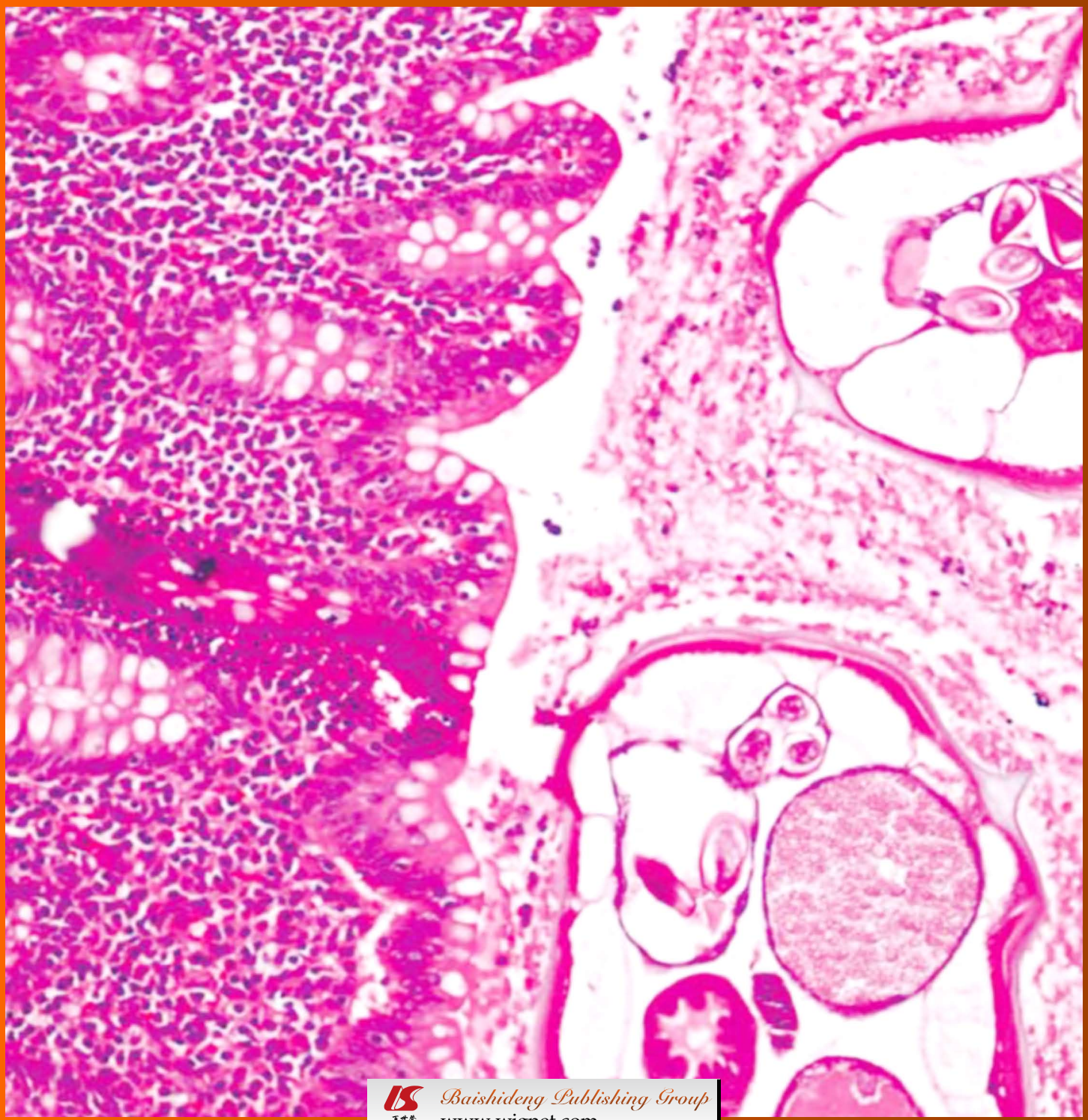
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2011 April 21; 17(15): 1927-2062



Baishideng Publishing Group
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World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

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AIM AND SCOPE		*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries. The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.
FLYLEAF	I-VII	Editorial Board

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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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
Beijing Baishideng BioMed Scientific Co., Ltd.,
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No. 62 Dongsihuan Zhonglu, Chaoyang District,
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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
April 21, 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,
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INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

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http://www.wjgnet.com/1007-9327office

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.1927

World J Gastroenterol 2011 April 21; 17(15): 1927-1938
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

An overview of occult hepatitis B virus infection

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Received: November 8, 2010 Revised: January 18, 2011
Accepted: January 25, 2011
Published online: April 21, 2011

Abstract

Occult hepatitis B virus (HBV) infection (OBI), alternatively defined as occult hepatitis B (OHB), is a challenging clinical entity. It is recognized by two main characteristics: absence of HBsAg, and low viral replication. The previous two decades have witnessed a remarkable progress in our understanding of OBI and its clinical implications. Appropriate diagnostic techniques must be adopted. Sensitive HBV DNA amplification assay is the gold standard assay for detection of OBI. Viral as well as host factors are implicated in the pathogenesis of OBI. However, published data reporting the infectivity of OBI by transfusion are limited. Several aspects including OBI transmission, infectivity and its relation to the development of chronic liver diseases and hepatocellular carcinoma have to be resolved. The aim of the present review is to highlight recent data on OBI with a focus on its virological diagnosis and clinical outcome.

Key words: Hepatitis B virus; Occult infection; Occult hepatitis B virus infection; Occult hepatitis B; Chronic liver disease; Hepatocellular carcinoma; Hepatitis B surface antigen

Peer reviewers: Damiao Carlos Moraes Santos, DCM, PhD, Department of Quality Control (DEQUE), Bio-Manguinhos - FIOCRUZ, Avenida Brazil, 4365, Manguinhos - Rio de Janeiro, 21045-900, Brazil; Dr. Jin-Town Wang, Professor, Department of Microbiology and Internal Medicine, National Taiwan University, College of Medicine, 1, Sec 1, Jen-Ai Rd, Taipei 10016, Taiwan, China

Said ZNA. An overview of occult hepatitis B virus infection. *World J Gastroenterol* 2011; 17(15): 1927-1938 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/1927.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.1927

INTRODUCTION

Hepatitis B virus (HBV) remains a major public health problem worldwide[1]. Among many transmission routes, transfusion is the one that should be prevented. Implementation of hepatitis B surface antigen (HBsAg) in routine screening of blood donors in the early 1970s has greatly enhanced transfusion safety. The incidence of transfusion-transmitted hepatitis B has been steadily reduced over the last four decades[2]. However, it was demonstrated that HBV transmission by blood components negative for HBsAg can still occur[3] and HBV transmission remains the most frequent transfusion-transmitted viral infection[4-6]; thus, the term occult hepatitis B virus infection (OBI) was introduced. OBI is simply defined as serologically undetectable hepatitis B surface antigen (HBsAg-ve), despite the presence of circulating HBV DNA[7,8]. OBI was reported for the first time almost 30 years ago in a case report of HBV infection through blood transfusion by an antibody to hepatitis B core antigen (anti-HBc) only positive donor[9]. The residual risk of HBV transfusion transmission is mainly related to blood donations negative for HBsAg that have been collected either during the pre-seroconversion "window period" (WP), defined as the time between infection and detection of a viral antigen or antibody marker, or during the late stages of infection[1]. Additionally, OBI has high significance in management of bone marrow and organ transplantations[10-13]. Implementation of HBV DNA screening has the potential to significantly reduce the WP and to reveal OBI or HBV carriage[14].

Allain[15] reported OBI in several clinical contexts including: (1) recovery from past infection indicated by the presence of hepatitis B surface antibody (anti-HBs); (2) chronic hepatitis with surface gene escape mutants that are not recognized by current assays; (3) chronic carriage without any marker of HBV infection other than HBV DNA (referred to as "seronegative"); and (4) most commonly in endemic areas, chronic carriage stage with HBsAg too low to be detected and recognized by the presence of anti-HBc as the only serological marker (referred to as "anti-HBc alone" or "isolated anti-HBc")[15].

DEFINITION OF OCCULT HEPATITIS B INFECTION

Several definitions for OBI have been proposed by many authors. Bremer *et al*[16] emphasized that the term "occult hepatitis B virus infection" has been introduced to describe a pattern with the presence of replication-competent HBV DNA in the liver but without detectable HBsAg in the serum. This often occurs after progressive disappearance of HBsAg in the years after infection[17] and persists in low-level carriers[14]. Early phase of HBV infection before appearance of HBsAg is not considered OBI, as the infection becomes eventually non-occult[18].

A more specific definition was provided by Allain[15] in 2004, who defined OBI as the presence of HBV DNA without HBsAg, with or without the presence of HBV antibodies outside the acute phase window period. This is in accordance with findings by Gerlich *et al*[19], who identified two blood donors whose donations tested HBsAg- and HBV DNA-negative, but transmitted HBV. Both subsequently developed HBsAg and acute hepatitis. It was confirmed that such cases are transient OBI and should not be considered as true OBI. A true OBI remains HBsAg-negative during the entire course[19]. Nevertheless, a 2008 international workshop on occult hepatitis B virus (HBV) infection (OBI), endorsed by the European Association for the Study of the Liver (EASL)[20], as well as The Taormina Consensus Conference in 2008, defined "OBI" as the "presence of HBV DNA in the liver of individuals testing HBsAg-negative with currently available assays"[10] and introduced a cutoff value for serum HBV DNA (< 200 IU/mL). Therefore, cases whose serum HBV DNA levels are comparable to those with different serologically evident (overt) HBV infection are generally due to infection with HBV escape mutants and should be labeled as "false" OBI[10]. As confirmed by Hollinger *et al*[21], this definition implies that infectious viral clones may be present. However, the detection of HBV DNA does not always correspond to infectivity or to the number of HBV progeny viruses released from hepatocytes; therefore, the authors suggested a more comprehensive term "occult hepatitis B (OHB)"[21] rather than OBI. Moreover, nosocomial sources should be carefully excluded before speculating that blood donors with OBI were involved in HBV viral disease transmission[22].

POSSIBLE MECHANISMS OF OBI

Several possible mechanisms have been hypothesized for the pathogenesis of OBI and the condition is probably multifactorial. Both host and viral factors are important in suppressing viral replication and keeping the infection under control[21,23,24]. The majority of OBI cases are secondary to overt HBV infection and represent a residual low viremia level suppressed by strong immune response together with histological derangements occurring during acute or chronic HBV infection[25]. It was previously suggested that long-term maintenance of an active anti-viral T cell response several years after clinical recovery from acute hepatitis B could be important, not only for protection against reinfection, but also for keeping the persisting virus under tight control where detection of minute amounts of virus in some recovered subjects was confirmed[26]. Also, in a study to characterize the features of the HBV-specific T-cell response in patients with OBI, 2 different profiles were defined. Anti-HBc-positive patients showed a T-cell response typical of protective memory, suggesting that this condition represents a resolved infection with immune-mediated virus control. In contrast, HBV-specific T cells in anti-HBc-negative patients did not readily expand, suggesting the possibility of a low-dose infection insufficient to allow maturation of protective memory[27]. Additional mechanisms not related to the host response were also extensively studied by many authors, where it was shown that the low level of viral replication was a result of the presence of defective interfering particles or of mutations in transcription control regions or the polymerase domain leading to decrease in HBV DNA replication and HBsAg expression[21,24,28-30].

