.1
AFP (≥ 40 ng/mL)	33.3	97.1	90.9	62.3	67.2
Combination[1]	46.7	94.1	87.5	66.7	71.9

[1]MnSOD ≥ 110 ng/mL and/or AFP ≥ 40 ng/mL. PPV: Positive predictive value; NPV: Negative predictive value; MnSOD: Manganese superoxide dismutase; AFP: α-fetoprotein.

Table 3 Correlation between serum manganese superoxide dismutase or thioredoxin levels and laboratory data in the hepatocellular carcinoma group

Factors	HCC group ($n = 30$)			
	Serum MnSOD levels		Serum TRX levels	
	Correlation coefficient	*P* value	Correlation coefficient	*P* value
Age (yr)	-0.97	0.61	0.11	0.55
Plt (× 10^4/μL)	0.03	0.89	0.66	< 0.001
PT (%)	-0.36	0.05	0.12	0.53
Alb (g/dL)	-0.63	< 0.001	0.19	0.33
T-Bil (mg/dL)	0.25	0.18	0.05	0.79
ALT (IU/L)	0.12	0.52	0.15	0.42
γ-GTP (IU/L)	0.30	0.11	0.28	0.13
AFP (ng/mL)	0.38	0.04	0.11	0.57
DCP (mAU/mL)	0.57	0.001	0.12	0.52

P values were assessed by Spearman's rank correlation analysis. MnSOD: Manganese superoxide dismutase; TRX: Thioredoxin; HCC: Hepatocellular carcinoma; Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: α-fetoprotein; DCP: des-γ-carboxy prothrombin.

Diagnostic value of serum MnSOD and TRX levels for patients with hepatocellular carcinoma and hepatitis C virus infection

Serum AFP and DCP concentrations are established diagnostic markers for HCC. To evaluate the utility of MnSOD and TRX for the diagnosis of HCC, we measured AFP and DCP expression in addition to MnSOD and TRX expression. In an AUC-ROC analysis, AFP was the strongest diagnostic marker for HCC (AUC-ROC, 0.90). AUC-ROCs for MnSOD, TRX and DCP were 0.73, 0.77 and 0.77, respectively. Additional analyses showed that the accuracy of AFP (≥ 40 ng/mL) for diagnosis of HCC was higher than that of MnSOD (≥ 110 ng/mL) (Table 2), while the combination of AFP and MnSOD was a more accurate marker of HCC than either marker alone.

Association of serum MnSOD or TRX levels with laboratory data in the HCC group

Serum MnSOD levels for the 30 patients in the HCC group were positively correlated with serum AFP and DCP levels and were negatively correlated with serum Alb levels (Table 3). Serum MnSOD levels were also significantly higher in patients with two or more HCC tumors than in patients with a single HCC tumor [average ± SD (ng/mL), 125.4 ± 50.9 *vs* 87.4 ± 48.8, $P = 0.008$], although HCC tumor size was not associated with serum MnSOD levels. In addition, HCC patient serum MnSOD levels increased in parallel with the Child-Pugh stage, CLIP score and JIS score (Figure 2A-C). In contrast, serum TRX levels were only associated with platelet counts (Table 3). Serum TRX levels were not associated with HCC tumor number or size. Furthermore, there were no significant correlations between serum TRX levels for various scores (Figure 2D-F).

Overall survival rate based on serum MnSOD or TRX levels in the HCC group

In the HCC group, the overall patient survival rate was significantly lower ($P = 0.01$) in patients with MnSOD levels ≥ 110 ng/mL compared to patients with levels < 110 ng/mL (Figure 3A). In addition, the overall survival rate tended to be lower ($P = 0.05$) in patients with TRX levels < 80 ng/mL compared to those with levels ≥ 80 ng/mL (Figure 3B). Furthermore, among all HCC groups, patients who had both serum MnSOD levels ≥ 110

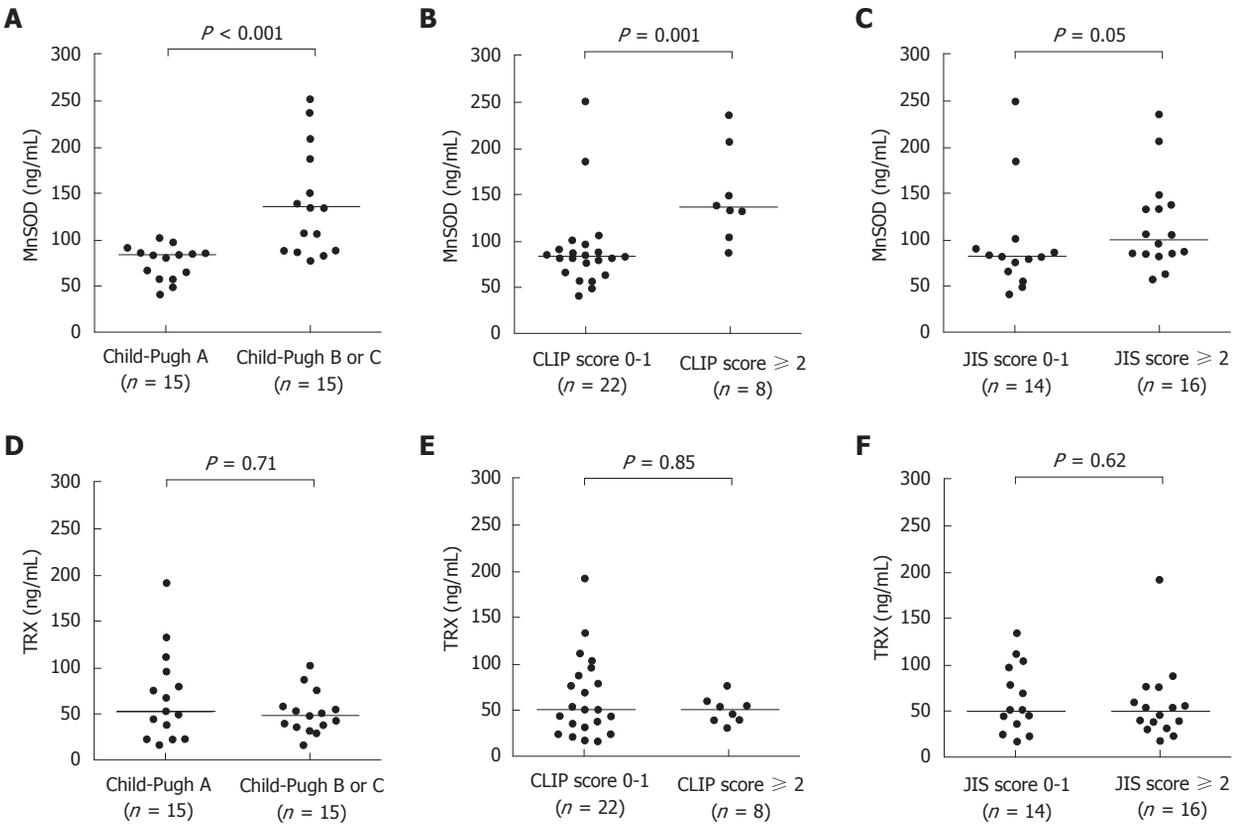

Figure 2 Clinical significance of serum manganese superoxide dismutase and thioredoxin levels in hepatocellular carcinoma. In the hepatocellular carcinoma (HCC) group, differences in serum manganese superoxide dismutase (MnSOD) and thioredoxin (TRX) levels were evaluated based on Child-Pugh stage, cancer of the liver italian program (CLIP) score and Japan integrated staging (JIS) score. A: Serum MnSOD levels were significantly higher in patients with Child-Pugh B or C compared to those with Child-Pugh A ($P < 0.001$); B: Serum MnSOD levels in patients with a CLIP score of 2 or greater were significantly higher compared to levels in patients with a CLIP score of 0 or 1 ($P = 0.001$); C: In addition, serum MnSOD levels tended to be higher in patients with a JIS score of 2 or greater compared to patients with a JIS score of 0 or 1 ($P = 0.05$); D-F: In contrast, serum TRX levels were not significantly different based on Child-Pugh stage, CLIP score or JIS score.

ng/mL and TRX levels < 80 ng/mL had a significantly poorer prognosis. Conversely, patients with a serum TRX level ⩾ 80 ng/mL had a favorable prognosis, regardless of their serum MnSOD level (Figure 3C).

In addition to serum MnSOD and TRX levels, other possible prognostic factors were also investigated in the HCC group. A univariate analysis (log-rank test) revealed that the survival rate was significantly different between patients with high and low levels of MnSOD, PT, Alb, AFP and DCP, but not other factors such as TRX (Table 4). A multivariate analysis using a Cox proportional hazard model and five markers (MnSOD, PT, Alb, AFP and DCP) selected based on the results of the univariate analysis revealed that MnSOD levels ⩾ 110 ng/mL and AFP levels ⩾ 40 ng/mL were independent risk factors that were associated with a poor patient prognosis (Table 5). In addition, similar results were obtained from a similar multivariate analysis using the same five factors and TRX, supporting the finding that TRX is not an independent risk factor associated with HCC prognosis. Furthermore, patient Child-Pugh stage, CLIP score and JIS score, which were calculated based on several factors including clinical symptoms and laboratory data, were also prognostic factors for patients with HCC (Table 4). A multivariate analysis using the three markers of MnSOD, Child-Pugh stage and CLIP score indicated that Child-Pugh stage was also a significant prognostic factor (risk ratio: 6.19, 95% confidential interval: 1.33-28.95, $P = 0.02$).

DISCUSSION

HCV infection is the most important known contributor to the etiology of HCC. An increasing incidence of HCC has been largely attributed to a rise in HCV infections in the general population during the last 50 to 60 years[26]. During HCV infection, ROS production increases and persists throughout the infection. In addition, ROS are thought to play a major role in the pathogenesis of chronic inflammatory changes in the liver, leading to increased hepatic fibrosis and decreased hepatic function. In this study, we have shown that both serum MnSOD and TRX levels are elevated in patients with HCV-related HCC, with no correlation between these two markers. In addition, serum MnSOD and TRX levels were a useful predictor of overall patient survival. Serum MnSOD and TRX levels are reported to be biomarkers of oxidative stress in several diseases, including liver disease[7,14,17,19,27-29]. There were a small number of enrolled patients in this study and other contributors to liver diseases such as chronic hepatitis B infection should be further evaluated. However, our

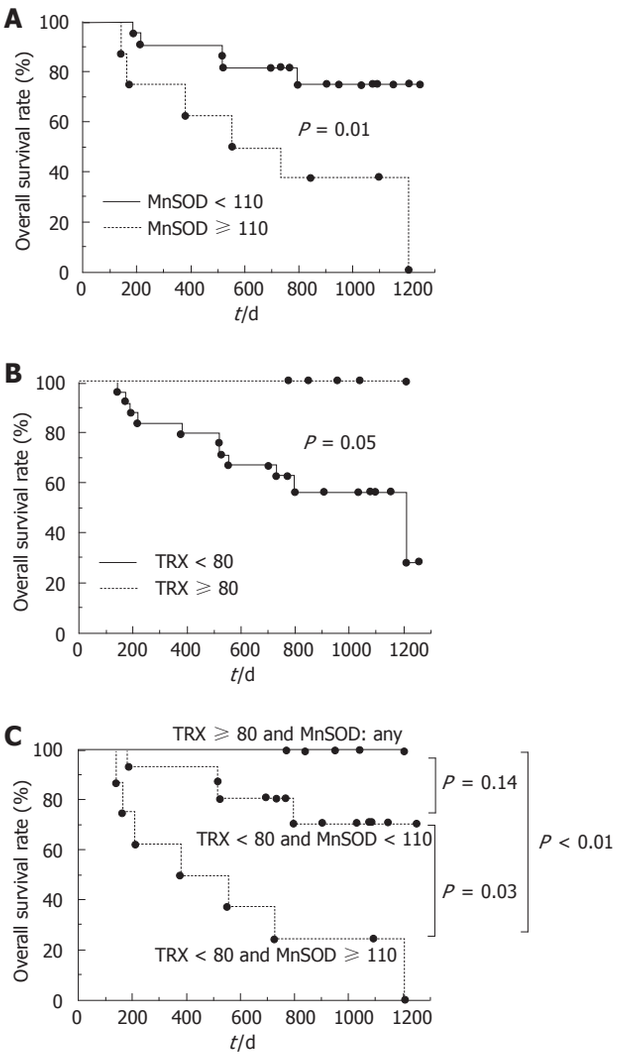

Figure 3 Overall hepatocellular carcinoma patient survival based on serum levels of manganese superoxide dismutase or thioredoxin. Overall survival was plotted using the Kaplan-Meier method after separation into two or three groups defined as follows: A: Manganese superoxide dismutase (MnSOD) < 110 ng/mL or ⩾ 110 ng/mL; B: Thioredoxin (TRX) < 80 ng/mL or ⩾ 80 ng/mL; TRX ⩾ 80 ng/mL, TRX < 80 ng/mL; C: MnSOD < 110 ng/mL, or TRX < 80 ng/mL and MnSOD ⩾ 110 ng/mL. The overall survival rate was lower in patients with MnSOD levels ⩾ 110 ng/mL (P = 0.01) (A). Also, cumulative patient survival rate tended to be lower in patients with TRX levels < 80 ng/mL (P = 0.05) (B). Among these groups, patients with serum TRX levels < 80 ng/mL and serum MnSOD levels ⩾ 110 ng/mL had the poorest prognosis (C).

Table 4 Univariate analysis of prognostic factors in the hepatocellular carcinoma group

Factors	Category	Number	*P* value[1]
Single marker			
MnSOD (ng/mL)	< 110/⩾ 110	22/8	0.01
TRX (ng/mL)	< 80/⩾ 80	24/6	0.05
Age (yr)	< 70/⩾ 70	12/18	0.23
Plt (× 10^4/μL)	< 10/⩾ 10	19/11	0.38
PT (%)	< 80/⩾ 80	15/15	0.02
Alb (g/dL)	< 3.5/⩾ 3.5	15/15	0.02
T-Bil (mg/dL)	< 1.5/⩾ 1.5	18/12	0.34
ALT (IU/L)	< 40/⩾ 40	11/19	0.58
γ-GTP (IU/L)	< 50/⩾ 50	17/13	0.98
AFP (ng/mL)	< 40/⩾ 40	20/10	< 0.01
DCP (mAU/mL)	< 40/⩾ 40	16/14	0.02
Staging system			
Child-Pugh stage	A/⩾ B	16/14	< 0.01
CLIP score	0-1/⩾ 2	22/8	0.01
JIS score	0-1/⩾ 2	14/16	0.41

[1]*P* values were assessed using the log-rank test. MnSOD: Manganese superoxide dismutase; TRX: Thioredoxin; Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: Alpha-fetoprotein; DCP: Serum des-γ-carboxy prothrombin; CLIP: Cancer of the Liver Italian Program; JIS: Japan Integrated Staging.

Table 5 Multivariate analysis of prognostic factors in the hepatocellular carcinoma group

Factors	Risk ratio	95% CI	*P* value
MnSOD (⩾ 110 ng/mL)	4.12	1.22-13.88	0.02
AFP(⩾ 40 ng/mL)	6.75	1.70-26.85	< 0.01

95% CI: 95% confidence interval; MnSOD: Manganese superoxide dismutase; AFP: α-fetoprotein.

study has clearly demonstrated the clinical significance of these markers in patients with HCV-related HCC.

Serum MnSOD and TRX levels should both reflect hepatic oxidative stress. The results of the current study showed that both of these markers were increased in the HCC group relative to levels in the non-HCC group and the HV group (Figure 1A and B). However, there was no correlation between these two markers in the HCC group (Figure 1C). MnSOD is primarily localized to the mitochondrial matrix[3] and abnormal mitochondrial morphologies are frequently observed in CHC[8]. Therefore, MnSOD may be an indicator of mitochondrial disorders that are induced by oxidative stress. On the other hand, there are two TRX proteins, cytoplasmic TRX1 and mitochondrial TRX2[30]. TRX1 negatively regulates the apoptosis signal-regulating kinase 1 (ASK1)-c-Jun N-terminal kinase/P38 apoptotic pathway by binding to and inhibiting the kinase activity of ASK1, which plays an important role in ROS-induced cellular responses[31]. TRX2 is an essential regulator of mitochondrial ROS levels that has been associated with mitochondrial outer membrane permeability[32]. In the present study, we examined the serum levels of TRX1, but not TRX2, using a sandwich ELISA. Thus, the MnSOD and TRX proteins that were examined in this study have different origins in the mitochondria and cytoplasm, respectively, which could contribute to the lack of correlation between these two markers.

Several studies have shown that the HCV core protein directly inhibits the electron transport system and modulates apoptosis, transcription, and cell signaling[33]. Abdalla *et al*[34] reported that expression of not only the HCV core protein but also the HCV NS proteins increases ROS and further showed that the presence of these proteins can increase endogenous expression levels of antioxidant enzymes and prooxidants such as MnSOD. Several reports have shown that serum MnSOD levels in patients with HCV-related CLD[35-37] are associated with

various clinical findings, such as fibrosis and hepatic oxidative stress. However, the significance of serum MnSOD levels has not been fully examined in patients with HCC. We previously reported that serum MnSOD levels may be correlated with fibrosis in patients with NAFLD[7]. In addition, serum MnSOD levels decreased in patients with CHC after administration of an interferon-based treatment (data not shown). These results indicate that serum MnSOD levels are likely associated with hepatic fibrosis or oxidative stress in patients with CHC. In the present study, however, MnSOD levels were not associated with platelet counts, which is a simple predictor of hepatic fibrosis in this patient population[38]. Thus, advanced hepatic fibrosis or oxidative stress may be one reason why serum MnSOD levels have diagnostic and prognostic utility with HCC, but other mechanisms should also be considered.

The present study revealed that serum MnSOD levels were significantly higher in the HCC group than in the non-HCC group (Figure 1A). In the HCC group, serum MnSOD levels were negatively correlated with serum Alb and tended to negatively correlate with PT (Table 3); these results showed an association between MnSOD and Child-Pugh stage (Figure 2A). It is known that in humans, MnSOD activity is comparatively higher in the liver compared to other tissues[39]. In addition, although a previous immunohistochemical study showed that MnSOD expression was higher in both cancerous and non-cancerous liver tissues from patients with HCC, this positive immunoreactivity was strongly observed in non-cancerous liver tissues, especially in normal hepatocytes surrounding HCC, regenerative small hepatocytes in the tumor boundary, and mononuclear inflammatory cells in necroinflammatory lesions[40]. Furthermore, ROS are overproduced by Kupffer cells and inflammatory cells in liver disease[5,41]. In the present study, serum MnSOD levels were also positively correlated with the serum tumor markers AFP and DCP (Table 3) and with Child-Pugh stage and CLIP score (Figure 2). These results indicate that increased MnSOD expression reflects hepatocyte oxidative stress and correlates with decreased hepatic function, increased hepatic fibrosis and ROS production by inflammatory cells in liver cirrhosis. These features comprise the main background characteristics leading to HCC and may be associated with the indirect effects of liver cancer progression. These associations may also explain why serum MnSOD levels predicted the overall survival of patients with HCC.

It was previously reported that serum levels of TRX, which is a stress-induced protein, increase relative to the degree of hepatic fibrosis, and that high serum concentrations of TRX may indicate advanced hepatic fibrosis[19,20]. In contrast, it has also been reported that a higher degree of hepatic fibrosis is associated with lower platelet counts[38]. Therefore, the present study may present a conflict, since results indicated that serum TRX level was positively correlated with platelet count. A previous report showed that the survival rate following LPS plus GalN-induced hepatitis was much higher in transgenic mice overexpressing TRX than in wild-type mice, and that thioacetamide-induced hepatic fibrosis was suppressed in TRX transgenic mice compared to wild-type mice[42]. Although it is still unclear why TRX and platelet counts are positively correlated, we speculate that elevated serum TRX in patients with HCC and advanced hepatic fibrosis potentially improves overall survival by suppressing oxidative stress[43]. In addition, patients with HCC, low levels of TRX, and high levels of MnSOD, which may be indicative of excessive oxidative stress without TRX attenuation, have the poorest prognosis. This result supports the hypotheses presented above. In order to better assess these findings, future studies are needed that incorporate sequential observations of serum TRX and MnSOD levels over time in patients with chronic hepatitis, cirrhosis and HCC.

Serum MnSOD and TRX may be useful biomarkers for HCC diagnosis (Figure 1). AFP is also a diagnostic marker for HCC, and the present results indicate that AFP can be used to distinguish between patients with and without HCC (Table 2). However, AFP is not a sufficiently sensitive marker for identification of the majority of patients with small HCCs[44,45], and AFP testing is not currently included in the recommendations for HCC surveillance in the updated HCC guidelines published by the American Association for the Study of Liver Disease[46]. Therefore, clinicians and clinical researchers should consider using MnSOD and TRX as diagnostic biomarkers for early HCC or as additional markers in a HCC surveillance program using ultrasonography or AFP. In addition, it is highly important to know whether these markers decrease in response to HCC therapy and reductions in tumor burden. These markers also may have utility in patients on a transplant waiting list who are treated with neo-adjuvant therapy for tumor downstaging.

Our study demonstrated that elevated serum AFP level is indicative of a poor prognosis for patients with HCC (Table 4), as was previously reported[47]. The CLIP score, which is calculated based on four factors such as the AFP value, was also useful to predict the prognosis of HCC patients in this study as well as in a previous report[48]. Other markers such as the protein survivin have been reported as poor prognostic factors for HCC[49]. Similarly, MnSOD was an independent predictive factor for overall survival in the HCC group (Figure 3A, Table 5). Although TRX was not an independent predictor of overall survival in patients with HCC (Table 4), we speculate that a combination assay using both MnSOD and TRX could be used to predict overall patient survival. It will be important to conduct further prospective evaluations of each individual marker as well as a combination of these markers using a large number of patients.