Humoral and cellular immune pressure on the HBV envelope proteins are major mechanisms generating OBI. Amino acid substitutions are significantly concentrated in the immunologically active parts of the Pre-S/S proteins affecting both cellular CD8 T-cell epitopes and B-cell neutralizing major hydrophilic region epitopes[31]. Escape mutation is one mechanism which also leads to decreased reactivity in HBsAg detection assays[32]. This is confirmed by Gerlich *et al*[19]. van Hemert *et al*[33] in 2008 proposed an evolutionary scenario for occult HBV infection. They identified a novel RNA splicing event (deleting nucleotides 2986-202) that abolishes surface protein gene expression without affecting polymerase, core or X-protein related functions. This 2986-202 splicing generates intracellular virus particles devoid of surface protein, which subsequently accumulate mutations due to relaxation of coding constraints. Such viruses are deficient in autonomous propagation and cannot leave the host cell until it is lysed[33].

Masking of HbsAg by HbsAg-anti-HBs immune complexes is another postulated mechanism for the development of OBI[34,35]. Also, coinfection with hepatitis delta virus or hepatitis C virus (HCV) which results in down-regulation of HBV replication and a reduction in HBsAg synthesis has been reported[21]. Sagnelli *et al*[36] showed an inhibitory effect of HCV on HBV replication. This inhibitory activity of HCV on HBV replication has also

been reported by other investigators in a follow-up study of 6 years duration, where it was shown that the rate of HBsAg clearance is 2.5 times higher in HBsAg/anti-HCV-positive cases than in those with HBV infection alone; it was suggested that HCV is the most important hepatotropic virus that enhances HBsAg clearance in chronic hepatitis B[37]. The underlining molecular mechanism responsible for this suppressive effect has been extensively studied both *in vitro*[38] and *in vivo* studies[39]. Indirect mechanisms mediated by innate and/or adaptive host immune responses have also been postulated as being involved[40]. In this regard, we have recently studied the prevalence of occult HBV among children and adolescents with hematological diseases with or without HCV in an area of high endemicity of HCV infection. It was shown that HCV RNA was a significant predictor for OBI ($P < 0.05$), with an increased frequency of HBV DNA in those who were HBsAg-negative and HCV RNA positive (63.2%) compared with patients negative for HCV RNA (25%) ($P = 0.009$)[41].

Additional mechanisms for OBI have been thoroughly investigated, emphasizing that integration of viral sequence may alter HBsAg expression and decrease HBV replication[42]. Meanwhile, reduced HBV viremia may result from extra-hepatic HBV replication such as that takes place in peripheral blood mononuclear cells (PBMCs)[42]. Patients with long-standing abnormal results of liver function tests with unknown etiology may have HCV RNA or HBV DNA in their PBMCs in the absence of anti-HCV antibodies, HBV markers, serum HBV DNA and serum HCV RNA[43].

EVALUATION OF DIFFERENT OBI DIAGNOSTIC TECHNIQUES

Most OBIs are asymptomatic and would only be detected by systematic screening of large populations[7]. No published guidelines are provided up till now, categorizing those who should be screened for OBI. However, such investigations should be considered in the following situations: (1) HCV-infected patients with flares in viral replication and liver damage[44]; (2) infected patients becoming immune deficient mainly by receiving immunosuppressive regimens for various clinical conditions[7]; (3) screening of blood donations for immunocompromised recipients[41]; and (4) subjects with unexplained liver diseases. Candotti *et al*[1] further clarified that OBIs are mainly found in older donors, nearly 100% carry anti-HBc, and approximately 50% also carry anti-HBs, suggesting that OBIs occur largely in individuals having recovered from the infection but unable to develop a totally effective immune control[31].

Liver biopsy

Detection of HBV DNA in liver biopsy is the best way for diagnosis of OBI. However, liver biopsy tissue is not always available, and standardized and valid assays for detection of HBV DNA in liver tissue are not FDA approved[21]. A recent Italian study investigated the prevalence of occult HBV in the general population by examining 98 liver specimens from liver disease-free individuals who were HBsAg-negative, and detected HBV DNA in sixteen of them (16.3%); 10/16 (62.5%) were anti-HBc positive[20].

HBsAg testing

The main target for antibodies used in diagnostic tests is the major hydrophilic loop (MHL, amino acids 100-160) that contains the "a" determinant (amino acids 124-147) and is coded by the envelope (S) gene. The existence of mutations in this region could cause diagnostic failure[45]. Current HBsAg screening assays are enzyme immunoassays (EIAs), including enzyme-linked immunosorbent assays (ELISAs), and chemiluminescence immunoassays (CLIAs)[1]. These different assays have sensitivity ranging between < 0.1 and 0.62 ng of HBsAg per mL (1 ng/mL corresponds to approximately 2 IU/mL)[1,46,47]. Performance of commercial assays would be improved by the incorporation of OBI mutants in reagent development[32].

The course of HBV markers during the early phase of true OBI is not well known, where, in spite of transient strong HBV replication, much less HBsAg in the serum than the normal courses is shown[16]. This has been previously confirmed in a Japanese study by Yoshikawa *et al*[48], where 17 million donations were tested for occult infection, and 328 HBV DNA-positive donations were found. From 26 of these donors, sequential samples were examined for the dynamics of viral markers in acute HBV infection. Six of the 26 donors were infected with mutant viruses, and 3 of these 6 donors did not develop detectable HBsAg during the entire observation period, despite a moderately high viral load of 10^4 to 10^5 HBV DNA copies per mL. The authors concluded that HBV nucleic acid amplification test (NAT), even in minipool (MP) configuration, is more effective than HBsAg testing and capable of excluding infected donors in the pre- and post-HBsAg window periods[48].

A novel immunoassay that detects simultaneously HBV PreS1 and/or core-related antigens was developed and evaluated for its potential value for detecting HBsAg variants. The detection limits of the assay were 10 (2.9 ± 0.5) copies/mL (mean ± SD) for HBsAg-positive sera with different genotypes, and 10 (3.5 ± 1.2) copies/mL for HBsAg variants containing sera. The specificity of the assay was 99.9% (95% CI: 99.7-99.9, 4551 healthy individuals). The sensitivities were 93.9% (95% CI: 92.8-94.9), 59.3% (95% CI: 38.7-77.6) and 80% (95% CI: 44.4-97.5) in three independent groups which included: 2065 hepatitis patients, 27 patients with OBI and 10 HBsAg variants, respectively. In addition, a novel premature stop code mutation at position 112 of HBsAg was observed in two patients with chronic hepatitis B with different genotypes[49].

Anti-HBc testing

Serological profiling of HBV infection showed that OBI may be antibody (anti-HBc alone or together with anti-HBs) positive (seropositive OBI) or antibody negative

(seronegative OBI)[13]. The HBV DNA detection rate is highest in subjects who are anti-HBc-positive but anti-HBs-negative, and these individuals are more likely to be infectious[21].

Recently, Urbani *et al*[50] illustrated that the serological assay for the long-lasting antibody response to the highly immunogenic HBV core antigen (anti-HBc) represents a qualified candidate as a surrogate for DNA amplification, or for increasing overall sensitivity when assessing the risk of occult hepatitis in peripheral blood. The risk of occult hepatitis associated with anti-HBc seropositivity has been demonstrated extensively, and the presence of antibody response to HBc can be considered a sentinel marker of occult HBV infection[50].

In a recent review conducted by Candotti *et al*[1] in 2009[1], it was emphasized that approximately 90% of blood donors carrying anti-HBc also carry anti-HBs, indicating recovered HBV infection[51]. The remaining 10% are either false-positive anti-HBc due to poor assay specificity and the lack of confirmatory assays, or true anti-HBc (anti-core antigen alone)[1,52,53]. Anti-HBc only samples may originate either from recovered infections having lost detectable anti-HBs or from late stage chronic infections having lost detectable HBsAg[1]. Recent studies have confirmed the existence of occult HBV infection in samples with anti-HBc alone[54,55]. Nevertheless, low levels of HBV DNA were reported not only in anti-HBc alone positive blood donations but also in some blood units carrying low-level anti-HBs[1]. A serologic testing algorithm with anti-HBc followed by anti-HBs (anti-HBs $\geqslant$ 100 IU/L probably non-infectious) or implementation of highly sensitive HBV DNA screening are adopted in different countries; however, this is still an area of debate by many authors. In our recent study, OBI was detected in blood units from healthy volunteer blood donors showing adequate level of anti-HBs (under publication).

OBI is observed in anti-HBc-positive patients with chronic HBV infection following the decline of HBsAg to an undetectable level that is sometimes associated with the appearance of anti-HBs. This serological pattern occurs at a rate of 0.7%-1.3% per year and is associated with older age and hepatitis B e antibody (anti-HBe) reactivity[21,56-58]. In an experimental study to determine the relationship between anticore detection and the molecular status of virus replication in a primary woodchuck hepatitis virus (WHV) surface antigen (WHsAg)-negative infection or long after resolution of WHV hepatitis, it was shown that the long-term presence of anticore antibodies alone is a consequence of sustained restimulation of the immune system by virus nucleocapsid produced during low-level hepadnaviral assembly[59]. On the other hand, it was shown that about 20% of OHB sera are negative for all serological markers of HBV infection except HBV DNA[21].

HBV nucleic acid (DNA) testing

The gold standard test for detection of OBI is the amplification of HBV DNA[50]. At present, the optimal standard for diagnosis is the analysis of HBV DNA extracts from plasma performed by real-time, nested polymerase chain reaction (PCR) techniques[21]. False results of these assays could be avoided by choosing PCR primers that span at least three genomic regions of the HBV genome such as the S, X and core genes, and validation should require detection from at least two regions of the genome[20]. Unfortunately, this suggestion is not usually fulfilled, and only one segment of a region is amplified. The preferred lower limit of detection (LLOD) for HBV DNA is 5 IU/mL[21]. Some investigators prefer to repeat extraction and testing under the assumption that according to Poisson distribution, repeated testing increases the chances of detecting a low number of template sequences[7]. Nucleic acid testing (NAT) for HBV DNA detection that combines simultaneous detection of human immunodeficiency virus (HIV) RNA, HCV RNA, and HBV DNA ("multiplex" NAT assays) and use of an automated testing platforms have made HBV NAT blood screening feasible[1]. In order to standardize these newly developed assays, the World Health Organization International Standard for hepatitis B virus DNA (NAT)-based assays was created (code 97/750) with a potency of 10^6 IU/mL (500 000 IU/vial)[60].

Biswas *et al*[46] showed that pooled-sample NAT would reduce the WP by 9 to 11 days; and single-sample NAT would reduce the WP by 25 to 36 d, compared to currently licensed HBsAg tests[46]. This leaves WPs of 40-50 d and 15-34 d with minipool (MP) and individual donor (ID) HBV NAT, respectively[1]. As emphasized by Candotti *et al*[1], the ability of NAT to reduce the WP depends not only on the sensitivity of both the molecular and serological tests, but also on the sample volume (200 or 500 μL) as well as the dilution factor introduced by pooling samples, the prevalent HBV genotype at the location and the level of HBV endemicity[7,46,61-63]. Beyond shortening the WP, NAT screening, particularly in individual units, has uncovered a relatively large number of HBsAg-negative "occult" HBV infection or carriage[11,14].

OBIs are usually characterized by very low HBV DNA load in plasma (< 200 IU/mL)[1]. Detection of OBI requires assays of the highest sensitivity and specificity with a lower limit of HBV DNA detection of less than 10 IU/mL and < 0.1 ng/mL for hepatitis B surface antigen (HBsAg)[21].