In conclusion, serum MnSOD and TRX levels increased as HCV-related chronic liver disease progressed, especially among patients with HCC. Although there was no correlation between serum levels of MnSOD and TRX, higher serum MnSOD levels and lower TRX levels in patients with HCC trended towards an indication of poor

patient prognosis. These results suggest that serum Mn-SOD and TRX levels are not only a potential biomarker for HCV-related progressed liver disease, but may also serve as prognostic markers in HCC.

ACKNOWLEDGMENTS

We thank Ms. Yuko Nakamura for technical assistance.

COMMENTS

Background

During hepatitis C virus (HCV) infection, production of reactive oxygen species (ROS) is persistently increased throughout HCV infection. ROS are thought to play an important role in the pathogenesis of chronic inflammatory changes in the liver, which may lead to the development of hepatic fibrosis, decreased hepatic function or hepatocellular carcinoma (HCC). However, there is little information currently available regarding serum oxidative stress markers in patients with HCV-related HCC.

Research frontiers

Cells are protected from oxidative stress by antioxidant enzymes such as superoxide dismutase (SOD) and by intracellular antioxidants such as thioredoxin (TRX). Serum manganese SOD (MnSOD) and TRX are thought to be biomarkers for various liver diseases, including HCV-related liver disease, but these possibilities have not been fully investigated. In this study, the authors demonstrated the clinical significance of serum levels of MnSOD and TRX in patients with HCV-related HCC.

Innovations and breakthroughs

Although there was no correlation between serum levels of MnSOD and TRX, serum levels of both markers increased as HCV-related chronic liver disease progressed, and in particular among patients with HCC. In addition, higher serum MnSOD levels and lower TRX levels tended to indicate a poor prognosis among patients with HCC.

Applications

Serum MnSOD and TRX levels are not only potential biomarkers for progression of HCV-related liver disease, but they may also serve as prognostic markers for patients with HCC. Therefore, clinicians should consider using serum levels of MnSOD and TRX as diagnostic biomarkers for early HCC or as additional markers in HCC surveillance programs. In addition, it will be important to know whether these markers change after therapy for liver disease, including HCC.

Peer review

Oxidative stress is closely associated with carcinogenesis. If oxidative stress markers could be useful in predicting clinical outcome in chronic hepatitis C and HCV-related HCC, they would provide us with a practical and informative tool. However, there are some limitations of this investigation, including a relatively small number of patients studied. Thus, the overall assessment is "good".

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Online Submissions: http://www.wjgnet.com/1007-9327office
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doi:10.3748/wjg.v17.i44.4899

World J Gastroenterol 2011 November 28; 17(44): 4899-4904
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Clinical presentation and management of Fasciola hepatica infection: Single-center experience

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Received: April 8, 2011 Revised: June 15, 2011
Accepted: June 22, 2011
Published online: November 28, 2011

Abstract

AIM: To identify the characteristic clinical, laboratory and radiological findings and response to treatment in patients with fascioliasis.

METHODS: Patients who were diagnosed with Fasciola hepatica infection were included in this prospective study. Initial clinical, laboratory and radiological findings were recorded. All patients were followed until a complete response was achieved or for 6 mo after treatment discontinuation.

RESULTS: Fasciola hepatica infection was diagnosed in 30 patients (24 females; mean age: 42.6 years) between January 2008 and February 2011. Twenty-two (73%) patients had hepatic phase fascioliasis, 5 patients had biliary phase, and 3 patients had biliary phase associated with acute pancreatitis. Of the 8 patients with biliary phase fascioliasis, 2 patients displayed features that overlapped with both hepatic and biliary phase. Abdominal pain and right upper abdominal tenderness were the most prominent signs and symptoms in all patients. Eosinophilia was the most prominent laboratory abnormality in both patients with hepatic and biliary phase (100% and 50%, respectively). Multiple nodular lesions like micro-abscesses on abdominal computerized tomography were the main radiological findings in patients with hepatic phase. Small linear filling defects in the distal choledochus were the main endoscopic retrograde cholangiopancreatography (ERCP) findings in patients with biliary phase. Patients with hepatic phase were treated with triclabendazole alone, and patients with biliary phase were treated with triclabendazole and had live Fasciola hepatica extracted from the bile ducts during ERCP.

CONCLUSION: Fasciola hepatica infection should be considered in the differential diagnosis of patients with hepatic or biliary disease and/or acute pancreatitis associated with eosinophilia.

Key words: Fasciola hepatica; Liver abscesses; Cholangitis; Pancreatitis; Triclabendazole

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Kaya M, Beştaş R, Çetin S. Clinical presentation and management of Fasciola hepatica infection: Single-center experience. *World J Gastroenterol* 2011; 17(44): 4899-4904 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4899.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4899

INTRODUCTION

Fascioliasis is an infection caused by a trematode of the liver, Fasciola hepatica, that particularly affects sheep, goats and cattle. The flukes are leak-like, flat worms, measuring 2-4 cm[1]. The number of reports of humans infected with Fasciola hepatica has increased significantly since 1980, and several geographical areas have been described as endemic for the disease in humans, with preva-

lence and incidence ranging from low to very high[2,3]. In humans, the infection begins with the ingestion of watercress or contaminated water containing encysted larva. The larva excyst in the stomach, penetrate the duodenal wall, escape into the peritoneal cavity, and then pass through the liver capsule to enter the biliary tree[1]. Human fascioliasis has two phases. The hepatic phase of the disease begins one to three months after ingestion of metacercariae, with penetration and migration through the liver parenchyma toward the biliary ducts[1,4,5]. Common signs and symptoms of the hepatic phase are abdominal pain, fever, eosinophilia, and abnormal liver function tests[1,4,6-8]. The biliary phase of the disease usually presents with intermittent right upper quadrant pain with or without cholangitis or cholestasis[9-11].

In non-endemic areas, diagnosis of fascioliasis can be difficult and usually is delayed because the disease is not often encountered and the symptoms may be confused with other hepatic or biliary disorders. Diagnosis of Fasciola hepatica infection has traditionally relied on detecting the presence of eggs in fecal samples, but this method is unreliable and complex[1,4]. Among human cases in non-endemic areas, low egg outputs, e.g., 1-2 eggs per g of feces (epg) and 1-4 epg were being considered rare. These egg outputs are much lower than those found among humans in endemic areas[3]. Computerized tomographic (CT) findings in patients with hepatic phase and ultrasonographic findings in patients with biliary phase are used for the diagnosis of fascioliasis[5,6]. Confirmation of the diagnosis is necessary and should be based on serological findings and parasitic tests[12]. Triclabendazole and bithionol are effective agents for the treatment of fascioliasis[8].

The aim of this prospective study was to identify the characteristic clinical, laboratory and tomographic findings and response to treatment during follow-up in patients with fascioliasis.

MATERIALS AND METHODS

Patients who were admitted to our clinic and were diagnosed with Fasciola hepatica infection between January 2008 and February 2011 were prospectively enrolled in this study. All patients received an initial complete clinical exam, laboratory tests (including complete blood counts and routine biochemical analyses), and abdominal CT. All of the CT scans were obtained using a 4-channel multislice CT scanner (Sensation 4; Siemens Medical Solutions, Erlargen, Germany). A specific indirect hemagglutination assay (IHA) using purified adult Fasciola hepatica F1 antigen (Laboratoires Fumouze Diagnostic, Levallois Perret, France; cut-off 1/320) was used for serological diagnosis of fascioliasis. The diagnosis of Fasciola hepatica infection with hepatic phase was based on: (1) the presence of characteristic findings on the abdominal CT examination, as previously described[5-8]; (2) exclusion of all other known diseases that cause hepatic lesions on tomographic examination; and (3) a positive specific IHA for Fasciola hepatica; and/or (d) the presence of Fasciola hepatica eggs in the fecal examination. The diagnosis of Fasciola hepatica infection with biliary phase was based on the extraction of live Fasciola hepatica during endoscopic retrograde cholangiopancreatography (ERCP). In all patients, clinical and laboratory response to treatment was assessed monthly. In patients with hepatic phase fascioliasis, radiological improvement was assessed at a 3-mo interval. All patients were followed until complete clinical and laboratory response or until 6 mo after treatment discontinuation.

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This prospective study was approved by Institutional Review Board, and all patients provided informed written consent for participation.

RESULTS

Fasciola hepatica infection was detected in 30 patients (24 females, mean age: 42.6 years, range: 19-79 years). In 22 (73%) patients, the diagnosis of fascioliasis was based on radiological findings on abdominal CT examination and positive IHA test (≥ 1/620). We did not perform ERCP in these patients because they did not have clinical or laboratory findings compatible with extrahepatic biliary obstruction; therefore, these patients were accepted as hepatic phase fascioliasis. In the remaining 8 (27%) patients, the diagnosis of fascioliasis was confirmed by extraction of live, mobile Fasciola hepatica from extrahepatic biliary ducts during ERCP; therefore, these patients were accepted as biliary phase fascioliasis. Microscopic examination of fecal specimens for Fasciola hepatica eggs revealed a positive result for only 2 (7%) of the 30 patients, one with biliary and one with hepatic phase.

Patients with hepatic phase fascioliasis

The mean antibody titer in the IHA was 1/2720 ± 1/549 (range: 1/640-1/5120) in the 22 patients with hepatic phase fascioliasis. Three patients were sisters and were admitted to the hospital on the same day. All patients were admitted at least five (mean: 7 ± 2) d before diagnosis. The mean duration of symptoms was 25 ± 36.6 (range: 3-144) wk. Abdominal pain was reported by all patients (100%), fever in 13 (59%), nausea in 3 (14%), chills in 4 (18%), weight loss in 4 (18%), pruritus and urticaria in 1 (5%), and recurrent oral aft and asthenia in 1 (5%) patient. On physical examination, there was mild right upper quadrant tenderness in 15 (68%) patients and hepatomegaly in 6 (27%) patients. Although 13 patients had a history of intermittent fever, only 3 (14%) patients had fever > 38 ℃ (2 of them were sisters), during clinical follow-up.

Table 1 shows the laboratory results for patients before treatment. Anemia was present in 6 (27%) patients, leukocytosis in 11 (50%), eosinophilia in 22 (100%) and elevations in the erythrocyte sedimentation rate (ESR) in 18 (82%) patients, alanine aminotransferase (ALT) in 6 (27%), aspartate aminotransferase (AST) in 2 (9%), alkaline phosphatase (ALP) in 13 (59%) , γ glutamyl transferase (GGT)

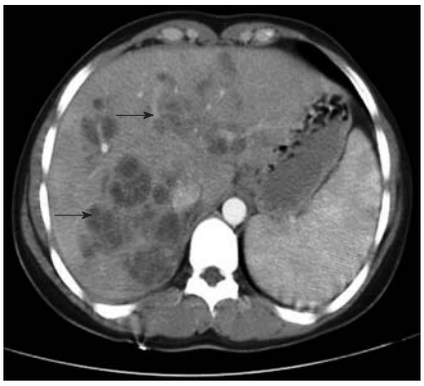
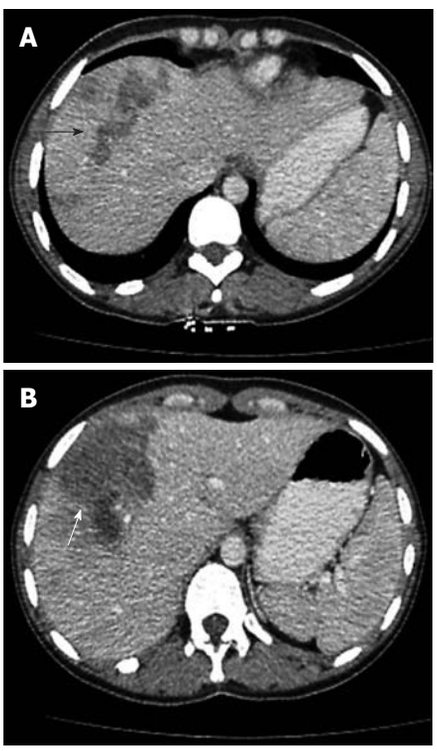


Figure 1 A 19-year-old female patient presented with right upper abdominal pain and fever lasting 3 wk. Abdominal computerized tomographic examination showed enlargement of the liver and extensive micro-abscesses (arrows).



Figures 2 A 30-year-old female patient presented with right upper abdominal pain lasting 8 wk. A: Tubular branching lesions in the right lobe (arrow); B: Abdominal computerized tomographic examination showed a sub-capsular low density area surrounded by a rim of parenchyma (arrow).

in 13 (59%), and total bilirubin in 1 (5%) patient. Platelet counts were normal in all patients.

On abdominal CT examination, the main abnormalities were multiple nodular lesions like micro-abscesses (Figure 1) in 21 (95%) patients, tubular branching lesions (Figure 2A) in 11 (50%), subcapsular low density areas surrounded by a rim of parenchyma (Figure 2B) in 7 (32%), solitary nodular lesions with hazy margins (Figure 3) in 5 (23%), lymph node enlargement in the portal area in 4 (18%), and localized perihepatic fluid accumulation in 2 (9%) patients.

After diagnosis of fascioliasis, triclabendazole was administered at a dose of 10-12 mg/kg for 1 d to all patients. We did not observe any side-effects related to triclabendazole administration. None of the patients with hepatic phase was administered antibiotics. Three months after treatment, we observed complete clinical and laboratory recovery in 18 (82%) patients and complete improvement on abdominal CT examination in 12 (55%) patients. Six months after treatment, there was complete clinical and laboratory recovery in all patients, but abdominal CT examination showed complete improvement in only 16 (73%) patients and residual hypo-dense lesions (Figure 4) in 6 (27%) patients.

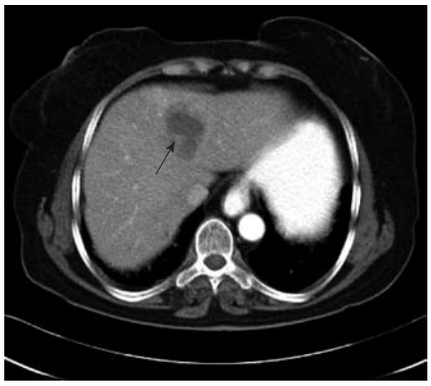


Figure 3 A 70-year-old female patient presented with right upper abdominal pain lasting 16 wk. Abdominal computerized tomographic examination showed low density masses with hazy margins located to medial segment of the left lobe (arrow).

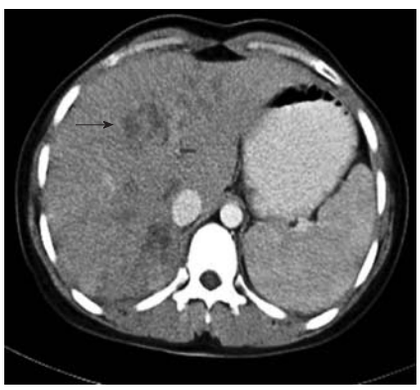


Figure 4 In the patient whose pre-treatment computerized tomographic image is shown in Figure 1, abdominal computerized tomographic examination showed residual lesions (arrow) and minimally enlarged spleen 6 mo after treatment with triclabendazole.

Patients with biliary phase fascioliasis

The mean duration of symptoms was 63.5 ± 80.6 (range: 1-208) wk in the 8 patients with biliary phase fascioliasis. One female patient underwent a cholecystectomy 6 mo ago after developing acute cholecystitis of unknown etiology. Abdominal pain was reported by all patients, fever in 2 (25%), nausea in 3 (38%), and weight loss in 1 (13%) patient. On physical examination, there was right upper quadrant tenderness in 5 (63%) and scleral icterus in 1 (13%) patient.

Table 1 Demographic features and laboratory results for patients with hepatic phase and biliary phase fascioliasis

Variables	Hepatic phase (mean ± SD)	Biliary phase (mean ± SD)
Age (yr)	40 (19-79)[1]	41 (29-49)[1]
Gender (M/F)	(5/17)	(1/7)
Hb (g/dL)	12.5 ± 1.4	12.7 ± 1.57
WBC (n/mm^3)	11862 ± 2829	8765 ± 1307
Eosinophil (% of total WBC count)	34.2 ± 16.2	14 ± 13.5
Plt (n/mm^3)	290 ± 62	273 ± 70
ESR (mm/h)	48 ± 26	17 ± 12
ALT (U/dL)	44 ± 49	220 ± 217
AST (U/dL)	26 ± 9	260 ± 357
ALP (U/dL)	157 ± 65	166 ± 85
GGT (U/dL)	64 ± 40	226 ± 128
Total bilirubin (U/dL)	0.57 ± 0.32	1.65 ± 2.02

[1]Age is represented using the median and range. Hb: Hemoglobin; WBC: White blood cell; Plt: Platelet; ESR: Erythrocyte sedimentation rate; ALT: Alanine aminotransferase (range, 10-40 U/L); AST: Aspartate aminotransferase (range, 10-35 U/L); GGT: γ glutamyl transferase (range, 9-64 U/L); ALP: Alkaline phosphatase (range, 40-150 U/L); Total bilirubin: range, 0.2-1.2 mg/dL; M: Male; F: Female.

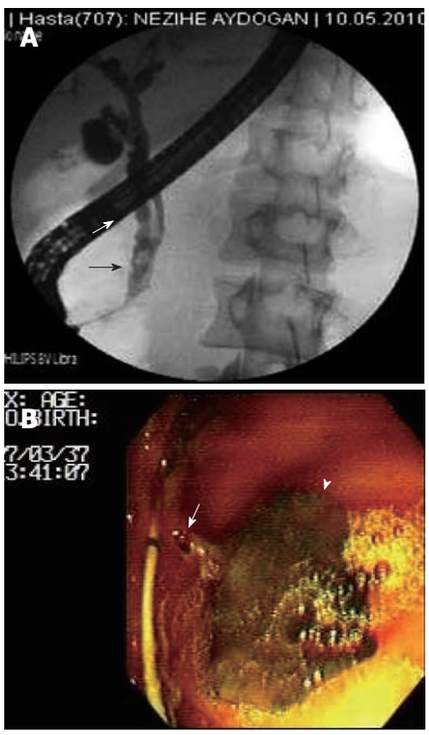


Figure 5 Live and mobil Fasciola hepatica removed from the choledochus by balloon catheter during endoscopic retrograde cholangiopancreatography. A: A 35-year-old female patient presented with acute pancreatitis associated with elevated liver enzymes. Endoscopic retrograde cholangiopancreatography image showed a radiolucent, roughly crescent-shaped shadow in the common bile duct (arrows); B: A 49-year-old female patient presented with acute cholangitis associated with eosinophilia. Arrow: Head of Fasciola hepatica; Arrowhead: Body of Fasciola hepatica.

The laboratory findings before treatment (Table 1) showed that mild anemia was present in 2 (25%) patients, eosinophilia in 4 (50%), and elevations in the ESR in 4 (50%), ALT in 7 (87%), AST in 6 (75%), ALP in 4 (50%), GGT in 7 (87%), and total bilirubin in 3 (38%) patients. Total white blood cell (WBC) and platelet counts were normal in all patients. One patient had a normal initial eosinophil count but elevated eosinophil count after the ERCP procedure (2.7% of total WBC before ERCP *vs* 23% after ERCP).

Abdominal CT examination showed no abnormalities in 6 (75%) and subcapsular low density areas surrounded by a rim of parenchyma in 2 (25%) patients. Although these 2 patients had clinical findings consistent with biliary phase fasciolisis, they had radiological findings consistent with hepatic phase fascioliasis. One of the patients was a 46-year-old woman who had cholangitis with a normal eosinophil count and live Fasciola hepatica extracted from the extrahepatic bile ducts. The other patient was a 29-year-old man who had acute pancreatitis, cholangitis, and eosinophilia (25% of total WBC); this patient also was one of the 3 patients who had biliary phase fascioliasis associated with acute pancreatitis.

Three (38%) patients (two females) with biliary phase fascioliasis also had acute pancreatitis. Two of these patients had elevated eosinophil counts, and all 3 patients had elevated liver enzymes and amylase (> 1275 U/dL). They also had acute edematous pancreatitis and were treated by extraction of live Fasciola hepatica by balloon during ERCP and conservative management.

ERCP was performed in all patients because there were clinical and laboratory findings of extrahepatic biliary obstruction. Before ERCP, we considered the diagnosis of fascioliasis in 4 (50%) patients with eosinophilia. Cholangiography showed slight extrahepatic and intrahepatic biliary dilatation in 1 (13%) patient with acute pancreatitis. In the remaining 7 (87%) patients, the intrahepatic and extrahepatic biliary systems were within normal diameter. ERCP demonstrated a radiolucent, roughly crescent-shaped shadow in the common bile duct in all patients (Figure 5A). After standard sphincterotomy, live Fasciola hepatica (3-5 Fasciola hepatica per patient) were removed using a balloon catheter from the extrahepatic bile ducts (Figure 5B).

We routinely administered 1 *g* ceftriaxone at least one hour before ERCP to prevent post-ERCP cholangitis. We did not administer antibiotics to any patient after ERCP. Triclabendazole was administered at a dose of 10-12 mg/kg for 1 d to all patients. Complete clinical and laboratory recovery was observed in all patients 3 mo after treatment. There was complete resolution on abdominal CT examination in the 2 patients who had initial lesions.

DISCUSSION

Fascioliasis is an emerging disease in humans. The epidemiological and transmission characteristics of fascioliasis suggest that the disease has a patchy distribution, with foci related to the local distribution of the intermediate snail host population in freshwater bodies as well as climatic conditions[2,3]. Epidemiological studies on the incidence of fascioliasis in our region have not been reported previously, but the results from this prospective single-center study suggest that Fasciola hepatica infection is not very rare in our region. Our hospital is a tertiary care cen-

ter in the southeast of Turkey that serves approximately 3 million people. All of the patients in this study resided in rural areas and had a history of consuming watercress grown in areas where sheep were raised. Twenty-four (80%) patients were female, and all of them were homeworking, suggesting that the females had more contact with watercress than the men.