Regarding estimation of HBV residual transfusion transmission risk, Candotti *et al*[1] in their recent review clarified that HBV DNA yield appears directly related not only to the analytical sensitivity and serum pool size used for the HBV NAT assay, but also to the analytical sensitivity of the HBsAg test used for screening and to the general HBV prevalence in the donor population. They further added that HBV NAT yields reported from countries with low, moderate, and high HBsAg prevalence range between 1:4 000 and 1:730 000[52,64-69], 1:4000 and 1:20 300[70-74], and 1:192 and 1:5200[51,75-80], respectively[1].

Role of Anti S

It is believed that occult HBV carriers without detectable

antibodies to the surface antigen could be infectious[45]. Indeed, Candotti *et al*[1] emphasized that the presence of anti-HBs following natural infection, vaccination, or passive immunoprophylaxis prevents *de novo* HBV infection in transplanted patients receiving anti-HBc positive livers[81-84]. Experiments in chimpanzees showed no HBV infection in animals transfused with blood from three anti-HBs positive human plasma samples, despite exposure to an HBV DNA dose known to be infectious in the absence of anti-HBs[85]. However, it has been reported by many authors that among individuals positive for anti-HBs, 0.5%-15% still tested positive for serum HBV DNA, though at a very low titer[3,86]. Countries such as Germany, Austria and Japan allow transfusion of units with anti-HBs titers higher than 100 IU/L[87].

CLINICAL SIGNIFICANCE

Continuous progress in molecular biology techniques has led to greater recognition and diagnosis of OBI. It has been reported in healthy blood donors, patients with chronic liver disease and patients with hepatocellular carcinoma (HCC)[21], in viral reactivation following immunosuppression, accidental transmission through transplantation, transfusion or experimental transmission to chimpanzees[42]. Therapy should be considered during reactivation and in cirrhotic settings[25].

As illustrated by Shi *et al*[88], a dynamic balance between viral replication and host immune response is pivotal to the pathogenesis of liver disease. Most HBV infections are spontaneously resolved in immunocompetent adults, whereas they become chronic in most neonates and infants who are at great risk of developing complications such as cirrhosis, chronic liver disease (CLD) and HCC. Those with chronic HBV infection may present in one of the four phases of infection: immune tolerance, immune clearance (HBeAg-positive chronic hepatitis B), inactive carrier state, and reactivation (HBeAg-negative chronic hepatitis B)[88].

OBI is a complex biological entity with possible relevant clinical implications, mainly related to the intrahepatic persistence of viral covalently closed circular DNA (cccDNA) and to a strong suppression of viral replication and gene expression[13]. Detection of virus-specific nucleic acid does not always translate into infectivity, and the occurrence of primer-generated HBV DNA that is of partial genomic length in immunocompetent individuals who have significant levels of anti-HBs may not be biologically relevant[21]. Several authors concluded that as a general rule, immune individuals who have recovered from acute hepatitis B have no clinical evidence of liver disease despite the detection of traces of HBV DNA in their blood, PBMC and/or liver decades later[20,21,23,26].

Cross-sectional studies across the spectrum of HBV infection have revealed a marked increase in OBI prevalence towards patients with cirrhosis or HCC[25,42,89]. However, data collected in Poland indicated that approximately 50% of OBIs occur in asymptomatic, apparently healthy blood donors carrying anti-HBs[70]. Levels of DNA and anti-HBs are variable[90].

OBI infectivity by transfusion

It is well known, and recently confirmed by Candotti *et al*[1], that the estimated residual risk of HBV transfusion transmission remains significantly higher than the risk of either HIV-1 or HCV. Whether residual risk estimates translate into true rate of infection is largely unknown since estimates are generally based on the simplification that all HBV DNA-containing donations are infectious[1].

All forms have been shown to be infectious in immunocompromised individuals, such as organ- or bone marrow-transplant recipients. In immunocompetent recipients, there is no evidence that anti-HBs-containing components (even at low titer) are infectious. Anti-HBc only, with HBV DNA, can be associated with infectivity, as can rare cases of HBV DNA without any serological HBV marker[14].

HBV transmission was previously reported from OBI donors who had circulating HBV DNA at a low level[74,91,92]. However, as reported by Candotti *et al*[1], in some cases units from WP and OBI donors were not infectious even though viral load ranging between < 20 and > 500 IU/mL (< 100 and > 2500 geq/mL) was transfused[86,91,93]. These authors emphasized that the lack of a clear relationship between infectivity and viral load in blood components may be related to immune factors affecting the susceptibility to infection in recipients. In addition, HBV infectivity is related to the amount of plasma transfused and the viral load in the product[1].

Few data regarding the infectivity of blood components or donated organs containing both anti-HBc and anti-HBs are available. Theoretically, if HBV particles are present in the peripheral blood of subjects with high-titer anti-HBs, the anti-HBs may neutralize the infectivity of the viral particles[3]. Nevertheless, an OBI carrier with anti-HBs was found to have transmitted HBV to two immunocompetent transfusion recipients[90]. Gerlich *et al*[94] reported five donors (4 genotype D, one genotype A2) with OBI, also carrying only anti-HBc, transmitting HBV to recipients. Candotti *et al*[1] examined the infectivity of HBV-containing blood products according to the immune status of recipients and concluded that: (1) WP and anti-HBs-positive and negative OBI units can transmit HBV; (2) the confirmed HBV transmission rate of WP-derived donations is higher than by occult carriers (81% *versus* 19%) but may be biased by the large number of Japanese cases identified, with a peculiar set of anti-HBc and DNA screening protocols[91]; (3) viral transmission can be associated with extremely low levels of HBV DNA in anti-HBc-positive only units (< 20 IU/mL) or blood collected during the very early phase of acute infection (eclipse phase) in which neither HBsAg nor HBV DNA is detectable[86,95]; (4) HBV DNA load is similar in infectious and non-infectious anti-HBc-positive donations, suggesting that viral load is not the only factor for infectivity; and (5) the presence of anti-HBs seems to largely protect from transmission[91,94], except in rare cases[1,90].

No transmission of HBV has ever been demonstrated in blood donors who developed anti-HBc and anti-HBs following acute hepatitis B[24]. Satake *et al*[91] in Japan found that no HBV infections occurred in 22 recipients of HBsAg-negative, HBV DNA-positive blood that contained anti-HBs compared to 10 HBV infections that occurred among 37 recipients (27%) of OHB units that were devoid of anti-HBs[21].

OBI in blood donors

It is generally admitted that pre-seroconversion WP infections are most likely to transmit HBV but transmission from occult HBV infection remains a debated subject[11]. Occult HBV is transmissible through blood transfusion in HBV-naive recipients[96]. Post-transfusion hepatitis B virus (HBV) infection still occurs, although its incidence has been found to be substantially reduced since the introduction of screening for HBsAg in blood donors[97]. A similar study was recently conducted in India and showed that a considerable number of HBV-infected donors remain undetected, if only HBsAg is used for screening[98].

Occult HBV in blood donors has a wide range of potential origin within the natural history of the infection. It may originate from previous infections with development of anti-HBs, but be accompanied by persistent, low-level, viral replication and/or escape mutants undetected by the HBsAg assays or healthy chronic carriage. The latter situation is mostly found with anti-HBc only. Over time, antibody markers may become undetectable leaving HBV DNA as the only marker of the infection[15].

A European study conducted by Candotti *et al*[31] confirmed that 91% of 77 donor samples of European origin were HBV DNA-positive/HBsAg-negative. Viral load ranged between unquantifiable and 5640 IU/mL (median 25 IU/mL).

A recent study conducted in Taiwan showed that in HBV hyperendemic areas, occult hepatitis B transfusion might not lead to HBsAg carriage or post-transfusion hepatitis. The risk of transfusion-transmitted HBV infection was probably lower than that in non-endemic areas because most recipients had already experienced HBV infection[96]. Infection of vaccinated individuals favors development of OBI, as was observed in 6 blood donors. HB vaccination may solve the problem of overt HBV infection but may favor OBI[19].

Addition of anti-HBc testing for donor screening, although leading to rejection of a large number of donor units, will definitely eliminate HBV-infected donations and help in reducing HBV transmission with its potential consequences, especially among the immunocompromised population[98].

OBI blood donors have very low HBV replication, and normal liver biochemistry and histology, conferring a favorable prognosis[99].

Donations carrying anti-HBc only and HBV DNA can be infectious and this is a threat where anti-HBc is not screened. Anti-HBc screening identifies most OBI but not all. HBV NAT needs either extreme sensitivity or to be performed on individual donations to eliminate HBV DNA-containing units[15]. Reduction of HBV residual risk depends upon developing more sensitive HBsAg tests, adopting anti-HBc screening when appropriate, and implementing HBV NAT, either in minipools or more efficiently in individual samples[1].

Liu *et al*[3] emphasized that anti-HBc screening has the potential to exclude the vast majority of OHBs, leaving only the probably rare cases with HBV DNA alone undetected. This approach, however, has two main drawbacks: it does not detect the seronegative WP infections; and most importantly, it would not be practical in most parts of the world where the prevalence of anti-HBc is > 10%, as too many otherwise healthy donors will be ineligible[3].

The transmission risk of OBIs is not well defined, although some cases of OBIs with anti-HBc only which were infectious by transfusion have been described[91,94]. HBV transmission by blood components from a single anti-HBs-positive OBI donation to two recipients was recognized and it was clearly illustrated that the neutralizing capacity of low-level anti-HBs is limited, reinforcing the validity of considering anti-HBs below 100 IU/L to be poorly protective from infectivity when HBV DNA is present[90]. Authors further emphasized that even in the presence of higher levels of anti-HBs in a severely immunodeficient recipient, HBV DNA-containing blood might be infectious and the clinical expression severe.

However, as emphasized by Candotti *et al*[1], iatrogenic sources of infection should be systematically investigated before concluding that HBV-infected blood donors are involved in viral transmission[22,100,101]. They further added that adequate donor follow-up and laboratory testing have to be performed, and more importantly, pre- and post-transfusion testing of recipients has to be completed[1]. Definitive evidence of transfusion transmission can be obtained by genomic analysis of the viral strains present in both donor and recipient[1]. In addition, sequencing, which might be informative, becomes very difficult to perform at levels of viremia below 200 IU/mL[7]. Limited but convincing evidence that OBIs can be infectious and can be detected by HBV DNA screening should be carefully considered by the health authorities of countries where neither anti-HBc nor HBV NAT are implemented[90].

Occult infection may have impact in several different clinical situations. Extensive studies have evaluated the risk of acquiring OBI in several clinical entities including the following.

OBI and chronic liver diseases

The long-lasting persistence of the virus in the liver may provoke a very mild but continuing necro-inflammation that (if other causes of liver damage coexist) may contribute over time to the progression of the chronic liver damage towards cirrhosis[13].

In studying the situation of OBI and HCV coinfection, Hollinger *et al*[21] reviewed several cross-sectional studies where it was suggested that HBV replication accounts for many of the ALT flares that occur in patients with HCV[40]. OHB is also known to decrease the

response to interferon therapy when employed in patients with chronic hepatitis C[102] and to accelerate the progression of cirrhosis, hepatic decompensation and HCC[40,103]. A strong association was noted between the presence of OHB in 204 patients with chronic hepatitis C and the development of HCC when compared to HCV monoinfected patients[21,104].

Chemin *et al*[42] previously put forward the theory that estimating the percentage of OBI among non-A-E hepatitis cases depends on several parameters including: (1) the method of detection, including PCR primer selection; (2) patient recruitment; (3) patients from countries highly endemic for HBV are more likely to develop occult HBV infections; and (4) prevalence may also vary depending on the nature of biological material tested, with a higher proportion for liver compared to serum specimen[42].