Fascioliasis has a hepatic phase and a biliary phase, each displaying different clinical signs and symptoms. The acute stage of fascioliasis (hepatic phase) begins with the slow migration of Fasciola hepatica through the liver parenchyma; the mature flukes digest and consume hepatocytes, dig tunnels and caves, and reside in the liver for months[1,7,13]. The hepatic phase is characterized by fever with chills, upper abdominal pain, hepatomegaly, mild hepatitis, weight loss and prominent eosinophilia[6-8,14]. Reports suggest that the clinical presentation of hepatic phase fascioliasis is similar to that of liver abscesses of other etiology[1,8]. In our patients with hepatic phase fascioliasis, the common clinical signs and symptoms were right upper abdominal pain, intermittent fever, right upper quadrant tenderness and hepatomegaly. Based on our clinical experience with pyogenic liver abscesses[15], patients with Fasciola hepatica infection had a longer duration of symptoms (25 ± 36.6 wk *vs* 5.7 ± 1.6 wk), a healthier condition, and less upper abdominal tenderness than patients with pyogenic liver abscesses. Although 13 (59%) of 22 patients with hepatic phase fascioliasis had a history of intermittent fever, we recorded fever in only 3 patients. These findings suggest that fever in hepatic phase fascioliasis is not a prominent finding.

In the biliary phase of the disease, patients often present with biliary colic, epigastric pain, jaundice and abdominal tenderness due to the obstruction of the bile ducts by adult worms and the resultant inflammatory response. In this stage, the main laboratory findings are cholestasis including predominantly elevated serum ALP, GGT and total bilirubin[9-11]. Adult flukes in the extrahepatic bile ducts are visualized as a filling defect on cholangiogram[1,6,9,10]. Because of the chronic inflammation, the thickened walls of the extrahepatic ducts and gallbladder are visible on abdominal CT examination[1,5,6]. Although our patients with biliary phase fascioliasis had clinical signs and symptoms of biliary obstruction, we did not find the typical cholestatic biochemical abnormalities in these patients. We also found no specific abnormalities on abdominal CT examination in our patients with biliary phase except for slight dilatation of intrahepatic and extrahepatic bile ducts in 1 patient and subcapsular low density areas surrounded by a rim of parenchyma in 2 patients. The absence of bile duct wall thickness on CT examination and the absence of biochemical findings indicative of cholestasis in our patients may reflect intermittent rather than chronic biliary obstruction or our patients may have had early stage biliary duct involvement. The 2 patients with parenchymal lesions probably had overlapping hepatic and biliary phase fascioliasis. The most specific cholangiographic finding in our patients with biliary phase fascioliasis was a radiolucent, roughly crescent-shaped shadow in the extrahepatic bile ducts without dilatation.

Major causes of acute pancreatitis include alcohol ingestion and gallstones[16-18]. A small number of patients who have Fasciola hepatica infection complicated with acute pancreatitis have been reported. The pathogenesis of acute pancreatitis secondary to fascioliasis is unknown. Intermittent biliary obstruction and cholangitis caused by adult Fasciola hepatica may be the principle mechanism involved in the development of acute pancreatitis[19-21]. In our case series, 3 (38%) of 8 patients (2 with eosinophilia) with biliary phase fascioliasis also had acute edematous pancreatitis. One of the 3 patients, had both hepatic and biliary phase fascioliasis. Although our number of cases is small, we suggest that Fasciola hepatica infection should be considered during differential diagnosis in patients with acute pancreatitis associated with cholangitis and eosinophilia.

Diagnosis of fascioliasis may be delayed because of the wide spectrum of the differential diagnosis and the low incidence of Fasciola hepatica infection[3]. The abnormal laboratory and radiological findings in Fasciola hepatic infection may represent viral hepatitis, liver abscess, malignancy, cholecystitis, sclerosant cholangitis, AIDS-related cholangitis, ruptured hydatic cyst, and infection with parasites such as ascariasis and clonorchiasis[1,5,8]. The specificity of the indirect hemagglutination test (*IHA*) using purified adult Fasciola hepatica antigen F1 is 96.9% for serological diagnosis of Fasciola hepatica infection[12]. Diagnosis is confirmed only by demonstrating live parasites or eggs in the bile or feces[1,5,8]. The disease cannot be ruled out by a negative stool examination[3,5,8]. A high index of suspicion and specific radiological findings are very helpful in the diagnosis. We suspected of the possibility of fascioliasis in all patients with hepatic phase because of the presence of eosinophilia, characteristic abdominal CT findings, and typical clinical sign and symptoms. We found eggs in stool samples in 1 of 22 patients with hepatic phase fascioliasis and 1 of 8 patients with biliary phase fascioliasis. Diagnosis in patients with hepatic phase was confirmed by the clinical, laboratory and radiological responses to triclabendazole treatment and the high titer in the IHA. Diagnosis in patients with biliary phase fascioliasis was confirmed by extraction of live Fasciola hepatica form bile ducts. We suggest that stool examination for eggs is not a reliable method and that both serological testing and extraction of live parasites form bile ducts are very reliable methods for the diagnosis of fascioliasis.

Treatment of human fascioliasis has been difficult for a long time. Today, triclabendazole is the drug of choice for its effectiveness against both adult and immature worms[7,13,21]. Its anti-parasitic effect is derived from the inhibition by an active sulfoxide metabolite of the synthesis of the tegumental ultra-structure of Fasciola hepatica[22]. Triclabendazole at a dose of 10 mg/kg body weight (single or split postprandial dose) reportedly is effective in about 80%-90% of patients and is well tolerated. The most common drug-related side-effects are nausea, vomiting and abdominal pain[23]. All of our patients with hepatic phase were treated with triclabendazole alone, and those patients with biliary phase were treated with endoscopic

sphincterotomy, extraction of live parasite from bile ducts, and administration of triclabendazole. We observed that triclabendazole improved both clinical and laboratory findings in a few weeks; radiological improvement, however, required a longer period.

In conclusion, in addition to classically defined hepatic phase and biliary phase fascioliasis, some cases may have overlap of these two phases with or without acute pancreatitis. In cases of right abdominal pain, elevated eosinophil count, and multiple micro-abscesses and/or tunnel-like hypo-dense lesions on abdominal CT examination, hepatic phase fascioliasis should be considered, and a serological test for Fasciola hepatica should be used for diagnosis. In cases of biliary colic and/or acute pancreatitis associated with eosinophilia, we suggest that biliary phase fascioliasis should be considered, and ERCP should be used for both diagnosis and treatment.

COMMENTS

Background

In non-endemic areas, diagnosis of fascioliasis is difficult and usually is delayed because the disease is relatively rare and the symptoms may be confused with other hepatic or biliary disorders. Confirmation of the diagnosis is necessary and patients should be followed for response to treatment.

Research frontiers

Fascioliasis has a hepatic phase and a biliary phase, each displaying different clinical signs and symptoms. In addition to classically defined hepatic phase and biliary phase fascioliasis, some cases may have overlap of these two phases with or without acute pancreatitis.

Innovations and breakthroughs

Fascioliasis may have different clinical presentations. In cases of abdominal pain and elevated eosinophil count, fascioliasis should be considered in differential diagnosis. Serological tests and abdominal computerized tomographic (CT) examination are the methods of choice for diagnosis. But, fecal examination for Fasciola hepatica eggs is not a reliable diagnostic method. It may take a long time for complete clinical and radiological improvement after triclabendazole administration.

Applications

Fasciola hepatica infection should be considered in the differential diagnosis of patients with hepatic or biliary disease and/or acute pancreatitis associated with eosinophilia.

Terminology

Fascioliasis is an infection caused by a trematode of the liver. Fasciola hepatica, particularly affects sheep, goats and cattle. The flukes are leak-like, flat worms, measuring 2-4 cm. In humans, the infection begins with the ingestion of watercress or contaminated water containing encysted larva.

Peer review

The authors investigated the characteristic clinical, laboratory, and tomographic findings and response to treatment during follow-up in patients with fascioliasis. They revealed that fascioliasis has different clinical presentations and in cases of right abdominal pain, elevated eosinophil count, and multiple micro-abscesses and/or tunnel-like hypo-dense lesions on abdominal CT examination, the diagnosis of hepatic phase fascioliasis should be considered, and a serological test for Fasciola hepatica should be used for diagnosis.

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S- Editor Wu X **L- Editor** Ma JY **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4905

World J Gastroenterol 2011 November 28; 17(44): 4905-4910
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Prognostic role of sensitive-to-apoptosis gene expression in rectal cancer

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Supported by Marmara University Research Fund, No. SAG-DKR-140305-0089
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Received: April 21, 2011 Revised: June 21, 2011
Accepted: June 28, 2011
Published online: November 28, 2011

Abstract

AIM: To investigate the association between prognosis of rectal cancer treated with chemoradiotherapy (CRT) and expression of sensitive-to-apoptosis (SAG), B-cell lymphoma-extra large (Bcl-X_L) and Bcl-2 homologous antagonist/killer (Bak).

METHODS: Real-time quantitative polymerase chain reaction was used to determine the expression of proteins of interest, namely SAG, Bcl-X_L, Bak and β-actin, in rectal carcinoma patients who had a follow-up period of 3 years after CRT. Biopsy specimens were excised from the rectal tumor preceding CRT.

RESULTS: SAG, Bcl-X_L and Bak proteins showed significant correlations with each other. In multivariate analysis, patients with high *vs* low SAG expression showed a statistically significant difference in 2-year survival rates: 56% *vs* 73%, respectively (P = 0.056). On the other hand, there were no significant correlations between the expression levels of all three genes and metastatic rates or tumor responses to CRT. Mean overall survival in the patients with elevated SAG expression was 27.1 mo ± 3.9 mo [95% confidence interval (CI): 19.3-34.9], and in patients with reduced expression, it was 32.1 mo ± 2.5 mo (95% CI: 27.3-36.9). The corresponding values for Bcl-X_L were 28.0 mo ± 4.1 mo (95% CI: 19.9-36.1) and 31.7 mo ± 2.9 mo (95% CI: 26.0-37.5), and those for Bak were 29.8 mo ± 3.7 mo (95% CI: 22.5-37.2) and 30.6 mo ± 2.4 mo (95% CI: 25.5-35.0), respectively.

CONCLUSION: Two-year survival rates significantly correlated with low SAG expression, and SAG may be a candidate gene for good prognosis, independent of therapeutic response of different individuals.

Key words: Sensitive-to-apoptosis gene; Sensitive-to-apoptosis; Rectal cancer; B-cell lymphoma-extra large; Bcl-2 homologous antagonist/killer; Apoptosis

Peer reviewer: Francis Seow-Choen, MBBS, FRCSEd, FAMS, Professor, Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, Singapore 238859, Singapore

Ozden SA, Ozyurt H, Ozgen Z, Kilinc O, Oncel M, Gul AE, Karadayi N, Serakinci N, Kan B, Orun O. Prognostic role of sensitive-to-apoptosis gene expression in rectal cancer. *World J Gastroenterol* 2011; 17(44): 4905-4910 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4905.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4905

INTRODUCTION

Colorectal cancer is one of the most common cancers worldwide, and 30% of patients experience local recurrence, thus posing a serious problem in treatment[1,2]. Currently, preoperative radiotherapy alone or in combination with chemotherapy is widely accepted to improve local control and overall survival.

The most frequently chosen treatment methods are either short-course irradiation (25 Gy in five fractions) followed by surgery after 1 wk, or conventional fractionated chemoradiotherapy (CRT) with delayed surgery[3]. Multidrug- and/or radiation resistance is one of the main causes of treatment failure in rectal carcinoma, as in other types of cancer. Predictors of treatment response are highly valuable, and they help to reduce the occurrence of undesirable treatment side effects, improve efficacy, and reduce costs, especially in long treatment schedules. Therefore, it is very important to identify those patients who will not show a good response to CRT. The success of CRT is strongly dependent on the molecular and cellular characteristics of the cells, therefore, identification of candidate molecular markers with significant prognostic power to estimate the response to CRT is crucial. Despite accumulation of data on prognostic markers, reliable markers for satisfactory clinical outcomes remain limited in number.

Sensitive-to-apoptosis gene (SAG)/regulator of cullins (ROC) 2/RING box protein (Rbx) 2/Hrt2 is a recently identified component of Skp/Cullin/F-box containing complex (SCF) E3 ubiquitin ligase, which controls cell-cycle progression by promoting ubiquitination and degradation of cell-cycle inhibitors. It has also been reported that SAG protects cells from apoptosis induced by redox agents such as hydroxyl radicals and radiation. The prognostic value of apoptotic activity in different cancer types has been previously described[4-7]. SAG overexpression has been shown in 60% of primary colon carcinomas; furthermore, significant correlation between SAG overexpression and poor survival has been demonstrated in non-small cell lung carcinoma[8,9]. Thus, it is proposed that SAG may regulate carcinogenesis *via* modulating both cell proliferation and apoptosis.

Ionizing radiation causes DNA damage that can lead cell to apoptosis and thus eradication of cancer cells. Antiapoptotic proteins such as SAG may have a major impact on the progress of cancer cell formation and proliferation. The present study investigated SAG expression as a potential molecular marker of ionizing radiation effect in rectal cancer. Two members of the Bcl-2 family, B-cell lymphoma-extra large (Bcl-Xl) and Bcl-2 homologous antagonist/killer (Bak), which are suggested as the most probable candidate biomarkers, were also analyzed. Our results indicate that SAG expression is a useful marker for early prognosis, regardless of local response to CRT, and that targeting SAG may have a potential role in the treatment of rectal cancer.

MATERIALS AND METHODS

Patients and tissue collection

This prospective study included 31 patients referred to the Kartal Education and Research Hospital with a diagnosis of stage Ⅱ and Ⅲ rectal cancer, according to the conventional tumor, node and metastases (TNM) classification[10]. The appropriate ethics committees related to the institution approved the study and all patients provided written informed consent before undergoing diagnostic colon biopsy. Prior to the start of treatment, all patients underwent examinations, including complete blood counts, liver and renal function tests, and tumor markers. Additionally, lung X-rays were evaluated before the start of treatment. Abdominal-pelvic magnetic resonance imaging or computed tomography was used for clinical staging and supplemented with transrectal ultrasound when needed. Biopsy specimens from the tumor and adjacent normal rectal tissues were obtained during colonoscopy. Freshly removed specimens were immediately immersed in RNAlater solution (Qiagen, Germany) and stored at -20 ℃ for RNA extraction.

Therapy

All patients received preoperative CRT. Patients were irradiated using the four-box-field technique and high-energy photon radiotherapy beams (15 mV), with a daily exposure of 1.8-2 Gy for five consecutive days. A cumulative dose of 45-50 Gy was administered during 5 wk and an additional 5.4-Gy boost was administered in three fractions. A short infusion of fluorouracil (320-400 mg/m^2) and of calcium folinate (20 mg/m^2) was administered on the first and last weeks, concomitantly[11,12].

Patients who were histopathologically diagnosed with rectal cancer underwent CRT prior to surgery. After a 4-6-wk interval, the patients underwent surgery. Patients were staged according to the TNM classification system[10], based on routine histopathological reports following surgery.

Real-time polymerase chain reaction

SAG, Bcl-Xl and Bak mRNA expression levels in tumor tissues and adjacent normal tissues were quantified by real-time polymerase chain reaction (PCR) analysis. Total RNA was extracted from RNAlater-conserved tissues using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's guidelines. RNA samples were treated with DNase I (MBI Fermentas, Burlington, Canada) to

Table 1 Clinicopathological factors and gene expression

		Survival (%)			Multivariate significance
	n	12 mo	24 mo	36 mo	*P* value
Age (yr)					
< 50	10	80	80	66	0.135
> 50	21	76	57	47	
Sex					
Male	17	64	52	45	0.073
Female	14	92	78	62	
preT					
T2	1	100	100	100	0.052
T3	24	79	70	59	
T4	6	66	33	16	
preN					
N0	18	77	66	66	0.036
N+	13	76	61	0	
Grade					
Low	4	100	75	75	0.0337
Moderate	22	77	68	58	
High	4	50	25	0	
pN					
N0	17	70	64	58	0.044
N1	6	83	83	56	
N2	3	66	83	67	
pT					
T0	6	83	66.7	66.7	0.067
T1	2	100	100	100	
T2	4	75	75	75	
T3	19	73	63	57	
Vascular invasion					
Negative	7	71	57	57	0.019
Positive	24	79	66.7	52.7	
Perineural invasion					
Negative	17	76.5	64.7	64.7	0.068
Positive	13	76.9	61.5	35.9	
Metastasis					
Negative	23	78	73	63	0.009
Positive	8	75	50	25	
SAG expression					
Increase	16	62	56	56	0.056
Decrease	15	93	73	49	
Bak expression					
Increase	14	73	62	31	0.731
Decrease	17	68	56	56	
Bcl-XL expression					
Increase	12	66	58	43	0.336
Decrease	19	84	68	61	

Clinical features of the 31 patients who received preoperative chemoradiotherapy and a summary of the relationship between protein expression level and patient survival after 1, 2 and 3 years of follow-up. SAG: Sensitive-to-apoptosis; CRT: Chemoradiotherapy.

remove possible genomic DNA contamination. RNA was quantified by measuring A_{260} using a conventional spectrophotometer. cDNA was synthesized from RNA using the 1st Strand cDNA Synthesis Kit for RT-PCR according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Following synthesis, 5 μg of cDNA was amplified using the appropriate primers. SAG expression was analyzed using following primers: forward (5'-CGGGATCCATGGCCGACGTGGAAG-3') and reverse (5'-CGAAGCTTTCATTTGCCGATTCTTTG-GAC-3'). Expression of two other apoptotic pathway genes (Bcl-X_L and Bak) was analyzed using the following primers: Bcl-X_L forward (5'-CCAGAAGGGACT-GAATCG-3') and Bcl-X_L reverse (5'-CCTTGTCTAC-GCTTTCCAC-3'); Bak forward (5'-GACCCAGAGA-TGGTCACCTT-3') and Bak reverse (5'-TCATAGC-GTCGGTTGATGT-3'). β-actin gene expression was used in parallel reactions as an internal PCR control. The β-actin primers were as follows: β-actin forward (5'-CT-GTGCTGTCCCTGTATGCC-3') and β-actin reverse (5' -GTGGTGGTGAAGCTGTAGCC-3'). Amplification products were 341, 361, 103 and 203 bp, respectively. Real-time PCR was performed using a Light-Cycler 480 system (Roche) and amplification conditions were set according to the instructions supplied with the Light Cycler FastStart DNA Master SYBR Green kit (Roche). The annealing temperature for amplifications was 56 ℃ for β-actin, 50 ℃ for Bcl-X_L and 60 ℃ for SAG and Bak proteins.

Statistical analysis

Multivariate analysis was performed using the logistic regression test. $P < 0.05$ was considered statistically significant. The relationship between protein regulations and disease-free survival up to 3 years was assessed by a log-rank comparison of Kaplan-Meier survival curves. All statistical analyses were conducted using SPSS 13 statistical software (SPSS, Chicago, IL, United States).

RESULTS

The study included 31 patients (17 males and 14 females) diagnosed with locally advanced rectal cancer. RNA was extracted from both normal and tumor tissues. Mean age of the patients was 59.9 years (range: 35-85 years). Survival, according to patient sex and age, was not significantly different (Table 1). Median follow-up was 3 years. In all, 8 of the 31 patients (34.8%) developed distant metastases. Table 1 summarizes the patients' 1-, 2- and 3-year survival rates, the corresponding data for some known prognostic factors, and the levels of SAG, Bcl-X_L and Bak protein expression.

There was an association between protein expression and survival following CRT. The 1- and 2-year survival rates were 93% and 73%, respectively, in patients with low SAG expression (fold change > 0.99), *vs* 62% and 56%, respectively, in patients with high SAG expression (fold change < 0.99); the correlation between SAG expression and survival was moderate ($P = 0.056$). A similar trend was observed in the expression of antiapoptotic protein Bcl-X_L; the corresponding survival values were 84% and 68%, respectively, in patients with low Bcl-X_L expression, *vs* 66% and 58%, respectively in patients with high Bcl-$X_L$expression. The levels of Bak were also in agreement with its apoptotic function. In accordance with our expectations, patients with high Bak expression had a higher 2-year survival rate (62%) than those with low Bak expression (56%). The expression patterns of SAG, Bcl-X_L and Bak proteins exhibited good correlations with the 1- and 2-year survival rates, in accordance with their anti- and pro-apoptotic roles, even though statistical

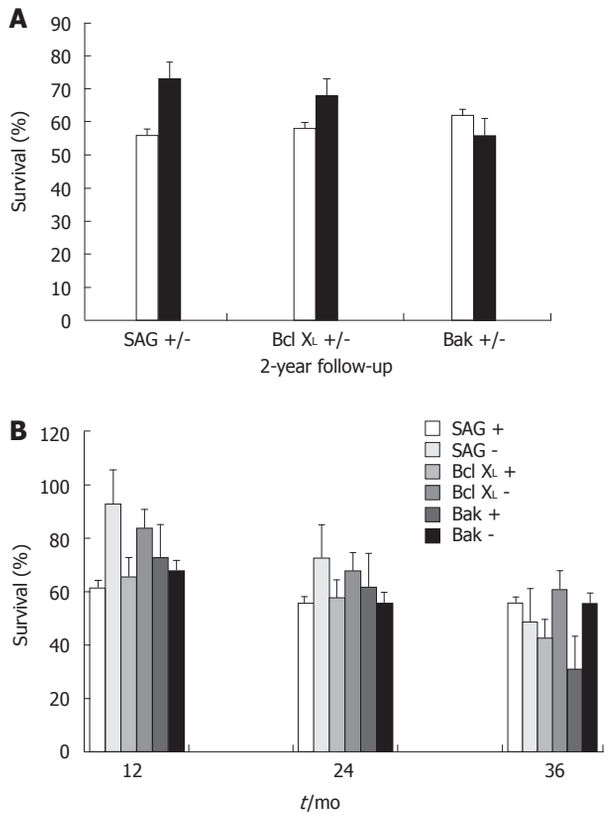

Figure 1 Dependence between gene expression and outcome of patients. A: Comparison for 2-year survival; B: Comparison after 1, 2 and 3 years of follow-up. All values were normalized with respect to β-actin expression. Calculations were based on the Pfaff method. +/- signs correspond to the subjects with increased or decreased expression with respect to their non-tumor tissues, respectively. SAG: Sensitive-to-apoptosis; Bcl-XL: B-cell lymphoma-extra large; Bak: Bcl-2 homologous antagonist/killer.

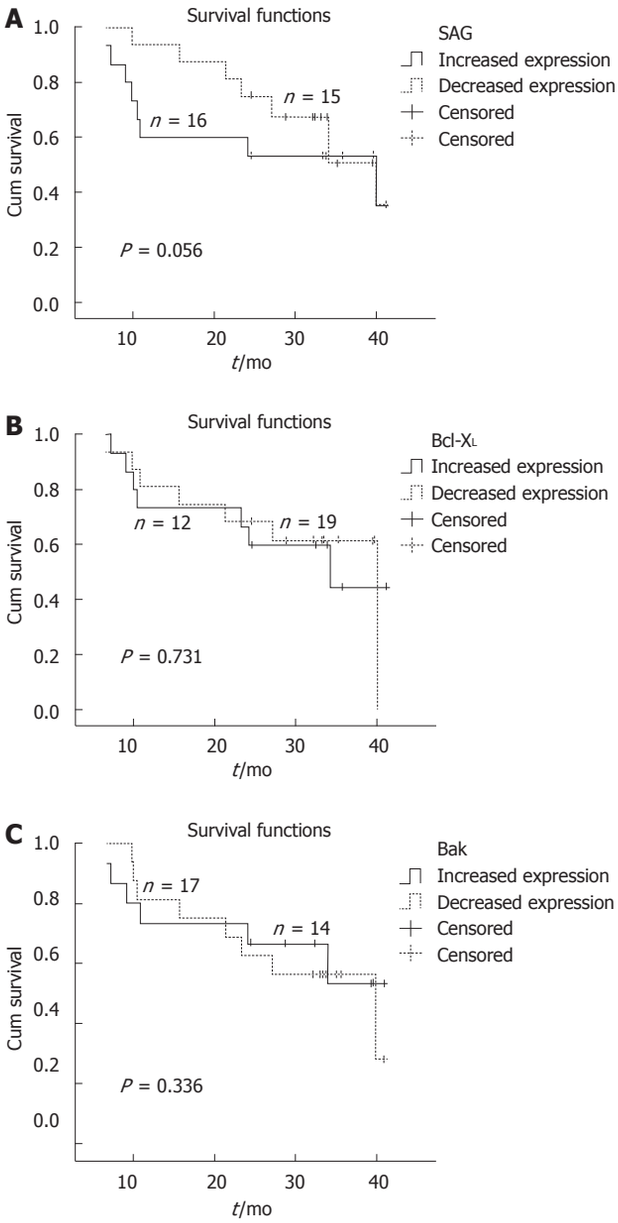

Figure 2 Kaplan-Meier survival curves with univariate log-rank comparisons at the end of 3 years for the three genes of interest. A: Sensitive-to-apoptosis; B: B-cell lymphoma-extra large; C: Bcl-2 homologous antagonist/killer. Metastasis was the major cause of death (seven cases). Information was not available for four of the patients. Other reasons of death were variable, but included advanced age, Alzheimer's disease and stroke. The median values for high- and low-level SAG expression in the study population were 24.5 and 30.4 mo, respectively. SAG: Sensitive-to-apoptosis gene, Bcl-XL: B-cell lymphoma-extra large, Bak: Bcl-2 homologous antagonist/killer.

significance was low, possibly due to the small number of patients included in the study (Figure 1A). There was no significant association in the expression of any gene with better survival at the end of the 3-year follow-up (Figure 1B).

Kaplan-Meier survival curves for SAG, Bcl-XL and Bak proteins are shown in Figure 2A-C, respectively. Mean overall survival in the patients with elevated SAG expression was 27.1 mo ± 3.9 mo [95% confidence interval (CI): 19.3-34.9]; and in patients with reduced expression, it was 32.1 mo ± 2.5 mo (95% CI: 27.3-36.9). The corresponding values for Bcl-XL were 28.0 mo ± 4.1 mo (95% CI: 19.9-36.1) and 31.7 mo ± 2.9 mo (95% CI: 26.0-37.5), and those for Bak were 29.8 mo ± 3.7 mo (95% CI: 22.5-37.2) and 30.6 mo ± 2.4 mo (95% CI: 25.5-35.0), respectively.

Tumor progression caused by distant metastases occurred only in eight patients (25.8%), of whom, seven had liver metastasis and one, who was alive at the time of evaluation, had bone metastasis. Mean overall survival was 41 mo. When the levels of SAG in tumor tissue were compared to the corresponding normal tissue, half of the patients expressed higher levels of SAG, whereas the other half had lower expression. Interestingly, patients with high SAG expression had a higher mean survival (28 mo) than did patients with low expression (18 mo). These results contradict those obtained in the non-metastatic patients. Among the non-metastatic patients, those with low SAG expression showed a higher survival (36 mo) and those with high SAG expression had lower survival (26 mo) (Table 1).

Pathological complete response was observed in five patients (5/31); three of whom (60%) had lower SAG expression and two (40%) had higher SAG expression.

DISCUSSION

Almost 50% of patients with rectal carcinoma who undergo potentially curative resection die from the disease, due to high recurrence rates. There is mounting evidence indicating that local control of disease and survival rates can be significantly improved with neoadjuvant CRT; however, responses to therapy vary widely among individuals and, as such, the ability to predict the response to CRT in patients with rectal cancer significantly improves therapeutic efficacy.

One of the immediate damaging effects of ionizing irradiation is the induction of cell-cycle arrest to provide time for DNA repair or apoptosis. Spontaneous apoptosis has been reported to be an important predictor of tumor regression in rectal cancer[13]. Reactive oxygen species, such as hydroxyl radical radicals, produced by ionizing radiation are highly reactive and easily trigger apoptosis, possibly by an indirect action on redox-sensitive molecules such as *p53* and *p27*[14,15]. Recently, SAG was identified as a redox-sensitive protein that protects cells from apoptosis, either by scavenging oxygen radicals or by acting on apoptosis-related proteins as part of the ubiquitin ligase complex SCF, thereby affecting the apoptotic sensitivity of cells. Moreover, SAG was identified as a potential regulator of *p27*, through inhibition of *p27* accumulation[16]. SAG overexpression has also been detected in a subset of human colon cancers and non-small lung carcinomas and has been shown to be associated with poor prognosis[8,9,17].

The present results, which are in agreement with above-mentioned studies, showed that low SAG expression in tumor tissue, as compared to normal tissue, had a positive effect on survival. The patients with low SAG expression had a median disease-free survival of 31.7 mo, as compared to 28 mo in those with high SAG expression. The clinical outcomes of the 31 patients revealed a strong discrepancy at the 2-year follow-up period between the groups with high *vs* low SAG expression, but this profile seemed to vanish at 3 years of observation.

Previously, Bak and Bcl-Xl, which are members of the Bcl-2 family, were also identified as candidate proteins for regulating chemotherapy-induced apoptosis[18]. Bcl-2 was shown to block γ-radiation-induced cell death[19,20]. Bak can form heterogenous dimers with Bcl-2 or Bcl-Xl to inhibit their antiapoptotic functions. Strong interaction between endogenous Bak and Bcl-Xl has been reported in hepatocytes as well as in other cells[21]. Furthermore, quantitative expression levels of Bcl-Xl and Bak have also been determined to correlate with apoptotic sensitivity of tissues in different carcinomas. Both genes have previously been shown to play a major role in colorectal carcinogenesis and tumor progression. Bcl-Xl, on the other hand, is suggested to be more crucial than Bcl-2 for regulation of apoptotic cell death in colon cancer[22]. Significant overexpression of Bcl-Xl mRNA has been observed in the majority of colorectal carcinomas as compared to the corresponding normal tissues. Krajewska *et al*[23] have also reported elevated Bcl-Xl and reduced Bak expression in colorectal adenocarcinoma. These studies reinforce the major roles of Bcl-Xl and Bak in colorectal carcinogenesis and tumor progression; furthermore, they also imply that expression levels of Bak and Bcl-Xl can be used as prognostic factors in rectal carcinoma, as pointed out by some other studies[24,25].

Thus, in addition to SAG, we also evaluated the expression of two other Bcl-2 family proteins, Bcl-Xl and Bak. The expression levels of antiapoptotic Bcl-Xl and proapoptotic Bak were well correlated with those of SAG, which lends further support to the role of apoptotic factors in the malignant potential of tumors. Higher survival rates for 2 years were recorded for increased expression of proapoptotic Bak, as opposed to SAG and Bcl-Xl levels.

We did not observe any correlation between development of metastases and expression of apoptotic factors; however, in patients who did not develop metastases, gene regulation, especially that of SAG, was closely associated with survival. In non-metastatic tumors, tissues with low-level SAG expression (n = 12) were associated with longer mean survival (36 mo), as opposed to those with high expression levels (27.9 mo, n = 11).

In conclusion, the present study shows that the level of expression of apoptosis-related genes may be associated with the degree of resistance to radiation exposure and may significantly affect therapeutic outcome. We observed an inverse correlation between SAG expression and survival. Furthermore, the data obtained in this study imply that SAG and Bak expression may serve as predictive parameters of disease progression. On the other hand, SAG and Bak expression levels did not significantly affect overall survival. This pilot study included a rather small number of patients, therefore, prospective studies with larger cohorts that include other proteins in the molecular apoptotic pathway are required to assess the roles of individual regulators in anticancer therapy.

ACKNOWLEDGMENTS

We thank Dr. Flemming Brand Sørensen for critical reading of the manuscript.

COMMENTS

Background

Apoptotic proteins have been reported to be important prognostic factors in various cancers. Sensitive-to-apoptosis gene (SAG) is a recently identified apoptotic protein, which may be a new candidate to estimate the outcome of treatment in rectal cancers.

Research frontiers

SAG was identified as a redox-sensitive protein that protects cells from apoptosis, either by scavenging oxygen radicals or by acting on apoptosis-related proteins as part of the ubiquitin ligase Skp/Cullin/F-box containing complex (SCF), thereby affecting the radiation sensitivity of cells. This study investigated the correlation between expression levels of SAG and survival rates of patients, who have advanced rectal carcinoma. In addition to SAG, this study also examined two other proteins, B-cell lymphoma-extra large (Bcl-XL) and Bcl-2 homologous antagonist/killer (Bak) proteins, which are important members of the mitochondrial apoptotic pathway.

Innovations and breakthroughs

The present study showed that the level of expression of some apoptosis-related genes may be associated with the degree of resistance to radiation ex-

posure and may significantly affect therapeutic outcome. The present research observed an inverse correlation between SAG expression and 2-year survival, although the overall survival rate was not affected significantly. There were no significant correlations between the expression levels of all three genes and metastatic rates or tumor responses to chemoradiotherapy (CRT).

Applications

The data obtained in this study imply that SAG and Bak expression may serve as predictive parameters of disease progression. This pilot study included a rather small number of patients, therefore, prospective studies with larger cohorts that include other proteins in the molecular apoptotic pathway are required to assess the roles of individual regulators in anticancer therapy.

Terminology

SAG/regulator of cullins 2/Rbx/Hrt2 is a recently identified component of SCF E3 ubiquitin ligase, which controls cell-cycle progression by promoting ubiquitination and degradation of cell-cycle inhibitors. It has also been reported that SAG protects cells from apoptosis induced by redox agents such as hydroxyl radicals and radiation. The Bcl-2 family proteins are members of the intrinsic apoptotic pathway. Bak is a proapoptotic member, while Bcl-X_L blocks apoptosis in many systems.

Peer review

This was an interesting and well conducted study which should be published because it confirms the usefulness of SAG in the prognostication of rectal cancer patients.

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S- Editor Wu X **L- Editor** Kerr C **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4911

World J Gastroenterol 2011 November 28; 17(44): 4911-4916
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Continuous regional arterial infusion and laparotomic decompression for severe acute pancreatitis with abdominal compartment syndrome

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Received: May 4, 2011 Revised: June 11, 2011
Accepted: July 11, 2011
Published online: November 28, 2011

Abstract

AIM: To evaluate the therapeutic effects of abdominal decompression plus continuous regional arterial infusion (CRAI) *via* a drug delivery system (DDS) in severe acute pancreatitis (SAP) patients with abdominal compartment syndrome (ACS).

METHODS: We presented our recent experience in 8 patients with SAP. The patients developed clinical ACS, which required abdominal decompression. During the operation, a DDS was inserted into the peripancreatic artery (the catheter was inserted from the right gastroepiploic artery until it reached the junction between the pancreaticoduodenal and gastroduodenal artery). Through this DDS, a protease inhibitor, antibiotics and octreotide were infused continuously. The duration of the regional artery infusion ranged from 8 to 41 d. The outcomes and the changes in the APACHE Ⅱ score, computed tomography (CT) severity index and intra-abdominal pressure (IAP) of the patients were retrospectively evaluated.

RESULTS: Eight patients with an initial APACHE Ⅱ score of 18.9 (range, 13-27) and a Balthazar CT severity index of 9.1 (range, 7-10) developed severe local and systemic complications. These patients underwent subsequent surgical decompression and CRAI therapy because of intra-abdominal hypertension (IAH). After a mean interval of 131.9 ± 72.3 d hospitalization, 7 patients recovered with decreased APACHE Ⅱ scores, CT severity indexes and IAP. The mean APACHE Ⅱ score was 5.4 (range, 4-8), the CT severity index was 2.3 (range, 1-3), and IAP decreased to 7.7 mmHg (range, 6-11 mmHg) 60 d after operation. One patient died of multiple organ failure 1 wk after surgery.

CONCLUSION: CRAI and laparotomic decompression might be a therapeutic option for SAP patients with ACS.

Key words: Severe acute pancreatitis; Arterial infusion; Laparotomy; Abdominal compartment syndrome

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Deng ZG, Zhou JY, Yin ZY, Peng YY, Wang FQ, Wang XM. Continuous regional arterial infusion and laparotomic decompression for severe acute pancreatitis with abdominal compartment syndrome. *World J Gastroenterol* 2011; 17(44): 4911-4916 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4911.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4911

INTRODUCTION

Acute pancreatitis is an inflammatory disease that is self-

limited in the majority of patients and resolves within 48-72 h. However, approximately 20% of the patients develop a more severe form of the disease with evidence of organ dysfunction and complications such as pancreatic necrosis, abscess or pseudocyst. This acute form is classified as severe acute pancreatitis (SAP) according to the Atlanta classification and has a mortality approaching 30%[1]. Previous studies have shown that 60% of the patients developed organ failure on admission, and those with persistent organ failure had the worst outcomes[2].

The current treatment paradigm calls for non-operative management of SAP as long as there is no evidence of infection. However, there is a subset of patients with acute pancreatitis who may need an urgent laparotomy in the absence of an infection to decompress the clinically significant abdominal compartment syndrome (ACS)[3].

SAP with ACS, which is defined as a sustained intra-abdominal pressure (IAP) greater than 20 mmHg that is associated with the development of organ dysfunction or failure[4], is the most severe form of acute pancreatitis and has a high morbidity and mortality. SAP with ACS injures not only the pancreas itself but also the surrounding organs. Despite various treatment protocols, including intensive care therapy and blood filtration, the mortality rate of SAP with ACS is still reported to be 30%-60%[2].

In a number of recent studies, continuous regional arterial infusion therapy (CRAI) for SAP using protease inhibitors and antibiotics has been shown to control the inflammation of the pancreas and to prevent the extension of the inflammatory process, which would decrease the rate of infection and mortality[5-11].

Accordingly, in this study, we used a new system during our decompressing surgical procedure in 8 patients suffering from SAP with ACS, and the therapeutic effects of continuous regional intra-arterial infusion were retrospectively evaluated.

MATERIALS AND METHODS

Eight patients, 6 men and 2 women, with a mean age of 51.5 year (35-66 year) were diagnosed with SAP at Xiamen University Zhongshan Hospital from April 2009 to July 2010. SAP was diagnosed based on clinical manifestation, biological findings and contrast-enhanced abdominal computed tomography (CT) within 3 d after admission. These patients presented with multiple organ dysfunction (MOD) or multiple organ failure (MOF) within 3 d of admission, and they continued to deteriorate under intensive medical support (Table 1).

Laparotomy was performed 3-9 d after the onset of SAP in these patients because of clinical deterioration despite intensive medical care and persistent abdominal hypertension. The abdominal pressure was measured by the bladder technique[4]. In contrast to the traditional surgical procedure for SAP, necronectomy (debridement) was not performed. For abdominal decompression and placement of wide-bore drains for continuous postoperative irrigation, the catheter of a drug delivery system (DDS) was inserted into the peripancreatic artery. Because the pancreas has a multiple-sourced blood supply, an appropriate drug distribution in the pancreas can not be achieved unless the catheter is properly placed. The proper position of the catheter tip was decided based on the methylthionine chloride injection. By injecting methylthionine chloride from the head of DDS, we could find the dyeing area, and adjust the position of catheter tip. In cases which it was difficult to find the right gastroepiploic artery, an intraoperative ultrasound was used for guiding. The injection head of the DDS was subcutaneously placed for postoperative drug delivery (Figure 1), and the patients subsequently received an arterial infusion with protease inhibitor, antibiotics and octreotide. In addition, patients with biliary stones had emergency surgery such as endoscopic sphincterotomy, endoscopic nasobiliary drainage, cholecystectomy or common bile duct lithotomy. If we had difficulties with the closure after laparotomy, we left the abdomen open and applied a temporary closure device until suturing again.

Table 1 Multiple organ dysfunction or multiple organ failure within 3 d after admission

Dysfunction (failure)	*n* (%)
Pulmonary insufficiency	8 (100.0)
Requiring mechanical ventilation	8 (100.0)
Renal insufficiency	7 (87.5)
Requiring dialysis	6 (75.0)
Shock	7 (87.5)
Requiring catecholamines	8 (100.0)
Sepsis (or SIRS)	8 (100.0)
Coagulopathy	7 (87.5)
Hepatic dysfunction (failure)	7 (87.5)
Cardiovascular disable	7 (87.5)
Gastrointestinal bleeding	7 (87.5)
Center nerve system problem	5 (62.5)

Ulinastatin (100 000 U), antibiotics (imipenem/cilastatin 0.5 g) and octreotide (0.3 mg) were dissolved into the saline (48 mL) and continually infused through the DDS twice a day. To ensure the therapeutic effects, the infusion was continued even after the patients' conditions had improved and until the Balthazar CT severity index had decreased to 3 or less.

All patients were treated in the intensive care unit (ICU). Fluid, electrolytes, albumin, and insulin were replaced dependent on central venous pressure (6-10 mm H_2O), hematocrit (30%-35%), urinary excretion, and blood glucose measurement. Assisted ventilation was begun if the partial pressure of oxygen could not be maintained at a level of > 60 mmHg with an oxygen mask. In patients with progressive renal failure (serum creatinine > 3.0 mg/dL), hemodialysis or continuous venovenous hemodialysis (CVVHD) was performed.

The clinical data of patients, transvesical measurement of IAP, CT severity index[12], APACHE Ⅱ score[13], presence of MOD, local-regional complications and outcome were examined and reported.

RESULTS

The severity of the pancreatitis was judged according to

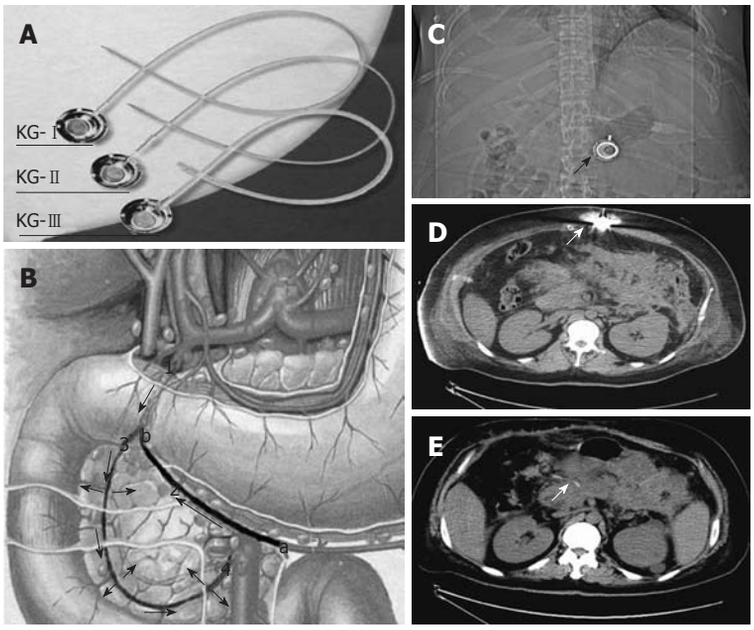

Figure 1 Procedure of placing drug delivery system and imaging after drug delivery system was inserted. A: Drug delivery system; B: Anatomy and how it is inserted; 1: Gastroduodenal artery; 2: Right gastroepiploic artery; 3: Superior pancreatico-duodenal artery; and 4: Inferior pancreatico-duodenal artery (arrows indicate the direction of drug delivery and blood flow); a: The inserting point; and b: The end in which the catheter should be inserted; C: Abdominal X-ray film showed the subcutaneous drug delivery system (DDS) head (arrow); D: Computed tomography (CT) scan showed the subcutaneous DDS and the drug delivery needle inside the DDS (arrow); E: DDS catheter around the pancreas (arrow) in the CT scan.