Occult hepatitis B and fulminant hepatic failure

The state of suppression of viral replication and gene expression may be discontinued when an immunosuppressive status occurs, leading to typical hepatitis B with severe - and sometimes fulminant-course[13,105]. Gerlich *et al*[19] studied 5 blood donors with OBI and 55 of their recipients. In 22 recipients, transmission was probable, but they remained healthy. However, in 3 recipients, who were immunosuppressed at the time of transfusion, fatal fulminant hepatitis B developed. The majority of anti-HBc-positive healthy individuals have HBV DNA in the liver which may start replication under severe immunosuppression.

Occult hepatitis B and hepatocellular carcinoma

OBI is supposed to be an important risk factor for HCC development since it maintains the pro-oncogenic properties typical of the overt infection[13]. It has been suggested that the occult viral strains, maintaining the transcriptional activity and the pro-oncogenetic assets of the clear HBV infection (HBsAg+), may harbor a potential risk for liver cancer development[8]. A recent study conducted in Japan confirmed the existence of serum HBV DNA in OBI as a predictor of a high hepatocellular carcinogenesis rate in a cohort of patients with non-B, non-C cirrhosis. Eighty-two consecutive Japanese patients with cirrhosis, who showed negative HBsAg and negative anti-hepatitis C virus, were observed for a median of 5.8 years. The carcinogenesis rates in the patients of the positive HBV DNA group and negative DNA group were 27.0% and 11.8% at the end of the 5th year, and 100% and 17.6% at the 10th year, respectively ($P = 0.0078$)[106]. The mechanisms leading to HCC in OBI seem similar to those in overt HBV-infected patients with low-grade but diagnosable HBV replication that retains its pro-oncogenic properties[13,42].

Occult hepatitis B infection and immune suppression

Patients with an OBI undergoing immunosuppression are at risk of HBV reactivation. As emphasized by Allain[7], the severity of the immunosuppression and its duration play a considerable role in triggering reactivation of HBV infection. Reactivation during relatively mild and short immunosuppression for homologous bone marrow transplantation or solid tumor chemotherapy elicits lower frequency of reactivation than more severe regimens such as employed in allogeneic bone marrow or organ transplantation[7]. The reactivation of OBI in hematological malignancies (< 5%), although at a lower rate than that of HBsAg-positive cases, carries a significant risk of mortality and morbidity[107], which is much higher in the setting of stem cell transplantation[108].

Occult HBV infection harbors potential risk of HBV transmission through hemodialysis. A recent study conducted in Italy showed that occult HBV infection is frequent among hemodialysis patients, particularly correlated to the presence of isolated anti-HBcAg and anti-HCV antibodies. The authors recommended that the presence of isolated anti-HBcAg should prompt the clinician to evaluate a possible occult HBV infection, especially if anti-HCV antibodies are also detectable[109].

Furthermore, another recent Iranian study assessed OBI in 289 hemodialysis patients with isolated hepatitis B core antibody (18 subjects). HBV DNA was detected quantitatively in 9 of 18 patients (50%) where plasma HBV DNA load was less than 50 IU/mL[110]. Meanwhile, a recent study conducted in Brazil found that OBI was not observed in hemodialysis patients and immunosuppression in HIV-positive patients was not a determining factor for occult HBV infection[111].

On the other hand, Demir *et al*[112] showed that the prevalence of occult HBV infection is higher in diabetics compared with healthy controls, which may contribute to the increased prevalence of primary HCC in diabetics[112].

OBI and organ transplantation

OBI often leads to HBV transmission and subsequent infection during organ transplantation[21]. In studying occult HBV infection in HBsAg-negative patients undergoing liver transplantation, Ghisetti *et al*[113] found that OBIis not associated with increased episodes of acute rejection, coinfection with hepatotropic viruses, different responses to HBV vaccination, or the development of *de novo* hepatitis B. In OBI, a particular virus-host interaction can explain the low intrahepatic HBV content and the lack of extrahepatic HBV replication, thus justifying the low risk of hepatitis B reactivation, in absence of specific prophylaxis, once the recipient liver is removed[113]. On the other hand, Hollinger *et al*[21] emphasized that liver transplant recipients with serological evidence of past infection with hepatitis B (anti-HBc-positive) may have reactivation of OHB under immunosuppression in the post-transplant period[21]. A recent systematic review by Cholongitas *et al*[114] covering the last 15 years, identified 39 studies including 903 recipients of anti-HBc-positive liver grafts. They found that liver grafts from anti-HBc-positive donors can be safely used, preferentially in HBsAg-positive or anti-HBc/anti-HBs-positive recipients. HBsAg-negative recipients should receive prophylaxis with lamivudine, while both anti-HBc- and anti-HBs-positive recipients may need no prophylaxis at all[114].

Transmission of HBV after kidney and heart transpla-

ntation from an anti-HBc-reactive donor occurs at a much lower rate[21]. A study in the USA which included 1067 cadaveric kidneys, 38 of them from HBsAg(-)/HBcAb(+) donors, showed that recipients of kidneys from HBsAg(-)/HBcAb(+) donors are at a small risk of hepatitis B seroconversion and are at no excess risk of graft failure or short-term morbidity or mortality[115]. This low viral transmission risk was also confirmed in transplantation of hearts from donors with hepatitis-B core antibodies[116].

Currently, the critical issue in transfusion safety is to identify blood or tissue donors with OHB, and then to block this transmission route. Liu *et al*[3] concluded that the strategy to prevent transmission of HBV by OHB carriers will be different in endemic and nonendemic areas; in low-endemic areas it is still a subject of debate whether anti-HBc screening should be implemented[117]. Whether ID-NAT would eventually be able to replace HBsAg or anti-HBc testing also remains to be studied[3]. On the other hand, in HBV endemic areas, the priority is to examine the prevalence of OHB in blood donors on a large scale, and so establish the cost-effectiveness of implementing sensitive ID-HBV NAT blood screening technology in order to reduce the risk of HBV transmission[3].

PREVALENCE OF OCCULT HEPATITIS B

The prevalence of occult HBV is unclear and depends in part on the sensitivity of the HBsAg and DNA assays used as well as the prevalence of HBV infection in the study population[117]. OHB varies significantly between different geographical regions[11]. Studies have shown that the prevalence of occult HBV infection is closely related to the endemicity of HBV infection[118,119]. Patients from countries highly endemic for HBV are more likely to develop occult HBV infections[42]. As in highly endemic countries, the majority of infections are contracted perinatally or in early childhood; a higher proportion of the infected adults have late chronic HBV with undetectable HBsAg. This may account for the higher rate of OHB in anti-HBc-positive populations in these areas[3]. Prevalence may also vary depending on the nature of biological material tested, with a higher proportion for liver compared to serum specimens[42].

Occult HBV infection has been reported in 0.1%-2.4% of HBsAg-negative, anti-HBc-positive (± anti-HBs) blood donors in Western countries such as the United States, where only 5% of the population has prior exposure to HBV, and in up to 6% of a similar cohort of donors who reside in endemic areas where 70%-90% of the population has been exposed to HBV[11,21]. When anti-HBc only data is evaluated, the rates range from 0% to 15% (median of 1.1%)[11]. In this regard, our recent unpublished data show that OBI is present among 15% of HBsAg-negative, anti-HBc-positive (± anti-HBs) healthy blood donors in an area of intermediate prevalence for HBV.

CONCLUSION

OBI is defined as the presence of HBV DNA in liver/serum with undetectable HBsAg. Advanced progress in molecular biology techniques helps in early detection of OBI and paves the way for implementing a detecting strategy to eliminate post-transfusion occult HBV infection, with consideration for the immune status of blood recipients. Evidence is accumulating supporting the prevalence of OBI among blood donors and in CLD patients. However, current data emphasize the low prevalence of OBI, implying a low impact on transfusion services. Detection of HBV DNA does not always indicate infectivity. Available data encourage testing for OBI in HCV-infected patients, in patients under immunosuppression, in people with unexplained liver diseases and in blood units for immunocompromised recipients where proper recruitment and selection of donors are highly recommended. Further work is needed to clarify the clinical significance of OBI, infectivity, possible transmission and its pathogenic consequences, reactivation and progression to chronic liver disease or hepatocellular carcinoma.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.1939

World J Gastroenterol 2011 April 21; 17(15): 1939-1946
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Perianal Crohn's disease: Is there something new?

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Received: December 1, 2010 Revised: January 17, 2011
Accepted: January 24, 2011
Published online: April 21, 2011

Abstract

Perianal lesions are common in patients with Crohn's disease, and display aggressive behavior in some cases. An accurate diagnosis is necessary for the optimal management of perianal lesions. Treatment of perianal Crohn's disease includes medical and/or surgical options. Recent discoveries in the pathogenesis of this disease have led to advances in medical and surgical therapy with good results. Perianal lesions in Crohn's disease remain a challenging aspect for both gastroenterologists and surgeons and lead to a greatly impaired quality of life for all patients affected by this disease. A multidisciplinary approach is mandatory to obtain the best results.

Key words: Crohn disease; Diagnosis; Biologic therapy; Surgery; Rectal fistula

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Ruffolo C, Citton M, Scarpa M, Angriman I, Massani M, Caratozzolo E, Bassi N. Perianal Crohn's disease: Is there something new? *World J Gastroenterol* 2011; 17(15): 1939-1946 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/1939.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.1939

INTRODUCTION

Perianal lesions are common in patients with Crohn's disease (CD); these may consist of anal skin tags, hemorrhoids, anal fissures and ulcers, anorectal strictures, perianal fistulas and abscesses, rectovaginal fistulas or ultimately carcinoma[1].

In the literature, the incidence of perianal inflammation in patients with CD ranges from 25% to 80%[2]. Risk factors for the development of disabling disease in CD patients are an initial need for steroids, an age below 40 years, and the presence of perianal disease[3]. Perianal lesions show a more aggressive CD phenotype, especially if perianal disease is present at the initial diagnosis[3-5].

In approximately 10% of patients perianal fistulization is the initial manifestation, usually preceding the diagnosis by several years[6]; less than 5% of patients have perianal disease as a unique manifestation of disease[7]. In a population-based study of fistulizing CD the incidence of perianal fistulas was 26%[8]. Perianal fistulizing CD should be considered as a distinct disease phenotype from luminal fistulizing disease, and it has a greater association with colonic and upper gastrointestinal rather than small bowel disease[9].

The pathogenesis of perianal fistulas, despite the prevalence of fistulas in CD, is poorly understood. There are 2 theories: the first suggests that fistulas begin as deep penetrating ulcers, and the second that fistulas result from an anal gland abscess[10]; but it is believed that the etiology of perianal CD involves microbiological, genetic (susceptibil-

ity locus on chromosome 5) and immunological factors[11]. This could explain the aggressive and chronic behavior of perianal lesions.

CYTOKINES

The success of antibodies towards tumor necrosis factor (TNF)-α has led to recent studies investigating other cytokines in perianal CD. In one study[12], the serum levels of TNF-α, interleukin (IL)-12, IL-1β, and IL-6 were analyzed in 12 patients with chronic perianal CD and a CD activity index (CDAI) score < 150 to exclude active intestinal disease, in 7 patients with indeterminate colitis (IC) after restorative proctocolectomy with perianal complications, in 7 patients with active intestinal CD without perianal manifestations, and in 19 healthy controls. Serum TNF-α levels were significantly higher in patients with IC than perianal CD patients and healthy controls. Serum TNF-α levels significantly correlated with perianal CDAI score and with the presence of anal fistulas. Serum IL-12 levels correlated with the presence of anal strictures and were similar in all groups. Serum IL-6 levels were significantly higher in the presence of perianal fistulas and lower in the presence of anal strictures. This study found that the efficacy of anti-IL-12 antibodies appeared doubtful in chronic perianal CD or IC without anal strictures while the role of IL-6 as a systemic mediator for active chronic inflammation was confirmed.

In a subsequent study[13], the cytokine profile was assessed in the rectal mucosa of patients affected by perianal CD in order to understand its relations with the systemic cytokine profile and inflammatory parameters and the need for surgery. Seventeen patients affected by perianal CD, 7 affected by CD without perianal involvement, and 17 healthy controls were enrolled and underwent blood sampling and endoscopy. During endoscopy rectal mucosal samples were taken and the expression of TNF-α IL-6, IL-1β, IL-12, and transforming growth factor (TGF)-1 was quantified by enzyme-linked immunosorbent assay. Local cytokine levels were compared and correlated with diagnosis, therapy, phenotype (fistulizing and stenosing), and disease activity parameters. In the group with perianal CD, rectal mucosal IL-1β, IL-6, and serum IL-6 and TNF-α were higher than in patients with small bowel CD and healthy controls. IL-12 and TGF-1 mucosal levels did not show any differences among the 3 groups. Mucosal IL-6 significantly correlated with the perianal disease activity index (PDAI) and mucosal TNF-α and IL-1. Mucosal TNF-α and IL-1β showed a direct correlation with the histological grade of disease activity. Furthermore, mucosal levels of IL-6 and IL-12 seemed to be predictors of recurrence and of need for surgery in perianal CD patients.

Further prospective and randomized studies are necessary to evaluate the use of these cytokines in this complex disease.

CLASSIFICATION

In 1998, the Vienna classification categorized CD phenotypes, considering age at onset, location and behavior[14], but only in the Montreal modification (2005) of this classification was perianal disease added as a subclassification of behavior; perianal fistulizing disease is not necessarily associated with intestinal fistulizing disease, and it was felt that perianal disease alone required separate subclassification[15].

At the present time, there are different classification systems for perianal CD, but no one has achieved a widespread agreement. In 1976 Parks *et al*[16] proposed a classification of perianal fistulas that uses the external sphincter as a landmark, describing 5 types: intersphincteric, trans-sphincteric, supra-sphincteric, extrasphincteric, and superficial. However, the value of this classification is limited because it does not consider the connection with other organs such as the bladder or the vagina. In 1978, Hughes proposed the Cardiff classification, an anatomic and pathologic classification in which each major manifestation of perianal CD (ulceration, fistula and stricture) is graded on a 2-point scale. This classification has never been globally accepted because it is considered of limited clinical relevance and difficult to use in daily practice[17,18]. In 2003, the American Gastroenterological Association (AGA) technical review[1] proposed an empiric approach that included: physical examination of the perianal area, endoscopic evaluation and a classification of fistulas as simple or complex: simple fistulas are low (superficial, low inter-sphincteric or low intra-sphincteric origin) with a single external opening and are not associated with perianal abscess, rectal stenosis or macroscopic proctitis and have no connection to the vagina or bladder; complex fistulas are high (high intersphincteric, high trans-sphincteric, supra-sphincteric or extra-sphincteric origin) and may have several external openings associated with perianal abscess, rectovaginal fistula, anorectal stenosis or macroscopic proctitis.

In 1995, Irvine described an index to evaluate perianal disease morbidity in CD patients, the PDAI, comprised of 5 categories: presence of fistula discharge, pain, restriction of daily activity, restriction of sexual activity, type of perianal disease, and degree of induration. Each category is graded on a 5-point scale, ranging from no symptoms to severe symptoms. It is widely used but it has never been compared with a reference standard[19,20]. Another method proposed to measure perianal disease activity is the Fistula Drainage Assessment: the presence of purulent drainage from the cutaneous opening after compression is considered an index of activity, but it does not consider the morbidity of the patient and the association with an abscess[20].

DIAGNOSIS

An accurate diagnosis is necessary for the optimal management of perianal lesions. Recently, the goal of treatment has changed from symptomatic improvement to cessation of drainage or even fistula healing. Therefore, the priority of diagnostic tools is to define the anatomy and the number of the fistulas, their complexity, and complicating features such as abscess and anal stenosis[6].

Besides physical examination (findings of skin tags, ulcers, fissures, abscesses, fistulas or anorectal stenoses), there are several other diagnostic modalities. Endoscopic examination is important to identify macroscopic inflammation or stenosis in the rectum; furthermore AGA and the European Crohn's and Colitis Organization (ECCO) agreed on the need to complete the study of perianal disease with other diagnostic methods such as examination under anesthesia (EUA), magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS)[21].

EUA is considered the gold standard for assessing fistulas; it has an accuracy of up to 90 for diagnosis and classification of fistulas and abscesses[22]. At the same time, it is possible to perform several surgical procedures to treat fistulas. However, as suggested by one author, anesthesia can produce a loss of tone and could compromise precise identification of underlying muscles[23]. MRI, an expensive modality, has an accuracy of between 76% and 100% and, combined with EUA, can obtain additional information in 15%-21% of patients; in contrast, EUS, known to be operator-dependent, has a diagnostic accuracy of between 56% and 100% and its findings can alter the surgical approach in 10%-15% of cases[21]. When any 2 modalities are combined, the accuracy is 100%, suggesting that EUA in combination with either EUS or pelvic MRI is the best approach for evaluating and classifying perianal fistulas[22]. The diagnostic accuracy of conventional fistulography and computed tomography (CT) does not exceed 50%-60%, which is considered too low to be clinically useful[1]. Even though fistulography was the first technique used to assess perianal fistula, nowadays it is rarely performed because of several weak points: extensions from the primary track may fail, the sphincter muscles are not directly imaged, the levator plane cannot be visualized, and there is dissemination of septic fistula contents and discomfort for patients[6,21]. Since CT exposes patients to not inconsiderable amounts of ionizing radiation, it may only be used for the diagnosis of fistulas associated with pelvic abscesses if other techniques are unavailable or cannot be tolerated[23,24].

THERAPY

Treatment of perianal CD includes medical and/or surgical options. The primary aim is to heal perianal lesions, but in many cases, because of the aggressiveness of the disease, the physician's role is to relieve symptoms and treat complications of the disease to improve the patients' quality of life. The percentage of spontaneous healing for perianal fistulas is very low, ranging from 6% to 13% in the placebo arm of 2 controlled studies[25,26].

MEDICAL THERAPY

Drugs with definite or potential efficacy for treating perianal CD include antibiotics (metronidazole and ciprofloxacin), immunosuppressors (azathioprine and 6-mercaptopurine), calcineurin inhibitors (cyclosporine and tacrolimus) and biologic agents (infliximab, adalimumab and certolizumab)[1,6].

Antibiotics

Antibiotics are used as first-line treatment for fistula healing, and also for abscesses and infection associated with fistulas. Despite the widespread use of antibiotics for the treatment of perianal CD, there is a lack of controlled studies in the literature and usually data consist of small sample size trials[27,28]. In these studies, the clinical response generally occurs after 6 to 8 wk, as a decreased drainage, while fistula closure is uncommon and symptoms may recur after the end of treatment. Recently, Thia *et al*[29] performed a randomized, double-blind, placebo-controlled trial to evaluate ciprofloxacin and metronidazole for the treatment of perianal CD, concluding that remission and response occurred more frequently in patients treated with ciprofloxacin, but the difference between the treatment arms was not significant. The limit of this study was probably the small sample size. Antibiotics are also used as a bridge to immunosuppressive therapy with azathioprine. In a prospective open-label trial, the use of metronidazole and/or ciprofloxacin at week 8 induced fistula closure in 25% of cases[30]. At week 20, patients treated with additional azathioprine had a better mid-term response (48% *vs* 15%). Antibiotics can be also used as an adjuvant to other drugs. In a recent placebo-controlled study, all patients received infliximab and were randomized to receive either 500 mg ciprofloxacin twice daily or a placebo for 12 wk. The response at week 18 showed a better result of ciprofloxacin in combination with infliximab compared to infliximab alone[31]. Recently, in a randomized controlled study[32], 74 patients with perianal CD received 0.7 g 10% metronidazole ointment or placebo ointment applied perianally 3 times daily. Metronidazole ointment was not effective in reducing the perianal DCAI score, but some secondary outcomes showed improvement, suggestive of a treatment effect and it was well tolerated, with minimal adverse effects.

Immunosuppressants

Azathioprine and 6-mercaptopurine are immunosuppressive agents that, as demonstrated in the literature, successfully treat intestinal CD inflammation[33]. A meta-analysis of 5 randomized controlled studies, in which the closure of various fistulas was considered, showed a complete closure or decreased drainage in 54% of the patients treated with azathioprine or 6-mercaptopurine compared with 21% in the placebo group[34]. However, in this meta-analysis the fistula response was a secondary endpoint in all of the studies considered and at the moment there are no controlled trials in which fistula closure is the primary endpoint. Azathioprine or 6-mercaptopurine could be used as a second-line treatment in patients in whom immediate surgery is not mandatory, and when other pharmacological treatments have already been initiated[6].

Cyclosporine selectively blocks T-helper and cytotoxic lymphocytes through the inhibition of the transcription of IL-2. Several uncontrolled case series reported the use of intravenous cyclosporine in perianal CD patients resistant to traditional therapy, but the initial response was rapidly lost on drug withdrawal[35]. The effects of tacrolimus,

which has a similar mechanism, on fistulizing CD have been evaluated in a randomized, double-blind, placebo-controlled, multicenter study: 43% of the tacrolimus-treated patients had fistula improvement compared with only 8% of the placebo group; however fistula remission was comparable in the 2 groups[36]. More studies are warranted and at the moment the use of cyclosporine and tacrolimus for treatment of fistulizing CD is not recommended[6].

Methotrexate is used as a third-line therapeutic agent for CD patients intolerant to azathioprine and 6-mercaptopurine. No prospective studies have investigated its use for the treatment of fistulizing CD; however, in a retrospective study, 44% of patients treated with methotrexate had partial or complete fistula closure after 6 mo[37].

Studies evaluating therapies such as sargramostim (a granulocyte-macrophage colony-stimulating factor), mycophenolate mofetil (an antimetabolite agent) and thalidomide concluded that these could be considered as potential treatments for perianal CD[38-40].

Biologic therapy

The use of anti-TNF-α agents has changed the approach to CD, especially in patients with severe and refractory disease; in fact TNF-α is believed to play a key role in the pathogenesis of this disease[41].

Infliximab is a murine/human chimeric monoclonal antibody directed toward soluble and membrane-bound TNF-α[42]. There are 2 randomized, double-blind, placebo-controlled trials that demonstrated the efficacy of infliximab in fistulizing CD[25,43]. Present *et al*[30] assessed infliximab induction therapy and reported that 3 infusions of infliximab, 5 or 10 mg/kg, at weeks 0, 2, and 6 resulted in complete perianal fistula closure in 46% of patients. The median length of time the fistula remained closed was 12 wk, and the response rate was higher with the 5 mg/kg dose. The ACCENT Ⅱ (Adjuvant Colon Cancer End Points) study evaluated infliximab as maintenance therapy with 5 mg/kg infliximab at weeks 0, 2 and 6[43]. Of those patients, 64% had a response to therapy at weeks 10 and 14. At week 14, responders were randomized to receive placebo or infliximab 5 mg/kg every 8 wk for 54 wk. The time to loss of response was 40 wk in the infliximab maintenance group *versus* 14 wk in the placebo group. Cessation of drainage at week 54 was maintained in 36% of the patients in the infliximab group compared with 19% of the placebo group. The regime proven to be efficacious in clinical studies comprises induction therapy with 5 mg/kg infliximab at weeks 0, 2 and 6; maintenance therapy can then be continued at 5 mg/kg every 8 wk and the dose may be increased to 10 mg/kg if loss of response is seen at the lower dose. Adverse events of infliximab include infusion reactions, an increased rate of infections, delayed hypersensitivity reactions, formation of antibodies to infliximab, formation of anti-double-stranded DNA antibodies and drug-induced lupus[1].