Table 2 Clinical data of 8 patients with severe acute pancreatitis treated by postoperative peripancreatic artery infusion

Patients	Sex/age (yr)	Area of pancreatitis	Operation	AI (d)	IAP		Apache Ⅱ		CT-SI (d)		ICU (d)	Hosp. (d)	Outcome
					Pre	Post	Pre	Post	Pre	Post			
1	M/35	Entire	3	31	35	11	27	6	10	3	45	259	Recovered
2	F/42	Entire	4	8	38	18	24	18	10	8	11	11	Dead
3	M/64	Entire	5	25	26	8	13	4	7	1	34	68	Recovered
4	M/53	Entire	8	18	30	7	16	5	9	3	64	101	Recovered
5	M/49	Entire	6	41	31	9	21	8	10	3	58	159	Recovered
6	F/66	Entire	3	35	24	6	19	4	10	3	44	186	Recovered
7	M/57	Entire	4	19	26	6	16	5	9	1	33	74	Recovered
8	M/46	Entire	5	33	23	7	15	6	8	2	40	76	Recovered

AI: Artery infusion; AP: Abdominal pressure (mmHg); CT-SI: CT severity index by Balthazar *et al*; ICU: Intensive care unit; Pre: Preoperation; Post: 60 d after operation or before the patient died (No. 2 patient).

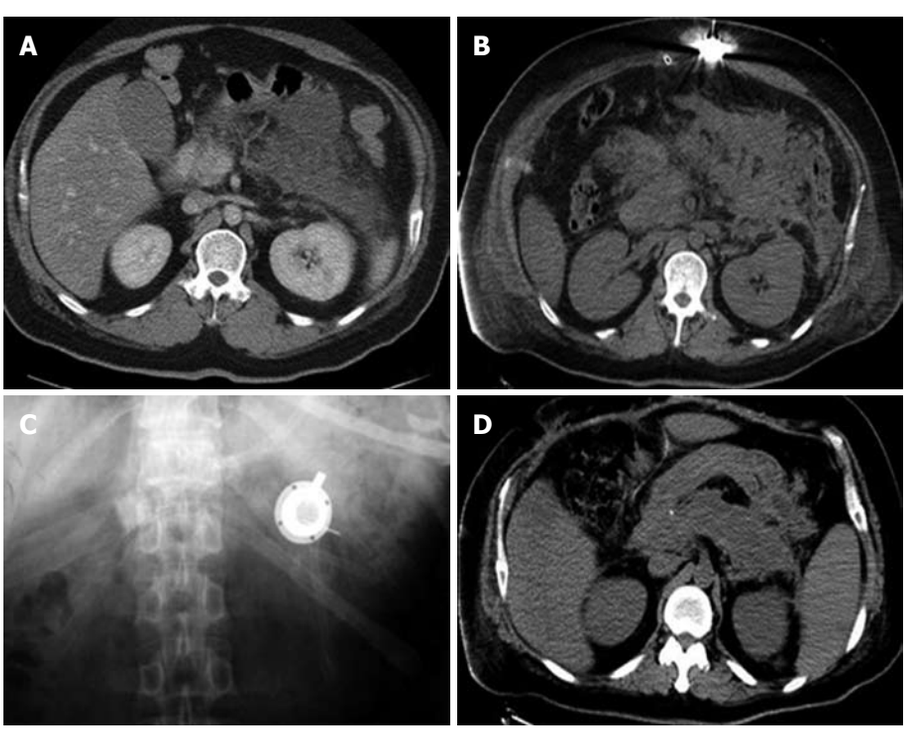

Figure 2 Imaging comparison between preoperation and postoperation. A: Abdominal computed tomography (CT) scan before the operation; B: Abdominal CT scan after the operation with the drug delivery system (DDS) inserted; C: Abdominal X-ray film showing the subcutaneous DDS head (white); D: CT scan showing the recovered pancreas. No more fluid could be seen around the pancreas (CT scan before discharge).

the APACHE Ⅱ score[1,14,15] , with a score of > 8 points considered indicative of severe disease[16]. In our patients, the APACHE Ⅱ score ranged from 13 to 27 points, with a mean of 18.9 points. The Balthazar CT severity index was evaluated at each CT study[12]. The CT severity index of the 8 patients ranged from 7 to 10 (average 9.1) before arterial infusion. The abdominal pressure of the patients was measured before operation, and the results suggested that every patient had high abdominal pressure and was diagnosed as having ACS. The average abdominal pressure was 29.1 mmHg (range, 23-38 mmHg). The cause of the pancreatitis was cholelithiasis in 4 cases, alcoholism in 1, hyperlipidemia in 2, and unknown in 1. The arterial infusion was started 3-9 d after the onset of SAP in these patients, and lasted 8-41 d (mean, 26.3 d).

The clinical conditions of the patients improved within 3 d after decompression and the initiation of the arterial infusion. The APACHE Ⅱ score was decreased from 13-27 (mean, 18.1) to 4-8 (mean, 5.4), and the CT severity index from 7-10 (mean, 9.0) to 1-3 (mean, 2.3), respectively, 60 d after the operation. IAP decreased to 7.7 mmHg (range, 6-11 mmHg). One patient died of MOF, and all other patients were discharged from the hospital within 68-259 d (mean, 131.9 d). The patients are all in good health now. The catheter was removed percutaneously 1 year after discharge, and there were no complications related to the procedure or drug delivery after long-term catheter placement. The clinical data are shown in Table 2 and Figure 2.

DISCUSSION

SAP injures not only the pancreas itself but also the surrounding organs, culminating in ACS and MOF in many cases. Despite the various treatment protocols, including intensive care therapy and blood filtration, SAP is still has a high mortality. The basic principles of the initial management of acute pancreatitis are adequate monitoring of vital signs, fluid replacement, correction of any electrolyte imbalance, nutritional support, and prevention of local and systemic complications.

Infected necrosis is generally accepted as an indication for surgery, but there is a subset of acute pancreatitis which may need an urgent laparotomy in the absence of infection to decompress abdominal pressure, which is unique as a compartment syndrome and virtually affects all organ systems within the body. Pathophysiologically, it deranges cardiovascular hemodynamics, respiratory and renal functions and may eventually lead to multi-organ failure. In addition, the gold standard for the treatment of established ACS is surgical decompression of the abdomen[17].

Our main purpose was to decompress the intra-abdominal hypertension (IAH). A double drainage tube was placed in peritoneal cavity around the region of pancreas, through which the patient's cavity was persistently douched using a large amount of saline solution. This can help drain the intra-abdominal hemorrhagic ascites, alleviate IAH, dilute the inflammatory mediators and activated amylase, and reduce toxin absorption through the peritoneum. In addition, we placed a DDS for postoperative treatment based on the positive results of regional infusion reported by other authors[5-11]. In contrast to their methods of vascular intervention using an angiocatheter, we inserted the DDS catheter into the pancreaticoduodenal artery from the right gastroepiploic artery to the gastroduodenal artery during the procedure. The DDS was subcutaneously fixed and did not result in any discomfort after drug delivery. In the ICU, the DDS could be easily used for the continuous injection of protease inhibitors, antibiotics and octreotide. This device was simple, safe and did not hinder the patient's movement. No complications related to the procedure or the device setting were observed, even after long-term placement.

Regarding the drug infusion, because acute pancreatitis is an autodigestive disease, protease inhibition has been the focus of experimental and clinical research. However, in clinical settings, the effect of protease inhibitors in the treatment of acute pancreatitis is still controversial. Some randomized, controlled trials failed to demonstrate any significant benefits[18,19]. For this reason, in Europe and the United States, protease inhibitors are not usually applied in the treatment of acute pancreatitis. In Japan, however, protease inhibitors are often applied, and in particular, it has been demonstrated that the CRAI of protease inhibitors and antibiotics are beneficial for severe acute necrotizing pancreatitis[5-11]. They believed that there were many reasons why the protease inhibitors were not as effective as expected in the experimental studies, such as the timing of administration, the concentration of the protease inhibitor in the pancreatic tissue, the diminution of the vasculature of the pancreas and so on. With intravenous administration, the concentration of the agent reaching the pancreas eventually becomes insufficient for controlling the inflammation. As a result of drug dilution as well as serum and hepatic metabolism, most of the aprotinin administered intravenously tended to go into the liver and thereafter accumulate in the kidneys, with only a small amount of aprotinin distributed in the pancreas. In contrast, with intra-arterial administration in experimental studies, the local concentration in the pancreas was high enough to improve the biochemical indices of inflammation and survival[20,21].

Because pancreatic and extrapancreatic infections are determining factors leading to death in patients with SAP, much attention has been paid to the potential role for antibacterial prophylaxis, especially in those patients with pancreatic necrosis. Many studies on the prophylactic effect of antibiotics have demonstrated that broad-spectrum antibiotics with good pancreatic tissue penetration decreased the incidence of infectious complications and mortality[22,23]. The antibacterial agent of first choice is likely to be imipenem because it reaches a higher distribution in the pancreatic tissue and provides higher bactericidal activity against most of the bacteria present in pancreatic infection compared with other types of antibiotics. An alternative antibiotic regimen is either ciprofloxacin

or ofloxacin in combination with metronidazole[24]. It also has been suggested that the effect was induced markedly by intra-arterial administration.

Octreotide reduces exocrine pancreatic secretion in acute pancreatitis, which would decrease pancreatic autodigestion, and it may also significantly prevent the bacterial translocation by preventing mucosal damage[25]. As a treatment guideline for acute pancreatitis, octreotide has been widely used in clinical practice, although the results of clinical investigations using somatostatin or its analogue are controversial. In a multicenter randomized controlled study with a large number of patients (n = 302) with an adequate level of disease severity, no benefit of octreotide on progression or outcome was found[26], but other research suggests that octreotide may have a beneficial effect in the treatment of SAP[27]. One study suggested that octreotide seemed to have a dose- and time-dependent effect on histopathology and lipid peroxidation: an early bolus application of octreotide reduced the severity of histopathological changes in acute pancreatitis and decreased lipid peroxidation in the pancreatic tissue samples. However, a late bolus application and continuous intravenous infusion did not influence pancreatitis or lipid peroxidation[28].

According to the above-mentioned clinical and experimental results, in this study, we used a combination of these three kinds of drugs *via* DDS as regional therapy in the 8 patients. Their clinical conditions improved after the treatment and the overall mortality was 12.5%, which is much lower than that reported in the literature. These results suggest that CRAI plus laparotomic decompression might be a therapeutic choice for SAP with ACS.

COMMENTS

Background

Severe acute pancreatitis (SAP), characterized by intricate mechanism, variant symptoms, poor prognosis and multiple complications, seriously threatens the life of patients. About 11% of SAP patients suffer from abdominal compartment syndrome (ACS). SAP complicated by ACS has a mortality rate of that is 30%-60%.

Research frontiers

ACS has been recognized as a contributing factor for the multiple organ failure commonly seen in SAP. Surgical decompression is the preferred method of treatment for ACS. Although decompression has a significant effect in lowering IAP, the mortality still remains high in SAP patients with ACS. Some recent studies showed that continuous regional arterial infusion of protease inhibitors and antibiotics may reduce the mortality rate and incidence of infectious complications in SAP.

Innovations and breakthroughs

SAP with ACS is the most severe form of acute pancreatitis and has a high morbidity and mortality which need an urgent laparotomy. In this study, a new system was applied during the decompressing surgical procedure in 8 patients. In addition to abdominal decompression and the placement of wide-bore drains for continuous postoperative irrigation, the catheter of a drug delivery system (DDS) was inserted into the peripancreatic artery for postoperative continuous regional arterial infusion, which increased the tissue concentration of the drugs in the inflamed pancreas and improved the biochemical indices of inflammation and survival.

Applications

The DDS applied for continuous regional arterial infusion (CRAI) in this study is simple, safe and easy to implement. These positive results suggest that CRAI plus laparotomic decompression might be a therapeutic choice for SAP with ACS.

Peer review

Even if it is not accepted by all, the presence of an ACS in severe pancreatitis is a very difficult complication to deal with. The paper reports a small group of patients with such a complication treated with a multidisciplinary approach (surgery, antibiotics, anti protease, *etc.*). It is difficult to understand the real value of each therapy, but the utility of the paper lies in reporting the complexity of such a disease.

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S- Editor Wu X **L- Editor** Ma JY **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4917

World J Gastroenterol 2011 November 28; 17(44): 4917-4921
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Plasma DNA methylation of Wnt antagonists predicts recurrence of esophageal squamous cell carcinoma

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Supported by Jiangsu University and Nantong Tumour Hospital
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Received: May 6, 2011 Revised: August 6, 2011
Accepted: August 13, 2011
Published online: November 28, 2011

Abstract

AIM: To detect the effects of plasma DNA methylation of Wnt antagonists/inhibitors on recurrence of esophageal squamous cell carcinoma (ESCC).

METHODS: We used methylation-specific polymerase chain reaction to detect hypermethylation of the promoter of four Wnt antagonists/inhibitors (*SFRP-1*, *WIF-1*, *DKK-3* and *RUNX3*) using DNA from the plasma of ESCC patients (n = 81) and analyzed the association between promoter hypermethylation of Wnt pathway modulator genes and the two-year recurrence of ESCC.

RESULTS: Hypermethylation of *SFRP-1*, *DKK-3* and *RUNX-3* was significantly associated with an increased risk of ESCC recurrence (P = 0.001, 0.003 and 0.001 for *SFRP-1*, *DKK-3* and *RUNX3*, respectively). Patients carrying two to three methylated genes had a significantly elevated risk of recurrence compared with those not carrying methylated genes (odds ratio = 15.69, 95% confidential interval: 2.97-83). The area under the receiver operating characteristic curve (AUC) was 77.1 for ESCC recurrence prediction (sensitivity = 66.67 and specificity = 83.3). When combining methylated genes and the clinical stage, the AUC was 83.69, with a sensitivity of 76.19 and a specificity of 83.3.

CONCLUSION: The status of promoter hypermethylation of Wnt antagonists/inhibitors in plasma may serve as a non-invasive prognostic biomarker for ESCC.

Key words: Plasma; Methylation; Esophageal Cancer; Recurrence

Peer reviewer: Dr. Yukie Sato-Kuwabara, Hospital A. C. Camargo, Teaching and Research International Center, Sao Paulo 01508-010, Brazil

Liu JB, Qiang FL, Dong J, Cai J, Zhou SH, Shi MX, Chen KP, Hu ZB. Plasma DNA methylation of Wnt antagonists predicts recurrence of esophageal squamous cell carcinoma. *World J Gastroenterol* 2011; 17(44): 4917-4921 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4917.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4917

INTRODUCTION

Esophageal cancer, predominantly esophageal squamous cell carcinoma (ESCC), is one of the most common malignancies in the world, with 482 300 incident cases and

406 800 deaths estimated in 2008[1]. Most patients with ESCC are diagnosed at an advanced stage. Although therapeutic advances have been designed to improve treatment outcome, the prognosis of patients with ESCC is still poor, with long-term survival rates between 5% and 20%[2]. The poor prognosis is partially due to the high rate of recurrence, which occurs in approximately half of patients after curative surgical resection. Therefore, development of accurate prognostic biomarkers for ESCC is imperative and crucial for improving ESCC prognosis and for guiding treatment.

DNA methylation is one of the most common epigenetic modifications[3]. One of the major changes caused by DNA methylation is transcriptional silencing of tumor suppressor genes resulting from hypermethylation of CpG islands in promoter regions[4]. This change occurs frequently during tumor pathogenesis and progression and has become widely recognized as a mechanism of gene inactivation in cancer[5-7]. The methylation profile may also help predict the response to a chemo-/radio-therapeutic agent and thus the prognosis of cancer[8,9]. In addition, detection of promoter CpG methylation in body fluid DNA is feasible and nearly non-invasive[10].

Wnt signaling operates across cell boundaries *via* secretion by cells of one tissue type, which results in activation of surface receptors on neighboring cells and tissues, leading to activation of transcription factors that regulate cell proliferation, survival, and differentiation[11]. Dysregulation of these processes in cancer results in aberrant activation of the Wnt pathway[11,12]. Several antagonists of Wnt signaling have been identified, including the secreted frizzled-related protein-1 (SFRP-1) and Wnt inhibitory factor-1 (WIF-1), which bind directly to Wnt proteins, and Dickkopf-3 (DKK-3), which binds to the LDL-receptor-related protein5 (LRP5)/LRP6 component of the Wnt receptor complex[13]. In addition, another Wnt inhibitor, runt-related transcription factor-3 (RUNX3), reportedly forms a ternary complex with β-catenin/transcription factor-4 (TCF4) to attenuate Wnt signaling, which regulates cell proliferation, apoptosis, and invasion[14,15]. Given the important roles of Wnt antagonists/inhibitors in cancer progression and prognosis, we evaluated the association between methylation of promoter CpG islands of the four tumor suppressor genes, *SFRP-1*, *WIF-1*, *DKK-3* and *RUNX3*, in the Wnt signaling pathway and ESCC recurrence using plasma DNA from 81 Chinese patients with ESCC.

MATERIALS AND METHODS

Study population

This study was approved by the institutional reviewer board of Nantong Cancer Hospital, and written informed consent was obtained from each patient or from the patient's representative. Briefly, patients with histopathologically diagnosed incident ESCC were recruited from Nantong Cancer Hospital from June to December 2008. Exclusion criteria included self-reported previous cancer history, metastasis from other organs, and surgical section, radiotherapy, or chemotherapy before blood collection. Patients were followed up at the 24th month after recruitment by personal or family contacts. All patients were genetically unrelated, ethnic Han Chinese, and each patient donated 5 mL of venous blood at their first admission to the hospital. As a result, 81 ESCC patients who had complete clinical information, adequate DNA samples, and successful follow-up were included.

Table 1 Primers for methylation-specific polymerase chain reaction

Gene		Primer sequence (5'–3')
WIF-1		
U	F	GGGTGTTTTATTGGGTGTATTGT
	R	AAAAAAACTAACACAAACAAAATACAAAC
M	F	CGTTTTATTGGGCGTATCGT
	R	ACTAACGCGAACGAAATACGA
RUNX-3		
U	F	TTATGAGGGGTGGTTGTATGTGGG
	R	AAAACAACCAACACAAACACCTCC
M	F	TTACGAGGGGCGGTCGTACGCGGG
	R	AAAACGACCGACGCGAACGCCTCC
SFRP-1		
U	F	GAGTTAGTGTTGTGTGTTTGTTGTTTTGT
	R	CCCAACATTACCCAACTCCACAACCA
M	F	GTGTCGCGCGTTCGTCGTTTCGC
	R	AACGTTACCCGACTCCGCGACCG
DKK-3		
U	F	TTAGGGGTGGGTGGTGGGGT
	R	CTACATCTCCACTCTACACCCA
M	F	GGGGCGGGCGGCGGGGC
	R	ACATCTCCGCTCTACGCCCG

M: Methylated sequence; U: Unmethylated sequence; F: Forward primer; R: Reverse primer.

DNA extraction and methylation-specific polymerase chain reaction

DNA was extracted from 200 μL plasma from each patient using an SG Spin Column Clinical Sample Genomic DNA MiniPreps kit (Shanghai ShineGene Molecular Biotech, Inc., Shanghai, China). The plasma DNA was modified with sodium bisulfite using the EZ DNA methylation™ kit (Zymo Research, Orange, CA, United States). The methylation status of CpG islands in the promoter region of *SFRP-1*, *WIF-1*, *DKK-3* and *RUNX-3* was determined by methylation-specific polymerase chain reaction (PCR)[16]. In brief, the first universal primer set covered no CpG sites in either the forward or the reverse primer but amplified a DNA fragment of the promoter region containing several sites. Then, a second round of nested methylation-specific PCR or unmethylation-specific PCR was performed using the universal PCR products as templates. Primer sequences are shown in Table 1.

Statistical analysis

Differences in demographic and clinical characteristics were evaluated with χ^2 tests (or the Fisher's exact test). The association between methylation and ESCC recurrence was

Table 2 Characteristics of patients

Variables	Recurrence		*P* value
	No	Yes	
Age (yr)			0.804
< 63	31	10	
⩾ 63	29	11	
Gender			0.569
Female	16	4	
Male	44	17	
Smoking			0.614
No	31	9	
Yes	29	12	
Drinking			0.799
No	26	8	
Yes	34	13	
Operation			0.614
No	38	12	
Yes	22	9	
Chemo-/radio-therapy			0.751
No	11	5	
Yes	49	16	
Stage			< 0.001
Ⅰ	25	3	
Ⅱ	22	3	
Ⅲ	13	15	

Table 3 Correlation between methylation and recurrence in esophageal squamous cell carcinoma patients

Gene	*n*	Recurrence		Crude OR (95% CI)	*P* value	Adjusted OR[1] (95% CI)	*P* value[1]
		No	Yes				
SFRP-1							
Negative	57	49	8	1.00	< 0.001	1.00	< 0.001
Positive	24	11	13	7.24 (2.42-21.68)		10.8 (2.54-46)	
WIF-1							
Negative	52	42	10	1.00	0.069	1.00	0.107
Positive	29	18	11	2.57 (0.93-7.11)		2.61 (0.8-8.47)	
DKK-3							
Negative	51	44	7	1.00	0.001	1.00	0.003
Positive	30	16	14	5.50 (1.88-16.08)		6.07 (1.73-21.28)	
RUNX-3							
Negative	52	45	7	1.00	< 0.001	1.00	0.001
Positive	29	15	14	6.00 (2.04-17.65)		7.81 (2.30-47)	

[1]Adjusted for age, gender, smoking, drinking, stage, surgical operation and chemo-/radio-therapy. CI: Confidence intervals; OR: Odds ratios.