Adalimumab is a fully humanized monoclonal antibody directed toward TNF-α and has proven effectiveness and efficacy in CD[44]. Its effects have been evaluated in 2 randomized, double-blind, placebo controlled, short-term (4 wk) induction trials. In the CLASSIC-1 trial (Clinical Assessment of Adalimumab Safety and Efficacy Studied as an Induction Therapy in Crohn's Disease), adalimumab was administered at 2 different doses during weeks 0 and 2; instead in the GAIN study (Gauging Adalimumab Efficacy in Infliximab Nonresponders), adalimumab was administered at a high dose and all participants were intolerant to infliximab or had experienced loss of response during week 4 of treatment[45]. In both studies, fistula closure was not significantly higher in patients treated with adalimumab compared with placebo. In the CHARM (Crohn's Trial of the Fully Human Antibody Adalimumab for Remission Maintenance) study, adalimumab was associated with an increased fistula closure compared with placebo. Closure of all fistulas that were draining at baseline was achieved in 30%-33% of adalimumab-treated patients compared with 13% of placebo-treated patients[46]. CD patients, including those with a fistula, should receive an induction dose of adalimumab (160 mg in the USA and 80 mg in Europe), with a second dose (80 mg in the USA and 40 mg in Europe) during week 2; the recommended maintenance dose in both the USA and Europe is 40 mg every other week, beginning at week 4 and the dose frequency can be increased to once weekly if there is no response[6].

Two randomized, double-blind, placebo-controlled trials that investigated the efficacy of certolizumab on fistula closure, for comparison with infliximab and adalimumab[47], were not sufficiently powered[48,49], and its effects require further study. Ng *et al*[50] evaluated CD perianal fistula closure after anti-TNF-α using MRI: even though fistulas appeared clinically healed, MRI demonstrated the persistence of the fistulous tracks as already demonstrated by previous studies[51]; so MRI fistula resolution could be useful to determine the duration of anti-TNF-α therapy.

A recent Japanese study investigated the effects of adsorptive carbon in fistulizing CD patients[52]. Thirty-seven percent of patients treated with an oral adsorptive carbon agent (AST-120) showed an improvement compared to 10% of the placebo group; the former group also had a significantly lower rate of remission (29.6% *vs* 6.7%). Probably adsorptive carbon reverses abnormalities in the luminal environment and gut microflora. In the ECCO consensus statement antibiotics and azathioprine or 6-mercaptopurine are considered the first-line therapy in complex perianal disease, and infliximab or adalimumab are reserved as a second-line treatment in case of failure[53]. In the AGA technical review infliximab is recommended for the treatment of complex perianal disease along with azathioprine or 6-mercaptopurine and antibiotics for the induction phase[1]. Maintenance is recommended with azathioprine or 6-mercaptopurine, and just in some cases in association with infliximab.

Surgical therapy

In the literature the incidence of perianal CD fistulas that require surgery ranges from 25% to 30%[54,55]. The primary goal of surgery is fistula healing and avoidance of sphinc-

ter damage. Patients with superficial or low perianal fistulas without proctitis can be treated by fistulotomy, which has reported healing rates of up to 85%[56,57]. Surgical treatment of complex perianal fistulizing disease requires abscess drainage and usually placement of non-cutting setons[58] before biologic therapy. Setons can be removed after 3 mo in the presence of fistula healing or can remain if the healing process has not been established. However, patients who were assessed 10 years after placement of a seton showed that complete healing was obtained in only 20% of patients[59]. Fistulectomy or fistulotomy are rarely indicated in complex fistulas because of the high rate of subsequent proctectomy due to closure failure or incontinence caused by the transection of both anal sphincters[53,58]. Endorectal flaps are useful when there are severe cases of high fistulas[58,60]. An advancement flap consists of incising a flap of tissue (mucosa, submucosa, circular muscle) around the internal opening of a fistula, excising the internal opening of the fistula tract, and pulling the flap down to cover the opening[61].

Makowiec *et al*[62] reported an initial healing rate of 89% in patients treated with an advancement-flap procedure, but fistulas recurred in 34% of cases during follow-up. If a second flap fails, the failure rate of subsequent flaps increases up to 75% and a temporary stoma might be necessary[63]. In patients with severe refractory disease, fecal diversion (loop ileostomy or end colostomy) is necessary and has an early response rate of 70%-80%[64,65]. Quality of life in symptomatic patients is rapidly improved by fecal diversion[53]. A recent study showed that patients with complicated perianal CD, colonic involvement, and a high rate of abdominal procedures carried a significant risk for a permanent stoma; the incidence of patients requiring a permanent stoma was 31%[66]. In another series of 86 patients with perianal CD disease, 49% of patients finally required permanent fecal diversion[67]. In the literature, proctocolectomy is necessary in only 18% of patients[66].

Primary closure after extended resection can be limited by scar tissue and healing can be impaired by contamination and immunosuppressive medication. Thus, myocutaneous flaps such as the gracilis and the distally based rectus abdominis muscle are used to repair perineal and vaginal defects that are too big to be closed directly.

The use of myocutaneous flaps are well described after proctectomy for cancer and there are only a few reports focusing on CD patients undergoing proctocolectomy and primary closure with myocutaneous flaps[68-70]. Schaden *et al*[69] concluded that a combined proctocolectomy and a perineal single-stage myocutaneous flap closure technique can reduce recovery time, obtain complete healing and improve patients' quality of life.

The treatment of rectovaginal fistulas in CD patients remains challenging. Rectovaginal fistulas seem to be a negative prognostic indicator for successful anti-TNF-α therapy[71]. In a study evaluating a series of 52 CD patients undergoing surgery for a rectovaginal fistula the outcome of surgery and the effect of anti-TNF therapy on healing were assessed[72]. Fistula closure was achieved in 81% of patients. Primary and secondary surgical success rates were 56% and 57% respectively. The primary healing rate was similar in patients who received anti-TNF treatment before the first operation (12 of 18 patients) and those who did not (19 of 34). In univariate analysis, duration of CD and previous extended colonic resection were significantly related to failure of primary surgery, but only the latter remained significant in multivariate analysis The authors concluded that fistula closure was achieved in most patients, but more than one operation was often required.

A recent systematic review was performed including 11 observational studies with a total of 219 flap procedures for rectovaginal fistulas in CD[73]. The pooled primary fistula closure rate was 54.2% after rectal advancement flaps and 69.4% after vaginal advancement flaps. Four studies were eligible for direct comparison between the 2 procedures. Although limited by the small number of studies at a low clinical evidence level, no significant difference in terms of outcome between rectal and vaginal advancement flaps was observed. The risk of recurrence after rectal advancement flaps compared with vaginal advancement flaps also seemed similar.

New therapies

New therapies include laser and adhesive treatment. In an uncontrolled study in perianal CD patients carbon dioxide laser ablation is considered an alternative treatment[74]. The injection of fibrin glue into fistulas is a simple and safe procedure[75]. The first series studies regarding this treatment reported good healing rates (52%-60%), while recent trials have not achieved the same success[76].

Fibrin glue variants include human granulocyte colony-stimulating factor[77] and autologous mesenchymal adult stem cells. Adult stem cells are obtained from adipose tissue with liposuction and initial studies have shown a complete response in 75% of perianal CD patients with complex fistulas[78,79].

More recently bioprosthetic plugs, incorporating porcine intestinal submucosa, have been used in the treatment of patients with anal fistulas[80], but in a retrospective review the use of anal fistula plugs was associated with a lower success rate (15%) than previously reported[81]. Finally, there are other local therapies which are under development. Tacrolimus is a macrolide compound isolated from *Streptomyces tsukubaensis*. Hart *et al*[82], in a randomized, double-blind, placebo-controlled trial showed that, although complete healing was not observed, improvement occurred rapidly, but there was no clear clinical indication that tacrolimus was helpful for fistulizing disease. Topical tacrolimus may have a role in patients who do not respond to infliximab. Similarly, infliximab[83,84], and more recently adalimumab[85], injected directly into the fistula seem to result in healing in some patients resistant to systemic therapy; the rationale of this approach is to avoid systemic toxicity.

CONCLUSION

Perianal lesions in CD remain a challenge for both gas-

troenterologists and surgeons and they lead to a greatly impaired quality of life for all affected patients. A multidisciplinary approach is mandatory to obtain the best results.

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S- Editor Sun H **L- Editor** Cant MR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.1947

World J Gastroenterol 2011 April 21; 17(15): 1947-1960
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Tetracycline-inducible protein expression in pancreatic cancer cells: Effects of CapG overexpression

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Supported by National Institute for Health Research Liverpool Pancreatic Biomedical Research Unit and the Pancreatic Cancer Research Fund (to Nedjadi T)
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Received: August 25, 2010 Revised: October 19, 2010
Accepted: October 26, 2010
Published online: April 21, 2011

Abstract

AIM: To establish stable tetracycline-inducible pancreatic cancer cell lines.

METHODS: Suit-2, MiaPaca-2, and Panc-1 cells were transfected with a second generation reverse tetracycline-controlled transactivator protein (rtTA2S-M2), under the control of either a *cytomegalovirus* (CMV) or a chicken β-actin promoter, and the resulting clones were characterised.

RESULTS: Use of the chicken (β-actin) promoter proved superior for both the production and maintenance of doxycycline-inducible cell lines. The system proved versatile, enabling transient inducible expression of a variety of genes, including GST-P, CYP2E1, S100A6, and the actin capping protein, CapG. To determine the physiological utility of this system in pancreatic cancer cells, stable inducible CapG expressors were established. Overexpressed CapG was localised to the cytoplasm and the nuclear membrane, but was not observed in the nucleus. High CapG levels were associated with enhanced motility, but not with changes to the cell cycle, or cellular proliferation. In CapG-overexpressing cells, the levels and phosphorylation status of other actin-moduating proteins (Cofilin and Ezrin/Radixin) were not altered. However, preliminary analyses suggest that the levels of other cellular proteins, such as ornithine aminotransferase and enolase, are altered upon CapG induction.

CONCLUSION: We have generated pancreatic-cancer derived cell lines in which gene expression is fully controllable.

Key words: Pancreatic cancer cells; Tetracycline-inducible; CapG; Suit-2; Panc-1; MiaPaCa-2

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Tonack S, Patel S, Jalali M, Nedjadi T, Jenkins RE, Goldring C, Neoptolemos J, Costello E. Tetracycline-inducible protein expression in pancreatic cancer cells: Effects of CapG overexpression. *World J Gastroenterol* 2011; 17(15): 1947-1960 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/1947.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.1947

INTRODUCTION

The ability to precisely control the levels of specific proteins in cells, either positively through overexpression, or negatively through siRNA-mediated depletion, provides an invaluable opportunity to examine their function. The most widely used technique for stringently controlling gene expression levels is the tetracycline-regulated system, originally proposed by Gossen and Bujard[1]. Using this approach, cells are transfected with a plasmid encoding a tetracycline-controlled transactivator protein (tTA), and subsequently transfected with a plasmid carrying a gene of interest that has a tetracycline response element (TRE) in its 5' regulatory region. The addition of a tetracycline derivative, doxycycline (dox) to the cell culture medium prevents the tTA from binding to the TRE, thus inactivating the system (tet-off).