Table 4 Combined analysis of methylation and recurrence in esophageal squamous cell carcinoma patients

No. of M gene	*n*	Recurrence		Crude OR (95% CI)	*P* value	Adjusted OR[1] (95% CI)	*P* value[1]
		No	Yes				
0	32	29	3	Ref.		Ref.	
1	25	21	4	1.84 (0.37-9.11)	0.454	1.71 (0.27-10.73)	0.570
2-3	24	10	14	13.53 (3.21-57.07)	< 0.001	15.69 (2.97-83.00)	0.001
Locus Trend				4.10 (1.92-8.75)	< 0.001	4.10 (1.73-9.72)	0.001

[1]Adjusted for age, gender, smoking, drinking, stage, surgical operation and chemo-/radio-therapy. CI: Confidence intervals; OR: Odds ratios.

estimated by computing odds ratios (ORs) and 95% confidence intervals (CIs) from logistic regression analyses for adjusted ORs and crude ORs, with or without adjustments for age, gender, smoking, drinking, clinical stage, surgical operation and chemo-/radio-therapy status. Receiver operating characteristic (ROC) curve analysis was conducted by using the "pROC" package in R. All the statistical analyses were performed with R software (version 2.11.1; The R Foundation for Statistical Computing).

RESULTS

Characteristics of patients

The clinicopathologic features of the patients are summarized in Table 2. A total of 81 ESCC patients were included in our current study. The mean age was 63 years (range, 46-80 years), and 61 patients were male (75.3%). Sixty-five (80.2%) patients underwent chemo-/radio-therapy, and 31 (38.3%) patients underwent surgical operation after blood collection. Among the 81 patients, there were 21 recurrences (26%) at the 24th month after recruitment. As expected, advanced stage was a risk factor for ESCC recurrence ($P < 0.001$). However, there were no significant differences in the recurrence rates among the subgroups of age, gender, smoking status, drinking status, with or without surgical operation, and/or chemo-/radio-therapy.

Single-gene analysis

The percentage of promoter methylation in the genes we analyzed was as follows: 29.6% for *SFRP-1*, 35.8% for *WIF-1*, 37.4% for *DKK-3*, and 35.8% for *RUNX-3*. Three genes showed significant associations with ESCC recurrence after adjusting for age, gender, smoking, drinking, stage, surgical operation, and chemo-/radio-therapy. As shown in Table 3, patients with methylated *SFRP-1* had a 10.8-fold increased risk of recurrence compared with those with unmethylated *SFRP-1* (95% CI: 2.54-46, $P < 0.001$). Similarly, methylated *DKK-3* was associated with a 6.07-fold increased risk of recurrence (95% CI: 1.73-21.28, $P = 0.003$), and methylated *RUNX-3* was associated with a 7.81-fold increased recurrence risk (95% CI: 2.30-47, $P = 0.001$). Methylation of *WIF-1*, however, was not significantly associated with an increased risk of recurrence ($P = 0.107$).

Combined analysis

We then performed combined analysis to assess the effect of methylation of the three genes (*SFRP-1*, *DKK-3* and *RUNX-3*) on ESCC recurrence. As shown in Table 4, compared with patients without methylated genes, those with two to three methylated genes had a significantly increased risk of recurrence (OR = 15.69, 95% CI: 2.97-83).

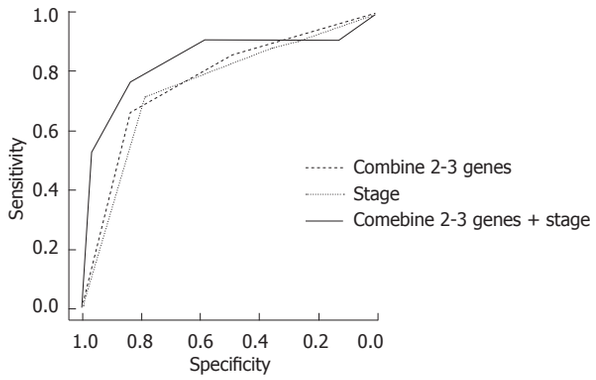

Figure 1 Receiver operating characteristic curve analysis to assess the sensitivity and specificity of the status of methylated genes in combination with the stage for predicting esophageal squamous cell carcinoma recurrence using the "pROC" package in R. Combination of 2-3 genes, area under curve (AUC) = 77.1, sensitivity = 66.67, specificity = 83.3, stage, AUC = 75.24, sensitivity = 71.43, specificity = 78.3, combination of 2-3 genes + stage, AUC = 83.69, sensitivity = 76.19, specificity = 83.3.

Furthermore, there was a dose-response effect between plasma DNA methylation status and ESCC recurrence (OR = 4.1, 95% CI: 1.73-9.72, *P* for trend: 0.001).

AUC analysis

We also conducted a ROC curve analysis and area under curve (AUC) analyses to assess the sensitivity and specificity of the status of methylated genes individually and in combination for predicting ESCC recurrence. For single genes, the AUC were 71.79 (*SFRP-1*), 70 (*DKK-3*) and 70.83 (*RUNX3*). When we considered two to three methylated genes, the AUC increased to 77.1, with a sensitivity of 66.67 and a specificity of 83.3. As a risk factor for ESCC recurrence, the AUC for stage was 75.24 (sensitivity = 71.43, specificity = 78.3). For the combination of methylated genes and clinical stage, the AUC was 83.69 (sensitivity = 76.19, specificity = 83.3) (Figure 1).

DISCUSSION

Wnt antagonists/inhibitors function as tumor suppressors, and thus hypermethylation of their promoters and subsequent silencing of these genes may be implicated in the pathogenesis or progression of a broad spectrum of human malignancies[17-19]. In our current study, we found that the methylation status of *SFRP-1*, *DKK-3* and *RUNX-3* promoters in plasma DNA can individually and jointly predict ESCC recurrence.

Most published studies on the association between hypermethylation of Wnt antagonist/inhibitor gene promoters and cancer development have focused on tumor tissues. Yu *et al*[17] found that epigenetic silencing of *DKK-3* is a common event in gastric cancer and is associated with a poor disease outcome. Urakami *et al*[18] used a methylation score to analyze the combined effects of hypermethylated Wnt antagonist family genes on bladder cancer detection, and they found that the score was significantly higher in bladder tumors than in bladder mucosa. Hamilton *et al*[9] assessed the methylation status of nine genes, including *RUNX-3*, in esophageal cancer patients and found that increased methylation of this gene correlated with poor responsiveness to therapy, suggesting potential clinical application of these biomarkers in guiding prognosis and management. A growing body of evidence suggests that the methylation signature is consistent between DNA derived from tissue and DNA derived from serum/plasma[19,20]. However, serum/plasma DNA has obvious advantages compared with DNA derived from tissue samples, i.e., with respect to the ease to obtain, non-invasiveness, and relative reproducibility. Therefore, serum/plasma DNA may be an excellent source of samples for assessing biomarkers, especially for patients who have not undergone surgery.

Previous studies have shown that methylation of various genes in serum/plasma can be a highly specific biomarker for human cancers[19,21-26]. Herbst *et al*[24] reported that *NEUROG1* is frequently methylated in sera of patients with colorectal cancers independent of the tumor stage and is a suitable non-invasive screening approach for detecting asymptomatic colorectal cancer. Göbel *et al*[25] found that methylated *PITX2* and *RASSF1A* in plasma are therapy-independent prognostic factors in breast cancer patients. Salazar *et al*[26] showed that the methylation status of *CHFR* in serum can influence the outcome of chemotherapy in stage Ⅳ non-small-cell lung cancer patients and that unmethylated *CHFR* predicts increased survival to EGFR TKIs. Urakami *et al*[19] reported that hypermethylation of Wnt antagonists can serve as an excellent epigenetic biomarker panel for detection, staging and prognosis of renal cell carcinoma using serum DNA. These studies all indicate the available and feasibility of using plasma DNA to establish the methylation status of gene promoters as a biomarker for cancer.

Given that ESCC is one of the most aggressive cancers in the world and that no non-invasive test is available, our preliminary study yielded a promising result. Limitations of our study include the lack of detection of DNA methylation in tissue and the unpredictability of the link between methylation status and gene expression. Nevertheless, the methylation status of Wnt antagonists/inhibitors may serve as a valuable biomarker for predicting ESCC recurrence.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies in the world. The prognosis of patients with ESCC is poor, which is partially due to the high rate of recurrence. Therefore, development of accurate prognostic biomarkers for ESCC is imperative and crucial for improving ESCC prognosis and for guiding treatment.

Research frontiers

Wnt antagonist/inhibitor genes function as tumor suppressors, and hypermethylation of their promoters and subsequent silencing of the genes, which can be detected in plasma DNA, may serve as a valuable non-invasive biomarker for predicting ESCC recurrence.

Innovations and breakthroughs

The association between methylation of promoter CpG islands of four tumor suppressor genes (*SFRP-1*, *WIF-1*, *DKK-3* and *RUNX3*) in the Wnt signaling pathway and ESCC recurrence was evaluated using plasma DNA from ESCC patients.

Applications

This result offers great potential for using plasma DNA to analyze methylation as a non-invasive biomarker for predicting ESCC recurrence.

Terminology

Epigenetic changes: Heritable changes in the gene structure that do not change the gene sequence. CpG islands: CpG-rich areas located in the promoter regions of many genes. CpG island methylation: The addition of a methyl group to a cytosine residue that is next to guanine.

Peer review

The study aimed to verify the promoter hypermethylation status of four Wnt [antagonist genes odds ratio (OR) antagonists] (using methylation-specific polymerase chain reaction with OR with the methylation-specific polymerase chain reaction technique using) DNA from plasma of 81 ESCC patients. The article reports interesting results that are important to the field.

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S- Editor Lv S **L- Editor** Cant MR **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4922

World J Gastroenterol 2011 November 28; 17(44): 4922-4927
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

SOX7 is involved in aspirin-mediated growth inhibition of human colorectal cancer cells

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Supported by The National Natural Science Foundation of China, No. 31071149; the Fundamental Research Funds for the Central Universities, No. 09ZDQD01 and No. 10QNJJ014; and the National Science Foundation of China, No. J0830627-2
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Received: March 22, 2011 Revised: June 21, 2011
Accepted: June 28, 2011
Published online: November 28, 2011

Abstract

AIM: To confirm the role of sex-determining region Y-box 7 (Sox7) in aspirin-mediated growth inhibition of COX-independent human colorectal cancer cells.

METHODS: The cell survival percentage was examined by MTT (Moto-nuclear cell direc cytotoxicity) assay. SOX7 expression was assessed by using reverse transcription-polymerase chain reaction and Western blotting. SB203580 was used to inhibit the p38MAPK signal pathway. SOX7 promoter activity was detected by Luciferase reporter assay.

RESULTS: SOX7 was upregulated by aspirin and was involved in aspirin-mediated growth inhibition of SW480 human colorectal cancer cells. The p38MAPK pathway played a role in aspirin-induced SOX7 expression, during which the AP1 transcription factors c-Jun and c-Fos upregulated SOX7 promoter activities.

RESULTS: SOX7 is upregulated by aspirin and is involved in aspirin-mediated growth inhibition of human colorectal cancer SW480 cells.

Key words: SOX7; Aspirin; p38MAPK; Colorectal cancer; SB203580

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Zhou X, Huang SY, Feng JX, Gao YY, Zhao L, Lu J, Huang BQ, Zhang Y. SOX7 is involved in aspirin-mediated growth inhibition of human colorectal cancer cells. *World J Gastroenterol* 2011; 17(44): 4922-4927 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4922.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4922

INTRODUCTION

In Western Europe and the United States, colorectal cancer is the second most common fatal cancer next to lung cancer. Aspirin is believed to have a chemopreventive role in colorectal cancer based on considerable observational data, which show that the rates of colorectal cancers and adenomas are 40%-50% lower in aspirin users[1,2]. Aspirin is a nonsteroidal anti-inflammatory drug (NSAID). There have been indications that use of NSAIDs can lead to the regression of colorectal adenomas[3]. In several rodent models of colorectal cancer, the NSAIDs indomethacin, aspirin, sulindac and piroxicam were shown to be able to reduce tumor growth[4]. However, the molecular mechanisms underlying the cancer preventive effects of NSAIDs are not well understood, and this has been an active issue of research interest.

Commonly, prevention of cancer may be implemented

through several means, including cell cycle arrest, induction of apoptosis and inhibition of angiogenesis. One most widely accepted mechanism for the anticancer effect of NSAIDs is the reduction of prostaglandin synthesis by inhibiting COX activity[5-7]. However, the importance of COX inhibition for the anti-proliferative effects of NSAIDs is controversial at the present time, since NSAIDs also manifest growth inhibitory effects against colon cancer cell lines that do not express COX-1 or COX-2 enzymes[8-11]. A common mechanism of NSAID action appears to be the induction of apoptosis, although several *in vitro* studies in CRC cells have proposed that different molecular pathways may be affected by distinct types of NSAIDs[9,12-14]. To date, little is known about the COX-independent molecular targets of NSAID action in cancer cells.

The mitogen-activated protein kinase (MAPK) superfamily, including the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, is involved in mediating the processes of cell growth and death[15,16]. JNK and p38 MAPK pathways are activated in response to chemicals and environmental stress[17], while the ERK cascade, activated by mitogenic stimuli, is critical for proliferation and survival[18]. Recent evidence indicates that the MAPK family proteins are important mediators of apoptosis induced by stressful stimuli[18,19]. JNK and p38 MAPK are collectively termed stress-activated protein kinases because they are activated by a variety of stress-related stimuli and also activated by chemotherapy drugs[20,21]. Activation of p38 MAPK distorts mitochondrial function *via* an increase in the ratio of Bax and anti-apoptotic (Bcl-2) members leading to an increased mitochondrial membrane permeability, the release of cytochrome c, and the activation of caspases[22]. Once activated, p38 regulates multiple cellular processes, including transcription, translation, cell cycle progression, and apoptosis. Schwenger *et al*[23] previously showed that sodium salicylate, the active component of aspirin, activated p38 to induce apoptosis. Also, aspirin activates the p38MAPK pathway, leading to the degradation of cyclin D1, nucleolar translocation of RelA, and apoptosis[24].

Sox genes encode transcription factors that possess strong homology to the high-mobility group (HMG box), which are homologous to sex-determining region of Y-chromosome in the HMG box. There are at least 30 Sox members expressed in many different cell types and tissues, and at multiple stages during development[25]. Sex-determining region Y-box 7 (Sox7), together with Sox17 and Sox18, belongs to the Sox group F subfamily. Sox7 encodes an HMG box transcription factor and has been implicated in parietal endoderm differentiation[26]. Our previous study demonstrated that the expression of SOX7 mRNA was frequently down-regulated in human colorectal cancer cell lines and in primary colorectal tumor tissues, and restoration of SOX7 induced colorectal cancer cell apoptosis, inhibited cell proliferation and colony formation[27].

Despite these available data, the mechanistic function of aspirin in inhibiting COX2 negative colorectal cancer cells awaits further investigation. In this study, we have examined the role of SOX7 in aspirin-mediated growth inhibition of COX2 negative SW480 cells, and we found that SOX7 is regulated by aspirin and the p38 MAPK pathway in SW480 cells. Our study has disclosed the involvement of SOX7 in aspirin-mediated growth inhibition of COX2 negative cancer cells, providing a new insight into the mechanism by which aspirin inhibits COX2 negative colorectal cancer.

MATERIALS AND METHODS

Cell lines and reagents

Human colorectal cancer cell line SW480 and human embryonic kidney HEK-293T cells were cultured in appropriate media with 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin, and kept in a humidified atmosphere of 20 mL/L CO_2. Genomic DNA was extracted using the standard Proteinase-K method. Total RNA was extracted by using the Trizol reagent (TAKARA).

Drug treatment

Aspirin (Sigma, St. Louis, MO) was dissolved in 1 mol/L Tris-HCl (pH 7.5) to a stock concentration of 1 mol/L and the pH adjusted to 7.2 with 4 mol/L HCl. SW480 cells were treated for 24, 48 and 72 h by adding various volumes of stock to obtain final concentrations of 1, 2 and 5 mmol/L of aspirin. Control cells were treated with an equivalent volume of Tris-HCl (pH 7.2).

The p38 inhibitor SB203580 (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mmol/L. SW480 cells were treated with 10 μmol/L SB203580 for 30 h. Control cells were treated with an equivalent volume of DMSO.

MTT assay

Cell proliferation was assessed by the MTT (Moto-nuclear cell direc cytotoxicity assay) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay. SW480 cells were plated at 1×10^3 cells/well on 96-well plates. After treatment with aspirin or transfection, 20 μL of MTT (5 mg/mL) was added to each well; the samples were incubated for 4 h at 37 ℃ and then subcultured in the medium with 100 μL DMSO. The absorbance of each well was determined at 492 nm. Survival percentage (%) was calculated relative to the control.

Plasmid constructs and transfection

SOX7 gene promoter (GenBank accession NM_031439) was cloned by PCR from the genome derived from normal human colorectal tissue using the following primers: 5'-CCCAAGCTTCTGCCGACTTTCATTCAGTAGGTG-3' (sense) and 5'-CCGCTCGAGGTAGGCTCCCAGCAGCGAAG-3' (antisense). To generate SOX7 promoter-luciferase (pGL3-SOX7-luc) construct, the PCR production was subcloned into the pGL3 enhancer vector *via* *Hin*dⅢ and *Xho*l Ⅰ sites. Short interfering RNA (siRNA) targeting SOX7 sequence (ACGCCGAGCTGTCGGATGG) was synthesized[27]. An oligonucleotide that represents the siRNA was cloned into the pSliencer4.1-CMV neo-vector (Ambion) between *Bam*H Ⅰ and *Hin*d Ⅲ sites following the manufacturer's instructions. c-Fos

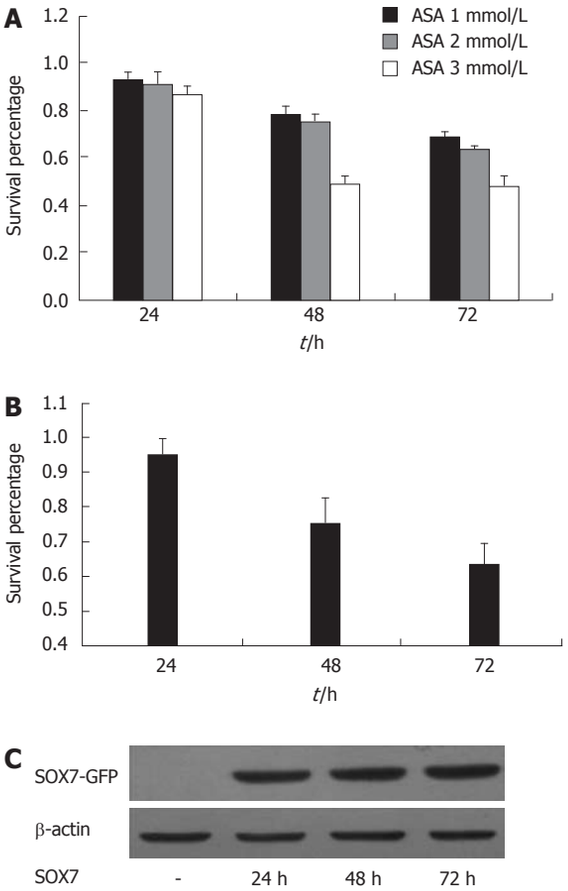

Figure 1 Aspirin and SOX7 inhibit the growth of SW480 cells. A: Dose- and time-dependent effects of aspirin on the growth rates of SW480 human colon cancer cells. SW480 cells were treated for 24, 48 or 72 h in aspirin-containing culture medium; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to estimate the survival percentage compared with untreated cells; B: Effect of SOX7 expression on cell proliferation. MTT assay was used to estimate the proliferation at different time points after transfection of SOX7 expression vector; C: Western blotting was used to verify the expression of SOX7 after transfection of SOX7 expression vector at indicated time points. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. ASA: Aspirin.

and c-Jun were gifts from Dr. Shizuo Akira (Osaka University, Japan); p38α was a gift from Dr. Roger David (University of Mass. Medical School); and SOX7 expression plasmid was previously constructed in this laboratory[27]. Plasmids were transfected using Lipofectamine™ 2000 (Invitrogen) follow the manufacturer's instructions.

Luciferase reporter assay

Reporter gene assays were done as previously described[27]. Briefly, 5×10^4 cells were seeded in 24-well tissue culture plates 24 h before transfection. The pGL3-SOX7-luc reporter vector was transfected at 500 ng/well and the *Renilla* luciferase control plasmid pREP7-RLuc was cotransfected at 50 ng/well as an internal control reporter. After treatment with indicated doses of aspirin or cotransfection with c-Fos and c-Jun expression vectors for 24 h, cells were washed and lysed in passive lysis buffer (Promega) and the transfection efficiency was normalized to the paired *Renilla* luciferase activity by using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction

For cDNA synthesis, 1 μg of total RNA was reverse transcribed using the RT-Systems supplied by Promega. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was carried out on an ABI Prism 7000 Sequence Detection System (Applied Biosystems), and SYBR Green (TOYOBO) was used as a double-stranded DNA-specific fluorescent dye. The PCR primer sequences were as follows: SOX7: 5'-ACCAACGGGTCCCACAGA-3'(sense) and 5'-CCACTCAAGGCACAAGAAGG-3' (antisense)[27]; β-actin: 5'-TCGTGCGTGACATTAAGGAG-3' (sense) and 5'-ATGCCAGGGTACATGGTGGT-3' (antisense)[28].

Western blotting assay

Western blotting was performed as described previously[29]. The primary antibodies used were the mouse anti-SOX7 (1:1 000, R&D system) and mouse anti-β-actin (1:10 000, Sigma).

RESULTS

Aspirin and SOX7 inhibit the growth of SW480 human colorectal cancer cells

We first demonstrated that aspirin treatment results in a profound concentration- and time-dependent reduction in the proliferation rate of SW480 cells (Figure 1A). Our previous experiments showed that SOX7 was frequently down-regulated in human colorectal cancer cell lines including SW480[27]. In order to investigate the relationship between SOX7 and aspirin, we tested the effect of SOX7 on the growth of SW480 cells, and we found that restoration of SOX7 inhibits SW480 cell proliferation (Figure 1B and C).

SOX7 is induced by aspirin in SW480 colorectal cancer cells

Since both aspirin and SOX7 were able to inhibit the growth of SW480 cells, we sought to examine whether SOX7 can be induced by aspirin in SW480 cells. Our reporter gene experiments demonstrated that aspirin upregulated the activities of SOX7 promoter, and a dose of 1 mmol/L aspirin was sufficient to induce SOX7 promoter activity (Figure 2A). This dose was then used in the later experiments throughout the study. RT-PCR and Western blot assays showed that the mRNA (Figure 2B) and protein (Figure 2C) levels of SOX7 were both upregulated by 1 mmol/L aspirin. These data indicate that the SOX7 expression is induced by aspirin in SW480 cells.

SOX7 is involved in aspirin-mediated growth inhibition of human colorectal cancer cells

The above data indicate that both aspirin and SOX7 inhibit the growth of SW480 cells, and aspirin upregulates the expression of SOX7 in SW480 cells. We next intended to determine whether SOX7 played a role in aspirin-inhibited growth of SW480 cells. First, we constructed a SOX7 siRNA plasmid and tested the interference efficiency of this plasmid. As shown in Figure 3A, transfection of SW480 cells with SOX7 siRNA plasmid resulted

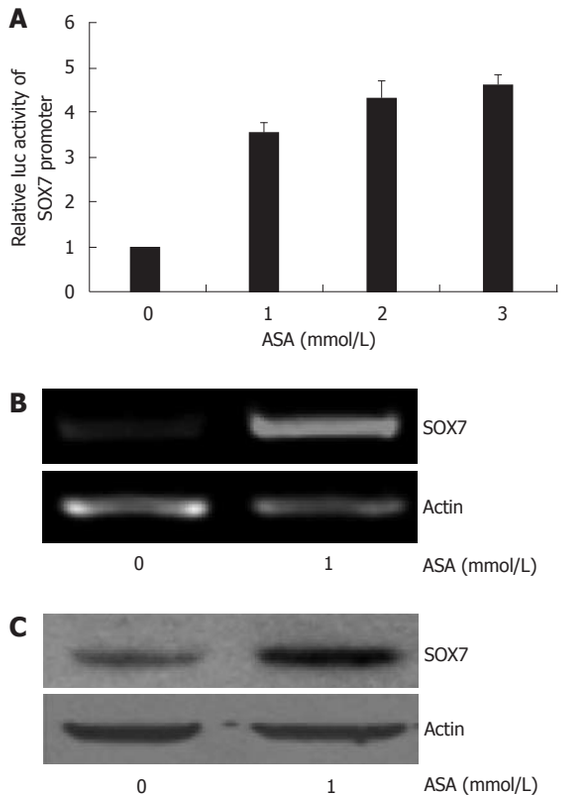

Figure 2 Aspirin upregulates SOX7 expression in SW480 cells. A: SW480 cells were transfected with pGL3-SOX7-luc plasmid, and relative luciferase activity was determined after treatment with different doses of aspirin for 24 h; B and C: Reverse transcription-polymerase chain reaction (B) and Western blotting (C) detected the expression of SOX7 after treatment with aspirin for 30 h; β-actin was used as the internal reference. ASA: Aspirin.

in a significant reduction in the SOX7 protein expression induced by aspirin, confirming the effective interference of this SOX7-siRNA. We then transfected SOX7 siRNA plasmid into SW480 cells, and we found that interference of SOX7 expression restored the growth rate of the aspirin-inhibited SW480 cells (Figure 3B). This indicated that SOX7 is involved in aspirin-mediated growth inhibition of human colorectal cancer SW480 cells.

Aspirin induces SOX7 expression through the p38MAPK pathway in SW480 colorectal cancer cells

We and others have shown that the p38 MAPK pathway can be activated by aspirin (data not shown)[24]. We thus sought to examine whether the p38 MAPK pathway is involved in aspirin-induced SOX7 expression. We demonstrated that SOX7 mRNA is significantly upregulated upon the overexpression of p38α in SW480 cells (Figure 4A). In contrast, in HEK-293T cells that express relatively high levels of SOX7, inhibition of p38 by SB203580 reduced the mRNA level of SOX7 (Figure 4B). We further examined the effects of the p38 inhibitor SB203580 on aspirin-induced SOX7 expression, and we discovered that chemical inhibition of p38 substantially abrogates the aspirin-induced upregulation of SOX7. We also found that upon the inhibition of the p38 MAPK pathway by SB203580, the upregulation of SOX7 by aspirin is counteracted at

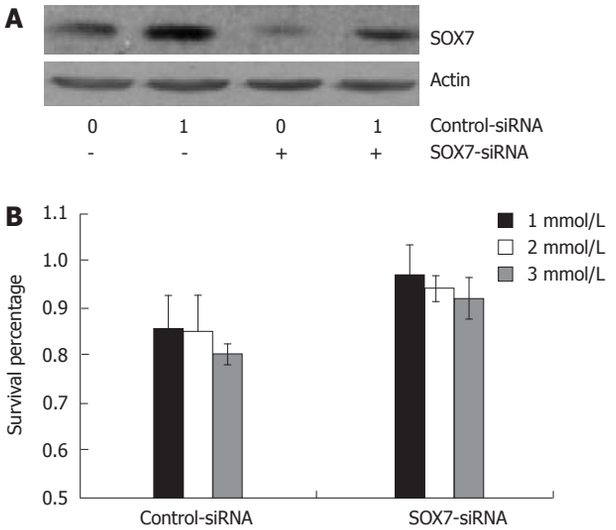

Figure 3 Knockdown of SOX7 counteracts the growth inhibitory effect of aspirin in SW480 cells. A: Western blotting verification of the interfering efficiency of SOX7 Short interfering RNA in aspirin-treated SW480 cells. β-actin was used as the internal reference; B: Effect of SOX7 knockdown on SW480 cell proliferation after treatment with different doses of aspirin for 48 h by moto-nuclear cell direc cytotoxicity assay assay. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

both mRNA and protein levels (Figure 4C and D). In addition, our data show that the AP1 transcription factors c-Jun and c-Fos upregulate SOX7 promoter activities in SW480 cells (Figure 4E).

DISCUSSION

The primary purpose of this study was to shed insights into the complex mechanistic action of aspirin in colorectal cancer inhibition and prevention. The results of the study show that SOX7 is upregulated by aspirin, and that SOX7 is involved in aspirin-mediated growth inhibition of human colorectal cancer SW480 cells. Moreover, we provided evidence that aspirin induces SOX7 expression through the p38 MAPK pathway.

Recently, aspirin and related NSAIDs have attracted considerable research attention as the compounds that might be of potential benefit in the chemoprevention of cancer[2]. However, the molecular mechanisms by which aspirin and related NSAIDs inhibit tumor formation and growth have largely remained unsolved; and hence the utilization of these compounds in cancer therapeutics is still a substantially disputed issue. One potential mechanism underlying the action of NSAIDs involves the inhibition of COX activity[5]; but there is evidence that in COX negative colorectal cancer SW480 cells, aspirin is also able to inhibit the growth of the cells[8]. Goel *et al*[30] reported that aspirin could act through COX-independent mechanisms that resulted in an increased expression of DNA mismatch repair proteins and subsequent apoptosis in SW480 cells. Our previous data suggested that the *SOX7* gene may play a crucial role in colorectal cancer development as a tumor suppressor[27]. The data from the present study show that SOX7 is upregulated by aspirin in SW480

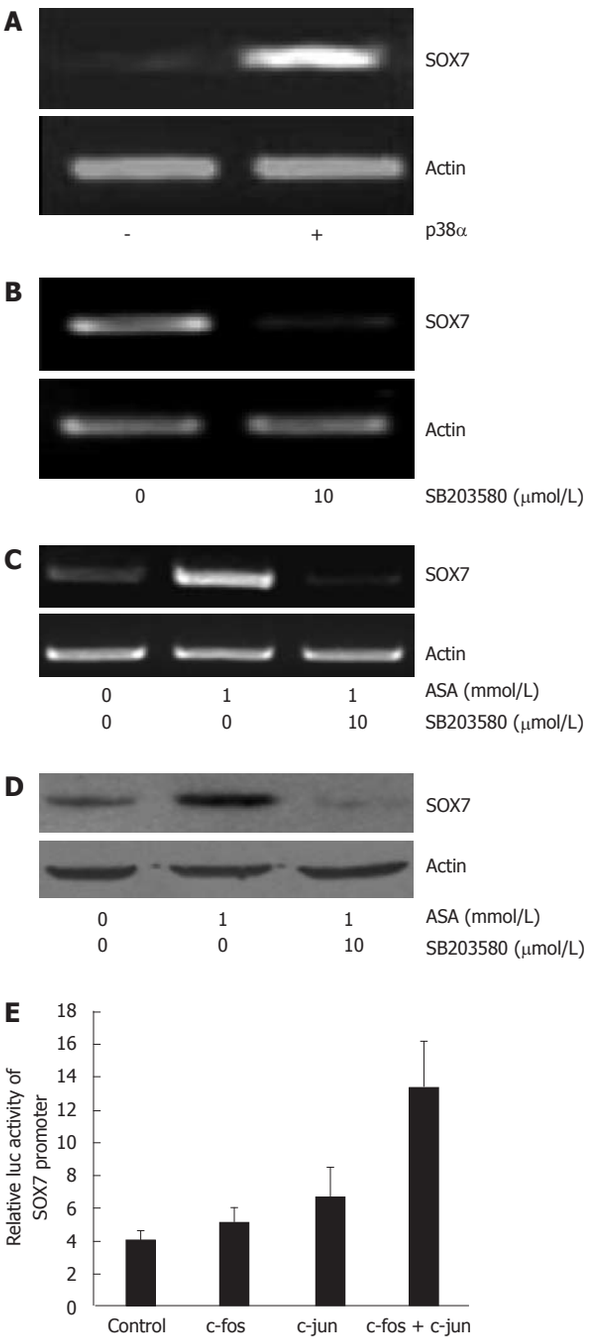

Figure 4 Role of the p38MAPK pathway in aspirin-induced SOX7 expression. A: HEK 293T cells were transfected with p38α expression vector; reverse transcription-polymerase chain reaction (RT-PCR) was used to detect SOX7 mRNA level 30 h after transfection; B: SW480 cells were treated with 10 μmol/L SB203580 for 30 h and SOX7 mRNA level was measured by RT-PCR; C: SW480 cells were treated with aspirin or aspirin combined with SB203580 for 30 h and SOX7 mRNA level was measured by RT-PCR; D: Cells were treated as in Figure 4 and SOX7 protein level was detected by Western blot; E: SW480 cells were transfected with pGL3-SOX7-luc plasmid and various expression vectors as indicated for 24 h and relative luciferase activity was examined. ASA: Aspirin.

cells and that it is involved in aspirin-mediated growth inhibition of SW480 cells (Figures 2 and 3), implying that SOX7 may be a target for aspirin in COX-independent cancer cells.

The p38 MAPK pathway plays pivotal roles in regulation of inflammation, proliferation, and cell death. Activation of the p38 pathway is generally mediated by conditions of cell stress and culminates in phosphorylation of p38 on a conserved regulatory domain by the upstream kinases MKK3 and MKK6[31]. In COX negative colorectal cancer SW480 cells, aspirin can activate the p38 MAPK pathway[24]. However, the mechanisms that are involved in this process need to be future investigated. Moreover, in the process of mouse preimplantation development, inhibition of the p38 MAP kinase pathway resulted in the suppression of SOX7 expression, implying that SOX7 may be a target gene of the p38 MAPK pathway[32]. Our experiments in this study indeed prove that the chemical inhibition of p38 substantially abrogates the upregulation of SOX7 upon aspirin treatment (Figure 4). These results indicate that SOX7 is regulated by aspirin and the p38 MAPK pathway in COX negative colorectal cancer cells.

The AP1 transcription complex consists of homodimers and heterodimers of the members from the fos (c-fos, fosB, fra-1 and fra-2) and jun (c-jun, junB and junD) families, and the complex activates target genes by binding with high affinity to particular DNA cis-elements, i.e., the 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (TRE)[33]. The AP1 activity is regulated at the transcriptional and post-translational levels by several external stimuli, mainly involving MAPK cascades[34]. Our Luciferase reporter assays showed that SOX7 promoter activity was moderately upregulated upon the overexpression of c-fos or c-jun, but markedly upregulated upon the simultaneous overexpression of both c-fos and c-jun (Figure 4E), indicating that c-fos and c-jun may form a dimer to regulate SOX7 promoter, though the details need to be further studied.

In summary, the experimental data presented in this report demonstrate that SOX7 is upregulated by aspirin and is involved in aspirin-mediated growth inhibition of human colorectal cancer SW480 cells. The regulation of SOX7 by aspirin is implemented through the p38MAPK pathway. Overall, this study will help to advance our understanding of the mechanisms of action of aspirin in inhibiting COX-independent colorectal cancer cells.

COMMENTS

Background

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide. Aspirin has been implicated to prevent CRC. The p38MAPK pathway is activated in aspirin-mediated apoptosis in a number of cancer cells. The sex-determining region Y-box 7 (Sox7) is a member of the high mobility group (HMG) transcription factor family, essential for embryonic development and endoderm differentiation.

Research frontiers

Aspirin is a nonsteroidal anti-inflammatory drug (NSAID), which has been implicated in preventing human colorectal cancer (CRC). However, the molecular mechanisms underlying the cancer preventive effects of NSAIDs are not well understood, and this has been an active issue of research interest. One most widely accepted mechanism for the anticancer effect of NSAIDs is the reduction of prostaglandin synthesis by inhibiting COX activity. To date, little is known about the COX-independent molecular targets of NSAID action in cancer cells.

Innovations and breakthroughs

The experimental data presented in this report demonstrate that SOX7 is upregulated by aspirin and is involved in aspirin-mediated growth inhibition of COX-independent human colorectal cancer SW480 cells. The regulation of SOX7 by aspirin is implemented through the p38MAPK pathway. Overall, this study will help to advance our understanding towards the mechanisms of action of aspirin in inhibiting COX-independent colorectal cancer cells.

Applications

By understanding how SOX7 is involved in aspirin-mediated growth inhibition of COX-independent human colorectal cancer cells, this study may represent a future gene target strategy for patients with COX-independent colorectal cancer.

Terminology

SOX7 is an HMG box transcription factor and has been implicated in parietal endoderm differentiation; recent reports have referred to the role of SOX7 in tumor suppression. mitogen-activated protein kinase (MAPK) is the mitogen-activated protein kinase superfamily, which includes the extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 MAPK, and is involved in mediating the processes of cell growth and death. Recent evidence indicates that the MAPK family proteins are important mediators of apoptosis induced by stressful stimuli.

Peer review

The authors examined the role of SOX7 in aspirin-mediated growth inhibition of COX2 negative SW480 cells, and found that SOX7 was regulated by aspirin and the p38 MAPK pathway in SW480 cells. The present results disclose the involvement of SOX7 in aspirin-mediated growth inhibition of COX2 negative cancer cells, providing a new insight to the mechanism as to how aspirin inhibits COX2 negative colorectal cancer.

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Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4928

World J Gastroenterol 2011 November 28; 17(44): 4928-4931
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Acute liver failure caused by drug-induced hypersensitivity syndrome associated with hyperferritinemia

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Author contributions: Miyazaki M wrote the paper; Tanaka M, Ueda A, Yoshimoto T and Nakamuta M managed medical treatment of this case; Kato M and Kotoh K analyzed the data; Takayanagi R supervised the paper.
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Received: April 9, 2011 Revised: August 1, 2011
Accepted: August 15, 2011
Published online: November 28, 2011

Abstract

Drug-induced hypersensitivity syndrome (DIHS) is a severe reaction usually characterized by fever, rash, and multiorgan failure, occurring 2-6 wk after drug introduction. It is an immune-mediated reaction involving macrophage and T-lymphocyte activation and cytokine release. A 54-year-old woman was diagnosed with rheumatic arthritis and initiated salazosulfapyridine by mouth. About 10 d later, she had a high fever, skin rash and liver dysfunction. She was admitted to hospital and diagnosed with a drug eruption. She was treated with oral prednisolone 30 mg/d; however, she developed high fever again and her blood tests showed acute liver failure and cytopenia associated with hyperferritinemia. She was diagnosed with acute liver failure and hemophagocytosis caused by DIHS. She was transferred to the Department of Medicine and Bioregulatory Science, Kyushu University, where she was treated with arterial steroid injection therapy. Following this treatment, her liver function improved and serum ferritin immediately decreased. We hypothesized that an immune-mediated reaction in DIHS may have generated over-activation of macrophages and T-lymphocytes, followed by a cytokine storm that affected various organs. The measurement of serum ferritin might be a useful marker of the severity of DIHS.

Key words: Acute liver failure; Drug-induced hypersensitivity syndrome; Ferritin; Human herpes virus 6; Macrophage

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Miyazaki M, Tanaka M, Ueda A, Yoshimoto T, Kato M, Nakamuta M, Kotoh K, Takayanagi R. Acute liver failure caused by drug-induced hypersensitivity syndrome associated with hyperferritinemia. *World J Gastroenterol* 2011; 17(44): 4928-4931 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4928.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4928

INTRODUCTION

Drug-induced hypersensitivity syndrome (DIHS) is one of the most severe drug eruptions, and is similar to toxic epidermal necrolysis and Stevens-Johnson syndrome[1]. It is characterized by maculopapular rash, high fever (≥ 38 ℃), hepatic dysfunction, leukocytosis with eosinophilia, and an increased number of atypical lymphocytes usually appearing 2-6 wk after exposure to certain drugs[2]. Although the pathology of DIHS remains unknown, in-

volvement of human herpes virus 6 (HHV-6) has recently been suggested[3,4]. DIHS is an immune-mediated reaction involving macrophage and T-lymphocyte activation and cytokine release associated with HHV-6 reactivation. However, there is no consensus on its etiology[5,6]. On the other hand, overactivation of macrophages is crucial for the development of other diseases, including hemophagocytic syndrome[7]. In patients with hemophagocytic syndrome, increased serum ferritin levels are thought to be secreted by activated macrophages[8]. However, to our knowledge, there are few reports that examine the correlation between the severity of DIHS and serum ferritin levels. In this report, we describe a case of acute liver failure (ALF) caused by DIHS associated with hyperferritinemia.

CASE REPORT

In January 2009, a 54-year-old woman presented to a clinic with morning stiffness in her hands. She was diagnosed with rheumatic arthritis and started treatment with oral salazosulfapyridine at 1 g/d on January 21. She developed a rash on her upper and lower extremities on February 2, with a fever of 37.6 ℃ on February 5. She presented to the clinic and discontinued salazosulfapyridine. Her blood test showed liver enzyme elevation [aspartate aminotransferase (AST), 50 U/L; alanine aminotransferase (ALT), 65 U/L]. However, her skin rash worsened. She was admitted to the Department of Dermatology, Kyushu Medical Center, National Hospital Organization on February 13. On admission, she had multiple areas of exudative erythema on her trunk and limbs. Laboratory data showed a white blood cell count of 14 700/μL with 9.0% eosinophils. AST and ALT levels were 59 U/L and 112 U/L, respectively.

She was diagnosed with drug eruption on the basis of clinical symptoms and the results of laboratory data, and treated with oral prednisolone 30 mg/d. After treatment, her liver function and rash improved gradually. However, she developed a high fever again on February 17. Her blood test showed ALF and cytopenia. Laboratory data on February 20 were as follows: white blood cell count of 6200/μL with 2.0% eosinophils; hemoglobin, 10.8 g/dL; platelet counts, 63 000/mm^3; AST, 1849 IU/L; ALT, 1623 IU/L; PT-INR, 1.79. Ultrasonography showed moderate ascites. A bone marrow aspiration demonstrated an increased number of macrophages with hemophagocytosis. The patient was diagnosed with ALF and hemophagocytosis caused by DIHS. She was transferred to the Department of Medicine and Bioregulatory Science, Kyushu University, for treatment of ALF.

Laboratory data on admission were as follows: total bilirubin, 5.8 mg/dL; AST, 2121 IU/L; ALT, 2231 IU/L; alkaline phosphatase, 1283 IU/L; γ-glutamyl transpeptidase, 736 IU/L; ferritin, 14 270 ng/mL; PT-INR, 1.79. Viral markers were negative for hepatitis A-C, Epstein-Barr virus, and cytomegalovirus. Serum anti-HHV-6 immunoglobulin G (IgG) titer was normal, but HHV-6 DNA was positive. She had multiple areas of exudative erythema on her trunk and limbs (Figure 1) and swelling of cervical lymph nodes. Abdominal computed tomography showed edematous change of the portal vein and moderate ascites (Figure 2).

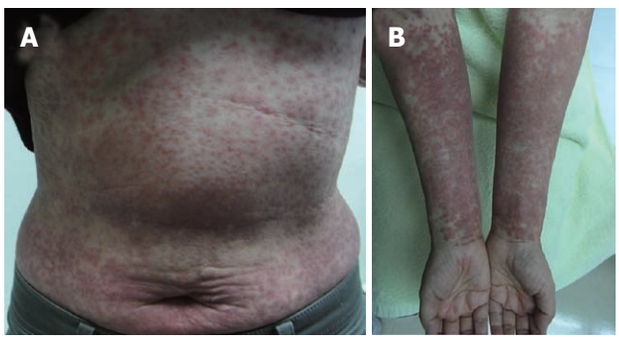

Figure 1 Multiple areas of exudative erythema were seen on admission. A: Body trunk; B: Forearm.