The tet system has undergone several improvements since it was first introduced. One improvement is the creation of a reverse tTA (rtTA), which can bind to the TRE in the presence of dox (Tet-on), obviating the need for the continuous presence of dox in the cell culture medium[2-4]. Further improvements came with the introduction of the second generation rtTA, termed rtTA2s-M2[5], which enhanced the transactivation potential and reduced the affinity for the TRE in the absence of dox. This overcame the problem of leakiness; low level gene expression in the absence of dox, sometimes observed with the earlier generation tTA[5]. The system continues to be modified for new applications in cell lines[6,7] and in animals[8].

For certain cell types, such as pancreatic cancer cells, there are remarkably few reports of dox-inducible cells[9,10]. This reflects the difficulty in obtaining such cell lines, and efficient methods for generating them are required. In this study, we attempted to derive dox-inducible pancreatic cancer cell lines, using vectors expressing rtTA2S-M2 under the control of either a viral (CMV) promoter[5] or a chicken (β-actin) promoter[11]. Using the resulting tet-inducible system, we went on to generate pancreatic cancer cells stably overexpressing CapG, an actin capping protein, which was previously shown to be upregulated in pancreatic ductal adenocarcinoma[12]. CapG is activated by calcium and caps actin filaments, a function that is inhibited by membrane polyphosphoinositides[13]. Depletion of CapG from a variety of pancreatic cancer cell lines has been associated with decreased cell motility and wound healing capacity[12]. The dox-inducible overexpression of CapG enabled further characterisation of its role in pancreatic cancer cells, and exemplified the utility of this model system in studying the role of specific genes in pancreatic cancer cells.

MATERIALS AND METHODS

Construction of vectors

Two Tet-on plasmids were used in this study. The rtTA2S-M2[5], which contains a viral CMV promoter driving the tet-transactivator rtTA2S-M2 (denoted here as CMV-rtTA2S-M2), was a kind gift from Professor W Hillen, University of Heidelberg. The pN1βactin-rtTA2S-M2-IRES-EGFP[11], in which the CMV promoter has been replaced with a strong chicken β-actin promoter, was a kind gift from Dr. A Welman, Paterson Institute for Cancer Research, Manchester. The full-length CapG coding sequence was amplified using a forward primer 5'-AGAACGCGTCAGCATGTACACAGCCAATTC-3', which includes an Mlu I restriction site, highlighted in bold, and a reverse 5'-AGAGCGGCCGCCACCCTCATTTCCAGTCCT-3' containing a *Not* I restriction site, in bold. The full size CapG amplicon was inserted directionally into the pTRE-2hyg vector (Clontech, Saint-Germain-en-Laye, France) using the *Mlu* I and *Not* I restriction sites. The full-length S100A6 sequence was amplified using the following forward 5'-TCAGCCCTTGAGGGCTTCAT-3' and reverse 5'-ATGGCATGCCCCCTGGATCA-3' primers. The amplicon was ligated into the EcoRV restricted pTRE2hyg vector. Vector inserts were verified by sequencing (Eurofins MWG Operon, London, UK) and aligned using the Basic Local Alignment Search Tool (Blast). PTRE2hygGST-P, containing the full length coding sequence of human glutathione S-transferase P (GST-P), and pTRE2hygCYP2E1, containing the full-length coding sequence of cytochrome P-450 2E1, were described previously[14].

Cell culture

Pancreatic cancer cells, Suit-2, Panc-1, and MiaPaca-2 cells were maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum, 10 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich, Gillingham, UK). Growth media for cell lines stably expressing the rtTA protein was supplemented with 50 μg/mL G418 sulphate (Invitrogen, Paisley, UK). The media for the transgenic cell lines stably transfected with the inducible CapG was supplemented with 100 μg/mL hygromycin B (Sigma-Aldrich, Gillingham, UK). Cells were maintained at 37℃and 5% CO_2. For the induction of CapG protein expression in clones harbouring stable inducible CapG, cells were cultured for 24 h with antibiotic-free medium, and for an additional 24 h with or without 500 ng/mL dox (Sigma-Aldrich, Gillingham, UK) in RPMI-1640 media, supplemented with 10 % FBS and L-Glutamine.

Cell transfection

Cells were transfected using Lipofectamine 2000 (Invitrogen, Paisley, UK) at a 1:3 DNA: lipofectamine ratio, following the manufacturer's protocol. Briefly, cells were plated at a density of 3×10^6 per 10 cm^2 dish and grown for 24 h, placed in serum- and antibiotic-free medium and transfected with 5 μg of CMV-rtTA2S-M2 or 20 μg pN1βactin-rtTA2S-M2-IRES-EGFP or with pTRE2hygCapG (5 μg or 20 μg). Cells were selected for integration of rtTA2S-M2 coding sequences by treating with G418 (300 μg/mL; Invitrogen, Paisley, UK) for two weeks. Single colonies were separated into 96-well

plates, and positive clones identified using a luciferase assay (described below), expanded, and cryopreserved. Stable CapG-inducible cell populations were selected by incubation with hygromycin B (200 μg/mL). After two weeks, single colonies were separated into 96-well plates, cultured until confluence, and subsequently transferred to 24- and 6-well plates, respectively. Tet-on CapG-overexpressing clones were identified by Western Blotting for CapG, using dox as an inducer (see below). The transient transfection of the tetracycline responsive vectors pTRE-2hygGST-P, pTRE2hygCYP2E1, and pTRE2hygS100A6 was undertaken as described above, and 24 h later expression was induced using 500 ng/mL dox in PBS. PBS alone was used as a vehicle control. Twenty-four hours following doxycycline induction, cells were lysed, and evidence of induction of protein expression was assessed using Western blotting, as described below.

Luciferase assay

To test CMV-rtTA2S-M2 or pN1βactin-rtTA2S-M2-IRES-EGFP-transfected clones for dox inducibility, cells were seeded into 96-well plates and transfected with 50 ng pTRE2hygluc vector (Clontech, Saint-Germain-en-Laye, France) using a 1:3 DNA: Lipofectamine ratio. Twenty-four hours later, clones were treated with 500 ng/mL dox in PBS or with PBS only as a control and incubated for 24 h. Cells were harvested in 50 μL 1 × Glo Lysis buffer (Promega, Southampton, UK) and assayed for luciferase activity according to the manufacturer's instructions, using a plate reader (PerkinElmer, Bucks, UK). Each clone was assayed in triplicate and experiments were repeated at least three times. Data were analysed statistically using Student's two-tailed paired *t*-test.

Western blotting

Cells were lysed in SDS lysis buffer (0.1 mol/L Tris/HCl pH 6.8, 2% SDS) containing protease inhibitor (Roche Diagnostics Ltd., Burgess Hill, UK). For separation into cytoplasmic and nuclear fractionations, the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Rockford, IL, USA) were used. For analysis of phospho-proteins, cells were lysed in RIPA buffer, supplemented with protease and phosphatase inhibitor (Roche Diagnostics Ltd., Burgess Hill, UK). Protein concentrations were determined using a Bradford assay (Bio-Rad, Hemel Hempstead, UK). For Western blotting, membranes were blocked with 5% milk (Bio-Rad, Hemel Hempstead, UK) in PBS containing 0.1% Tween 20 (PBST) for 1 h at RT. As primary antibodies, polyclonal chicken anti-CapG (1:7000, GenWay Biotech, San Diego, USA), monoclonal mouse anti-β-actin (1:200 000, Sigma-Aldrich, Gillingham, UK), rabbit anti-GAPDH (1:400, Santa Cruz Biotechnology, Heidelberg, Germany), monoclonal mouse anti α-tubulin (1:3000; Sigma Aldrich, Gillingham, UK), monoclonal mouse anti-GST-P (1:2500; Sigma Aldrich, Gillingham, UK), rabbit anti-CYP2E1 (1:2500; Sigma Aldrich, Gillingham, UK) and rabbit anti-S100A6 polyclonal antibody (1:3000; DAKO UK Ltd., Ely, UK) were used. The primary antibodies were incubated overnight at 4℃ with gentle shaking. As secondary antibodies, goat anti rabbit HRP (1:3000, Dako UK Ltd., Ely, UK), goat anti mouse HRP (1:3000, Dako UK Ltd., Ely, UK), or goat anti chicken HRP (1:14 000, GenWay Biotech, San Diego, USA) were used. For Cofilin and Ezrin the Actin-Reorganization Kit (Cell Signalling Technology Inc., Danvers, Maryland, USA) was used according to the manufacturer's protocol. Bound horse radish peroxidase was detected using enhanced chemiluminescence western lightning reagent (Perkin-Elmer Life Sciences, Waltham, USA). For quantification purposes, X-ray films were scanned using a GS-800- densitometry scanner (Bio-Rad, Hemel Hempstead, UK) and evaluated using the Quantity One software (Bio-Rad, Hemel Hempstead, UK), or directly developed using a Kodak Gel Logic 1500 imaging station and analysed using Kodak Molecular Imaging software (Carestream Health UK, Hemel Hempstead, UK).

Two-dimensional electrophoresis

The dox-inducible CapG-expressing clones, Sβtet29Cap35, Sβtet29Cap43, and negative control Sβtet29 were incubated for 18 h with or without 500 ng/mL dox and solubilised in lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate, 40 mmol/L Tris base and 1% dithiothreitol) by sonication. Extracted proteins (200 μg) were focused on pH 3-10 non-linear strips, 18 cm in length, as described[15], and then separated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels. Gels were stained with colloidal Coomassie Blue. Gel images were scanned using a GS-800 scanner, acquired using PDQuest software (Bio-Rad, Hemel Hempstead, Herts, UK) and analysed using Progenesis Workstation software V2002.1 (NonLinear Dynamics, Newcastle, UK).

MTS proliferation assay

The proliferation of Sβtet29Cap35, Sβtet29Cap43 and control Sβtet29 clones was determined by MTS assay (EZ4U nonradioactive cell proliferation and cytotoxicity assay, Fa. Biomedica, Vienna, Austria) following the manufacturer's protocol. This assay is based on the reduction of the nontoxic yellow tetrazolium salt (450 nm) to an intensively coloured red formazan derivate (620 nm). Reduction requires functional mitochondria, an indicator of cell viability and cell proliferation. Cells (n = 3000) were plated in 100 μL antibiotic free RPMI medium onto 96-well plates. Dox was added at a final concentration of 0 or 500 ng/mL. After 48 h, 100 μL substrate was added to each well, and cells incubated before absorbance readings were measured at 0, 1, 2, 3, and 4 h at 450 and 620 nm in an Multiskan EX plate reader (Thermo Scientific, Basingstoke, UK). Within each experiment, each condition was plated in twelve replicates and experiments were repeated on three independent occasions. Data were analysed statistically using Student's two-tailed paired *t*-test.

Cell cycle analysis

For cell cycle analysis, cells were plated in triplicate 12 h prior to treatment with or without 500 ng/mL doxycycline for an additional 24 h or 72 h. Cells were harvested, washed twice with ice-cold PBS, and fixed with ice-cold 70% ethanol for 1 h. Cells were centrifuged at 1000 r/min for 4 min, and cell pellets washed twice with PBST. Cell pellets were resuspended in PBS containing 100 μL RNAse A (10 mg/mL) and incubated for 5 min at 4℃. Cells were then stained with 900 μL of a PBS solution containing propidium iodide (55 μg/mL) and 10 000 cells analysed by flow cytometry.