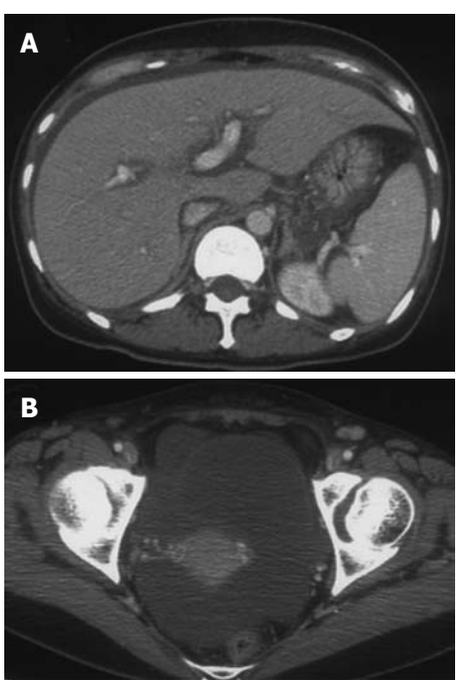

Figure 2 Abdominal computed tomography. A: Edema of portal vein; B: Moderate ascites.

Our diagnosis was DIHS based on high fever, rash, liver dysfunction, swelling of cervical lymph nodes, and eosinophilia. Hyperferritinemia in her blood samples led us to think that activated macrophages in the liver might play a key role in ALF. Therefore, we decided to use arterial steroid injection therapy to suppress macrophage activation seen in ALF[9].

A 5-frame catheter was inserted from the right femoral artery to the common hepatic artery and the tip of the catheter was set in the proper hepatic artery. After insertion of the catheter, 1000 mg of methylprednisolone was infused for 2 h per day. We also performed plasma exchange therapy on the first day of admission, because

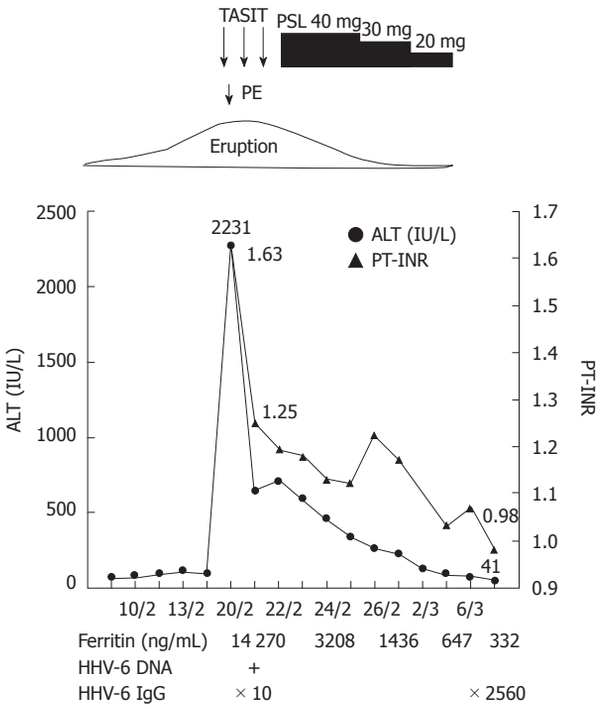

Figure 3 Clinical course of the patient. PE: Plasma exchange, TASIT: Transarterial steroid injection therapy.

the level of PT-INR was decreased. The arterial steroid injections were continued for 3 d, and the catheter was removed just after injection on the third day. Following arterial steroid injection therapy, her liver function improved immediately (Figure 3). Laboratory data on the fourth day of admission were as follows: AST 180 IU/L; ALT 597 IU/L; PT-INR, 1.18. After arterial steroid injection therapy, we started treatment with oral prednisolone at 40 mg/d and gradually decreased the dose. When liver dysfunction resolved, she was discharged from hospital (on March 3). In a serum sample taken 2 wk after hospitalization, anti-HHV-6 IgG titer had increased from 1:10 to 1:2560. She was diagnosed with typical DIHS according to criteria proposed by the Japanese Consensus Group; the patient had 7 items.

DISCUSSION

It is well-known that many patients with DIHS suffer liver injury and some progress to fatal liver failure. Until now, however, there has been no explanation of what causes ALF in some patients. Recently, it has been widely accepted that reactivation of herpes viruses such as HHV-6 and Epstein-Barr virus play a key role in the development of DIHS[3,4]. The question then becomes whether the reactivation of these viruses also contributes to the progression to ALF.

Hashimoto *et al*[10] evaluated clinical symptoms in 100 patients with DIHS and found that those with increasing serum anti-HHV-6 IgG titers suffered from severe organ involvement and a prolonged course of illness. In addition, they noted that flaring of symptoms, such as fever and hepatitis, was closely related to HHV-6 reactivation. However, none of the subjects in the study had ALF. In an investigation of case reports on the progression to ALF from DIHS, none indicated a correlation between viral reactivation and the development of ALF.

Since DIHS is triggered by some drugs, we should evaluate the possibility of drug-induced reactions that might directly harm hepatocytes and cause ALF. To our knowledge, there is no evidence that ALF is easily caused by specific drugs or in a dose-dependent manner. Therefore, we believe that the drugs causing DIHS do not directly injure numerous hepatocytes.

Some authors, speculating on the mechanism of progression to ALF, have indicated that DIHS develops through an immune-mediated reaction involving macrophages and T-lymphocytes[11]. In the last decade, several studies have suggested that activated intrahepatic macrophages play a key role in the development of ALF[12,13]. We also observed over-activation of intrahepatic macrophages in most of the patients with ALF in Japan[14]. One of the findings supporting this hypothesis is the markedly elevated serum ferritin concentration in those patients.

The patient presented in this report also had a markedly elevated serum ferritin concentration of over 20 000 ng/mL. One hypothesis is that intrahepatic over-activation of macrophages contributes to the development of ALF from DIHS as well as other etiologies. However, none of the past reports describing ALF from DIHS have referred to the elevation of serum ferritin concentration. To evaluate this hypothesis, cohort studies have focused on the correlation between the clinical course of patients with ALF from DIHS and the factors reflecting immune-mediated reactions.

Patients with DIHS can suffer not only from liver failure, but also various patterns of multiple organ failure, however, it remains unclear what induces these complications. We have hypothesized that an immune-mediated reaction in DIHS might generate over-activation of macrophages and T-lymphocytes, followed by a cytokine storm that affects various organs. In fact, this patient had elevated serum cytokines on admission (IL-6 10.8 pg/mL; IL-10 21 pg/mL). Such an immune reaction in bone marrow would result in pancytopenia, and liver involvement would cause liver failure. Both of these were seen in our patient.

It is well known that corticosteroids can suppress the activity of macrophages[15,16]. Therefore, it seems appropriate to use high-dose corticosteroids for DIHS. However, past reports show that corticosteroids are not always effective for ALF from DIHS. We speculate that intravenous injection of corticosteroids could be ineffective because the disturbance of hepatic microcirculation would prevent the drug from diffusing to the whole liver. Even in such a situation, injection *via* hepatic artery might be a useful option.

In this case report, we described a patient with ALF caused by DIHS associated with hyperferritinemia. In conclusion, we speculate that activated macrophages and

a cytokine storm were associated with DIHS. The measurement of serum ferritin might be a useful marker of the severity of DIHS.

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

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i44.4932

World J Gastroenterol 2011 November 28; 17(44): 4932-4936
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Pneumatosis cystoides intestinalis

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Received: January 13, 2011 Revised: April 19, 2011
Accepted: April 26, 2011
Published online: November 28, 2011

Abstract

Pneumatosis cystoides intestinalis (PCI) is a rare condition that may be associated with a variety of diseases. The presenting clinical picture may be very heterogeneous and represent a challenge for the clinician. In the present paper we describe both a common and an uncommon clinical presentation of PCI and review the pertaining literature. Our cases confirm that, apart from asymptomatic cases, the clinical presentation of PCI may be widely different and suggest that a new onset of stipsis might be the presenting symptom. Diagnosis might be suggested by a simple X-ray of the digestive tract showing a change in the characteristics of the intestinal wall in two-thirds of these patients. However, one third of the patients do not have a suggestive X-ray and require a computed tomography (CT) scan/nuclear magnetic resonance that may reveal a thickened bowel wall containing gas to confirm the diagnosis and distinguish PCI from intraluminal air or submucosal fat. CT also allows the detection of additional findings that may suggest an underlying, potentially worrisome cause of PCI such as bowel wall thickening, altered contrast mucosal enhancement, dilated bowel, soft tissue stranding, ascites and the presence of portal air. Our results also point out that clinicians and endoscopists should be aware of the possible presentations of PCI in order to correctly manage the patients affected with this disease and avoid unnecessary surgeries. The increasing number of colonoscopies performed for colon cancer screening makes PCI more frequently casually encountered and/or provoked, therefore the possible endoscopic appearances of this disease should be well known by endoscopists.

Key words: Pneumatosis cystoides intestinalis; Pneumoperitoneum; Treatment; Hyperbaric oxygen; Endoscopy

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Azzaroli F, Turco L, Ceroni L, Sartoni Galloni S, Buonfiglioli F, Calvanese C, Mazzella G. Pneumatosis cystoides intestinalis. *World J Gastroenterol* 2011; 17(44): 4932-4936 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i44/4932.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i44.4932

INTRODUCTION

Pneumatosis cystoides intestinalis (PCI) is a rare disease characterized by the presence of gaseous cysts containing nitrogen, hydrogen and carbon dioxide[1] in the intestinal wall that may be iatrogenic[2-5] or associated with a wide variety of conditions[6-9].

In particular, the cysts are located beneath the serosa and mucosa of the intestine with an increase, in recent years, of cases of colonic localization due to an increase in the number of examinations with barium and colonoscopies[10].

Jamart[11] in a study of 919 cases in 1979 found a prevalence of 42% for ileal localization, and 36% for the colon, in the remaining 22% of cases both the small and the large intestine were involved.

The exact etiology of the disease is still unknown. PCI may appear in association with ileal surgery[12], colonoscopies[5], chronic pulmonary disease[13], connective tissue disorders[14] and ingestion of sorbitol[15] or lactulose[16].

Various theories have been proposed: mechanical, bacterial and pulmonary. According to the mechanical theory, the bowel gas is pushed through a mucosal defect into lymphatic channels and is then distributed distally by peristalsis[17]. This may happen secondarily to a bowel obstruction that may be caused by trauma, surgery and colonoscopy leading to increased intraluminal pressure[18] and this could explain the association between these maneuvers and PCI. However this theory does not explain the high content of hydrogen present in the cysts[19].

The bacterial theory proposes that submucosal localization of fermenting *Clostridia* and *Escherichia Coli* leads to the production of gas which is retained by the submucosa and lymphatic channels. In fact, in animal experiments the introduction of bacteria in the gut wall by injection causes the pneumatosis and these cysts have a high content of hydrogen[13]. This theory is also supported by the resolution of pneumatosis with the use of metronidazole for bacterial overgrowth[20].

The pulmonary theory is demonstrated in patients with asthma and chronic bronchitis and argues that the gas freed by the rupture of the alveoli, travels through the mediastinum into the retroperitoneal space and then comes through the perivascular spaces in the intestinal wall[21].

Some recent reports[22] show an association between PCI and treatment with alpha-glucosidase inhibitor. The explanation would be the fermentation of carbohydrates by the intestinal bacterial flora with production of intestinal gas. The absorption of these carbohydrates is inhibited by αGI. Here we describe two cases of pneumatosis cystoides intestinalis.

CASE REPORT

Case 1

A 49 years old male (S.A.) presented to the gastroenterology outpatient clinic for abdominal pain. The pain was crampy and diffuse with no clear localization in the abdomen and had no clear relationship with meals or evacuation. Bowel habit was characterized by chronic constipation; the abdomen showed no relevant physical findings, in particular there was no palpable mass. The patient also suffered from a chronic obstructive pulmonary disease and had finger clubbing. Routine biochemical examinations were within normal values as well as inflammation indices. The physician prescribed a colonoscopy revealing melanosis coli, a sessile polyp 2 cm in diameter and two sessile formations with a large base and a reddened but regular overlying mucosa (Figure 1). The formations appeared soft when touched with a closed biopsy forceps and collapsed when biopsied suggesting the presence of air. The endoscopist performed multiple biopsies along the colon. The pathologist described the presence of optically empty spaces in the biopsies confirming the hypothesis of pneumatosis cystoides intestinalis. The patient was then referred to the pneumologist for oxygen therapy. At a subsequent colonoscopy the air cysts appeared reduced in volume and the patient referred pain reduction to the endoscopist. The patient did not present to subsequent control visits.

Case 2

N.F. 44 years old presented to the GI Unit for the re-evaluation of a known celiac disease. Despite adequate gluten free diet the patient complained of a worsening of his symptoms dominated by stipsis and abdominal distension. The patient suffered from recurrent subocclusive episodes and referred recent tetanic crisis from hypocalcemia on treatment with calcium and vitamin D. Biochemical analysis on admission revealed a megaloblastic anemia related to a Vitamin B12 deficiency and hypomagnesemia. Physical examination was consistent with abdominal distension and revealed hyperreflexia of the extremities. The patient had no fever nor any other remarkable finding.

The first postulated hypothesis, based on patient history was the complication of a celiac disease with intestinal lymphoma. A plain X ray of the abdomen revealed distension caused by dilatation of colonic loops leading to diaphragm elevation. The patient was then prescribed an upper GI endoscopy and an abdomen computed tomography (CT). The esophagogastroduodenoscopy revealed a normal endoscopic appearance of the mucosa and orientated biopsies were done in the distal duodenal mucosa. Histological examination revealed normal villi with a mild, non significant, lymphocytic infiltrate (below 25%) consistent with a celiac disease in remission responding to gluten free diet. The abdomen CT scan showed a complex picture including fecal stasis in the colon, severe distension of the large bowel with the presence of free air under the diaphragm (Figure 2A), and small air bubbles in the rectum wall (Figure 2B). Despite the radiologic picture, the patient was feeling well, with distended but tractable abdomen and no Blumberg sign. Therefore, the patient was kept fasting and received liquids and antibiotics iv. Subsequent radiologic evaluation of the small and large bowel, using gastrografin as contrast media, revealed no perforation suggesting that the free air might have come from the rupture of a subserosal air cyst. The patient then started hyperbaric therapy (5 treatments of 90 min: 2.5 atmospheres, 75 min of oxygen respiration divided in 3 cycles) with prompt amelioration of bowel movements and subjective feeling. The patient became asymptomatic and apyretic with normal bowel movements and was dismissed with antibiotic therapy.

DISCUSSION

There is no characteristic clinical presentation of pneumatosis. Patients may be asymptomatic or complain of

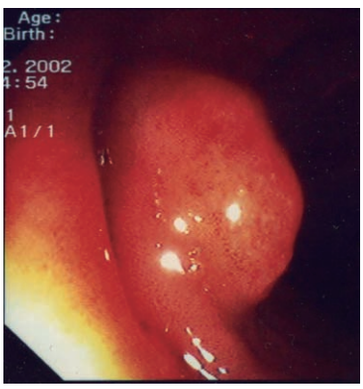

Figure 1 This photograph shows a typical endoscopic appearance of a larger cysts with a reddened overlying mucosa.

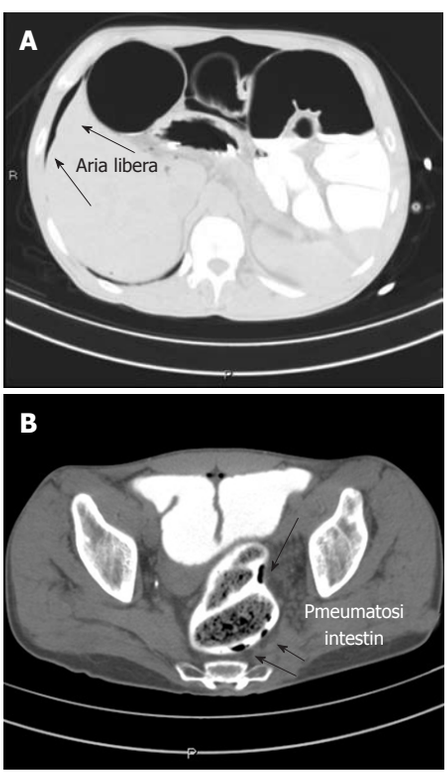

Figure 2 Computed tomography scan image. A: Presence of free air (arrows) in the abdomen; B: Presence of air in the bowel wall (arrows).

pain and abdominal distension, diarrhea and rectal blood loss with a mortality rate that may reach 75%[23]. Apart from the cases associated with chronic intestinal pseudo-obstruction[20,24,25], the majority of cases reported in the literature present with diarrhea; in the present manuscript we described two cases of PCI that presented with stipsis. The first was diagnosed after a colonoscopy that put the suspect of PCI, while the second required a CT scan because of the atypical presentation and the misleading anamnesis. In fact, the history of celiac disease lead the clinicians to hypothesize a complication of the pre-existent disease more than the onset of a new pathology. It is difficult to say whether the motility defects are a cause or a result of the pathologic condition and we are not aware of any longitudinal study evaluating such a question. However, on a purely hypothetical basis, it seems more reasonable to think that motility defects are secondary to PCI or to underlying pathological process that may have lead to PCI (i.e., ischemia, diverticular disease, *etc.*).

Colonoscopy is frequently requested to exclude colonic lesions. The endoscopic appearance of PCI is typically dual: multiple white small cysts coupled to a sub-atrophic mucosa or larger cysts (up to 3 cm) with a reddened overlying mucosa[26]. The cysts usually collapse when biopsied. Nowadays, given the increasing number of colonoscopies performed because of the colon cancer screening programs, the endoscopists should be aware of the endoscopic appearance of this rare pathology. In fact, some patients may be asymptomatic and in such cases the clinical suspect may rely on the endoscopist performing the procedure. In our case 1, the endoscopist cautiously biopsied the cyst because of his unusual appearance that was not suggestive of a typical polyp. This allowed a confirmation of the suspect and avoided an unnecessary snare polypectomy with the related costs and complications.

A simple X-ray of the digestive tract may show a change in the characteristics of the intestinal wall in two-thirds of these patients leading to further investigations. However, one third of the patients do not have a suggestive X-ray and require a CT scan/magnetic resonance imaging, showing a thickened bowel wall containing gas to confirm the diagnosis[27]. Suggestive images on plain radiography comprise different pattern of radiolucency: linear, small bubbles or collection of larger cysts[27]. CT is more sensitive than plain radiography in distinguishing PCI from intraluminal air or submucosal fat. In fact, CT more easily visualizes the presence of air in the bowel wall. Furthermore, CT allows the detection of additional findings that may suggest an underlying, potentially worrisome cause of PCI, i.e., bowel wall thickening, altered contrast mucosal enhancement, dilated bowel, soft tissue stranding, ascites, and the presence of portal air[28].

The intestinal pneumatosis may experience various complications, in particular, Goel *et al*[29] described the complications of pneumatosis of the small intestine which may be intestinal or extra-intestinal. Intestinal complications are obstruction caused by the cysts (i.e., fecal impaction) and perforation from stercoral ulceration. The extra-intestinal complications are adhesions or compression of adjacent structures by large masses of cysts.

For the resolution of these complications surgical treatment is often required because sometimes we have a picture of pneumoperitoneum due to rupture of cysts.

To determine the need for surgical therapy Knechtle *et al*[23] found a correlation between the clinical presentation, the need for surgery and the final outcome. It is necessary to evaluate six physical parameters, like pain, diarrhea, fever, tenderness, rectal blood loss and hypotension, and their severity coupled to clinical laboratory tests including white blood cell count, aspartate aminotransferases, alanine aminotransferases, alkaline phosphatase, pH, bicarbonate, lactic acid and amylase.

Surgical therapy is still a second-line therapy, chosen especially for complications, the first approach is oxygen therapy. It is also our opinion that the clinical decisions should not rely only on the radiologic picture of pneumoperitoneum, but should be coupled to the clinical symptoms (i.e., the positivity of the Blumberg sign). In fact, when the mucosa is intact surgery may be avoidable as in our case 2.

The rationale of oxygen treatment is based on increasing partial pressure of oxygen in blood and thus increasing the pressure gradient of the gas in the cysts. Cysts release gases contained within them and refills with oxygen which is then metabolized leading to resolution[26].

Oxygen therapy can be made through humidified oxygen administered by Venturi mask (6 L/min) or nasal cannula (4 L/min). However, treatment with oxygen at high doses can be toxic. The patient may experience a narcotic effect and therefore lung function should be monitored closely (during therapy) by measuring the vital capacity, daily blood gas estimations and chest radiography. A decrease in lung vital capacity can be a early parameter of oxygen toxicity[30].

To reduce the duration of oxygen administration hyperbaric oxygen can be used at a pressure of 2.5 atmosphere for up to 2 h a day [31]. To decrease the recurrence rate oxygen therapy should be continued until two days after the disappearance of cysts[32].

In conclusion, our cases confirm that the clinical presentation of PCI may be very heterogeneous and suggest that a new onset of stipsis might be the presenting symptom. Furthermore, it should be taken into account that the patients may also be totally asymptomatic. The clinicians and the endoscopist should be aware of the possible presentations of PCI in order to correctly manage the patients affected with this disease and avoid unnecessary surgeries. It is possible that with the increasing number of colonoscopies performed for colon cancer screening PCI is casually encountered and/or provoked, therefore the possible endoscopic appearances of this disease should be known.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2011 November 28; 17(44): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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World J Gastroenterol 2011 November 28; 17(44): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Events Calendar 2011

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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Online Submissions: http://www.wjgnet.com/1007-9327office
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World J Gastroenterol 2011 November 28; 17(44): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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Name of journal

World Journal of Gastroenterology

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

Indexed and Abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifer, and Directory of Open Access Journals. ISI, Thomson Reuters, 2010 Impact Factor: 2.240 (35/71 Gastroenterology and Hepatology).

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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 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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

Patent (list all authors)

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

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