In vitro wound healing assay

Sβtet29Cap35, Sβtet29Cap43, and, as the control, Sβtet29 cells were plated into 12-well plates at 5×10^6 cells per well and incubated for 24 h with or without 500 ng/mL doxycycline. Wounds were made using a P200 plastic pipette tip and photographed ($t = 0$ h) before being returned to 37℃. Wounds were photographed again eight hours later. The distance migrated by the cell monolayer was measured and the results expressed as a migration index (i.e. the distance migrated by the experimental condition, e.g. dox stimulated CapG inducible cells, divided by the distance migrated by the control treated cells, e.g. dox stimulated control cells). Experiments were performed in triplicate and repeated on three independent occasions. Statistical significance between the different conditions was determined using Student's paired *t*-test, with significance set at $P \leqslant 0.05$.

In vitro motility assay

Motility assays were performed using 24-well cell culture inserts (modified Boyden chambers, BD Biosciences, Oxford, UK) with 8 μm pore size. CapG-inducible Sβtet29Cap35, Sβtet29Cap43, and, as the control, Sβtet29 cells were plated in T25 flasks in RPMI media supplemented with 10% FBS and incubated with or without 500 ng/mL dox for 24 h. Cells were counted and 5×10^4 cells were plated into 24-well Boyden chamber inserts in 500 μL serum-free RPMI medium. The medium in the lower chamber was supplemented with 1% FBS. For cells undergoing dox induction, treatment was continued by supplementing both upper and lower chambers with 500 ng/mL dox. The Boyden chambers were incubated at 37℃, 5% CO_2 for 18 h. Cells that had migrated to the lower side of the transwell insert were fixed and stained using Diff-Quik (Siemens Healthcare Diagnostics, Deerfield, USA) and counted on a Leica CME microscope (Leica, Microsystems UK, Milton Keynes, UK) at × 40 total magnification. All experimental conditions were performed in triplicate with three independent repeats. Experiments were statistically analyzed using paired Student's *t*-test (StatView, version 5.0.1, Adept Scientific Plc., Letchworth, UK).

Immunohistochemistry

For immunohistochemistry (IHC), cells were grown on glass chamber slides (LabTek II, Nunc GmbH, Langenselbold, Germany) overnight at 37℃ and cultured for an additional 24 h with or without 500 ng/mL dox. Cells were fixed using acetone which had been equilibrated at -20℃ for 10 min and washed with PBST. Cells were permeabilized by incubation with 0.1% Triton X-100 in PBS for 10 min at RT. Peroxidase was blocked by a 3% H_2O_2/methanol treatment for 30 min in the dark. Slides were washed with PBST and unspecific binding blocked by incubation in 1:10 diluted goat serum in PBST for 30 min at RT. The primary antibody, chicken anti CapG antibody (1:1000; GenWay Biotech, San Diego, USA), was incubated in 1% BSA/PBST overnight at 4℃ in a humidified chamber. Slides were washed with PBST and incubated with the secondary anti-chicken HRP conjugated (1:1000; GenWay Biotech, San Diego, USA) for 1 h at room temperature in 3% BSA/PBS. The staining was developed with DAB and counterstained with hematoxylin.

RESULTS

Development of dox-inducible pancreatic cells

Pancreatic cancer cells, Suit-2, Panc-1 and MiaPaca-2 were transfected with plasmids CMV-rtTA2S-M2 and pN1βactin-rtTA2S-M2-IRES-EGFP, in which rtTA2S-M2 expression is under the control of the CMV and β-actin promoters respectively. Cells were selected for resistance to G418. Emergent clones were tested for dox inducibility by transient transfection with a luciferase expression plasmid under the control of a TRE (pTRE2hygluc). Elevated luciferase activity in the presence of dox when compared to the untreated (-dox) control was taken as evidence of expression of the rtTA protein. Of the Suit-2 cells transfected with CMV-rtTA2S-M2, 167 G418-resistant clones were screened, although only three of these clones (29, 85 and 109) showed evidence of statistically significant dox-inducible luciferase activity (Figure 1A, $P < 0.05$, Student's paired *t* test). Of the Suit-2 cells transfected with pN1βactin-rtTA2S-M2-IRES-EGFP, 49 G418-resistant Suit-2 clones were screened. Nineteen of these clones showed a greater than five-fold dox-inducibility of luciferase activity compared to the untreated (-dox) control. The luciferase measurement for twelve representative clones is shown in Figure 1B. The induction of luciferase was significantly increased in all clones shown ($P < 0.05$, Student's paired *t* test) except for clone 18 ($P < 0.09$).

Stably-transfected Panc-1 (Figure 2A) and MiaPaca-2 (Figure 2B) cell clones harbouring the pN1βactin-rtTA2S-M2-IRES-EGFP Tet-on plasmid were also identified. Thirteen G418-resistant CMV-rtTA2S-M2 Panc-1 clones emerged; however, none showed evidence of dox-inducible luciferase activity. No G418-resistant CMV-rtTA2S-M2 MiaPaca 2 clones were obtained despite four transfection and selection attempts.

Doxycycline-inducible pancreatic cells are suitable for the induction of a variety of genes

We next wished to determine the versatility of stable dox-inducible pancreatic cells for the expression of a variety of

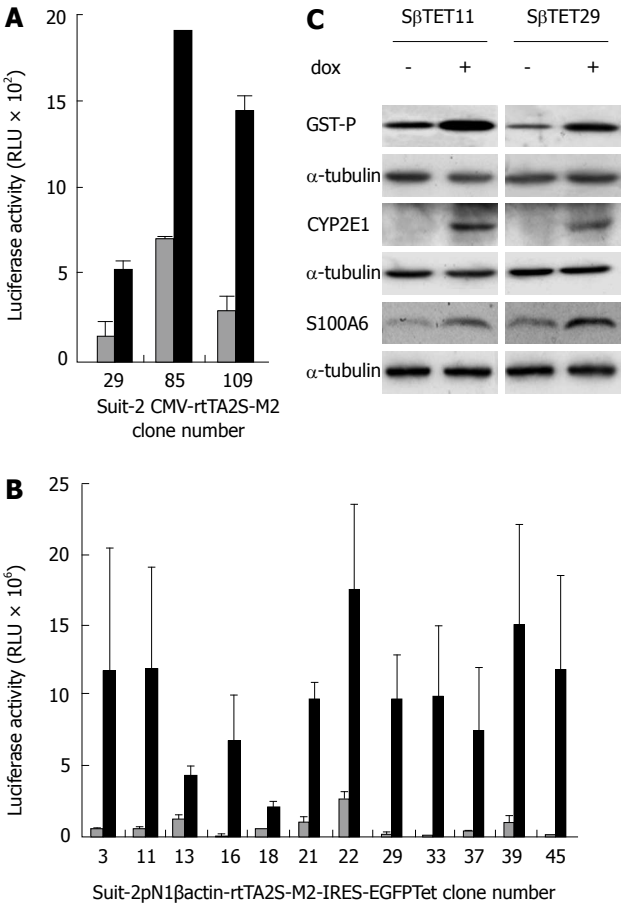


Figure 1 Identification of clones transfected with CMV-rtTA2S-M2 or pN1βactin-rtTA2S-M2-IRES-EGFP-SUIT-2 cell clones. Suit-2 cells were transfected with CMV-rtTA2S-M2 (A) or pN1βactin-rtTA2S-M2-IRES-EGFP (B) and stable clones selected using 300 μg/mL G418. Clones were isolated and transfected with pTRE2hygluc, used as an indirect measure of rtTA activity. Cells were treated for 24 h with 500 ng/mL doxycycline (black bars) or an equivalent volume of PBS (white bars), and luciferase activity measured in 50 μg protein (1 mg/mL protein; 50 μL was assayed per sample). Error bars represent standard errors for three experiments. C: Cells were transiently transfected with pTRE2hygGST-P, pTRE2hygCYP2E1, and pTRE2hygS100A6 and treated with 500 ng/mL doxycycline or PBS as control for 24 h. Protein lysates were prepared and subjected to Western Blotting using anti-GST-P, CYP2E1 and S100A6 antibodies. α-tubulin was used as a loading control.

heterologous genes. To do this, two pN1βactin-rtTA2S-M2-IRES-EGFP-derived Suit-2 clones (Sβtet11 and Sβtet29), which showed good inducibility, were transiently transfected with plasmids containing the genes for glutathione S transferase P (GST-P), cytochrome P450 2E1 (CYP2E1), and S100A6, each under the control of a TRE. These genes were chosen because of our previous experience of their inducible expression in hepatoma cells (GST-P, CYP2E1)[14] or because of our interest in them as genes that are overexpressed in pancreatic cancer (S100A6 and CapG)[12,16,17]. Their inducibility was measured using dox. Western blotting (Figure 1C) revealed that dox treatment of the transiently transfected Sβtet11 and Sβtet29 clones resulted in induction of GST-P, CYP2E1, and S100A6. Two inducible pN1βactin-rtTA2S-M2-IRES-EGFP-derived Panc-1 clones and two pN1βactin-rtTA2S-M2-IRES-EGFP-derived MiaPaca-2 clones were also evaluated by transient transfection of a plasmid carrying a pTRE-dependent *CapG* gene, and induction of expression with dox. Both Panc-1 and MiaPaca-2 clones showed increases in the CapG protein following dox treatment (Figure 2C).

Development of stable Suit-2 derived pTRE2hygCapG clones

Having observed good transient inducible gene expression, we wished to determine whether stable inducible CapG overexpressing cells could be derived. Transgenic cells stably expressing the inducible form of CapG (pTRE-2hygCapG) were constructed using Suit-2 pN1βactin-rtTA2S-M2-IRES-EGFP derived Tet-on cell clones (Sβtet11 and Sβtet29). Hygromycin B-resistant clones (n = 49) were screened for plasmid integration by treating with 500 ng/mL dox for 24 h and measuring CapG protein overexpression by Western blotting. The fold increase in CapG relative to expression of actin is shown in Figure 3A. Six CapG clones (clones 30-35) are shown on a representative Western blot presenting the dox-dependent CapG inducible expression (Figure 3B). From the 49 clones analysed, 20 (40%) showed no change in CapG expression, whereas 22 (44%) showed a 1.5 to 3 fold increase in CapG expression following dox treatment. For eight clones (16%), a greater than three-fold increase in CapG protein level was observed. For a proportion of clones (38%), actin levels appeared to be reproducibly induced following dox treatment/CapG induction. Since CapG is an actin-capping protein, it was decided that subsequent experiments would use alternative internal control proteins, such as tubulin or GAPDH.

Stable pTRE2CapG expressing cells show time- and dose-dependent increases in CapG expression

The time and dose-dependent inducibility of CapG protein expression was investigated. Concentration dependency was examined by inducing CapG overexpression with different concentrations of dox (0-1000 ng/mL) for 36 h (Figure 3C). CapG expression was not induced at low concentrations of dox (1-10 ng/mL), but was induced at concentrations of dox above 50 ng/mL. There was no concentration-dependent increase in the amount of CapG protein induced. For the time course study (Figure 3D) and further experiments, a concentration of 500 ng/mL dox was chosen. An increase in CapG protein level was observed after 12 h of dox treatment and was stable for at least a further 60 h (from 12 to 72 h) (Figure 3D).

Alteration in cellular protein expression associated with CapG overexpression

We next sought to determine whether artificially increasing the levels of CapG would alter the levels or activation status of other actin-modulating proteins, such as Cofilin and Ezrin/Radixin. We found that neither protein was changed in level or in phosphorylation status when CapG levels were increased (Figure 4A and B). This led us to ask

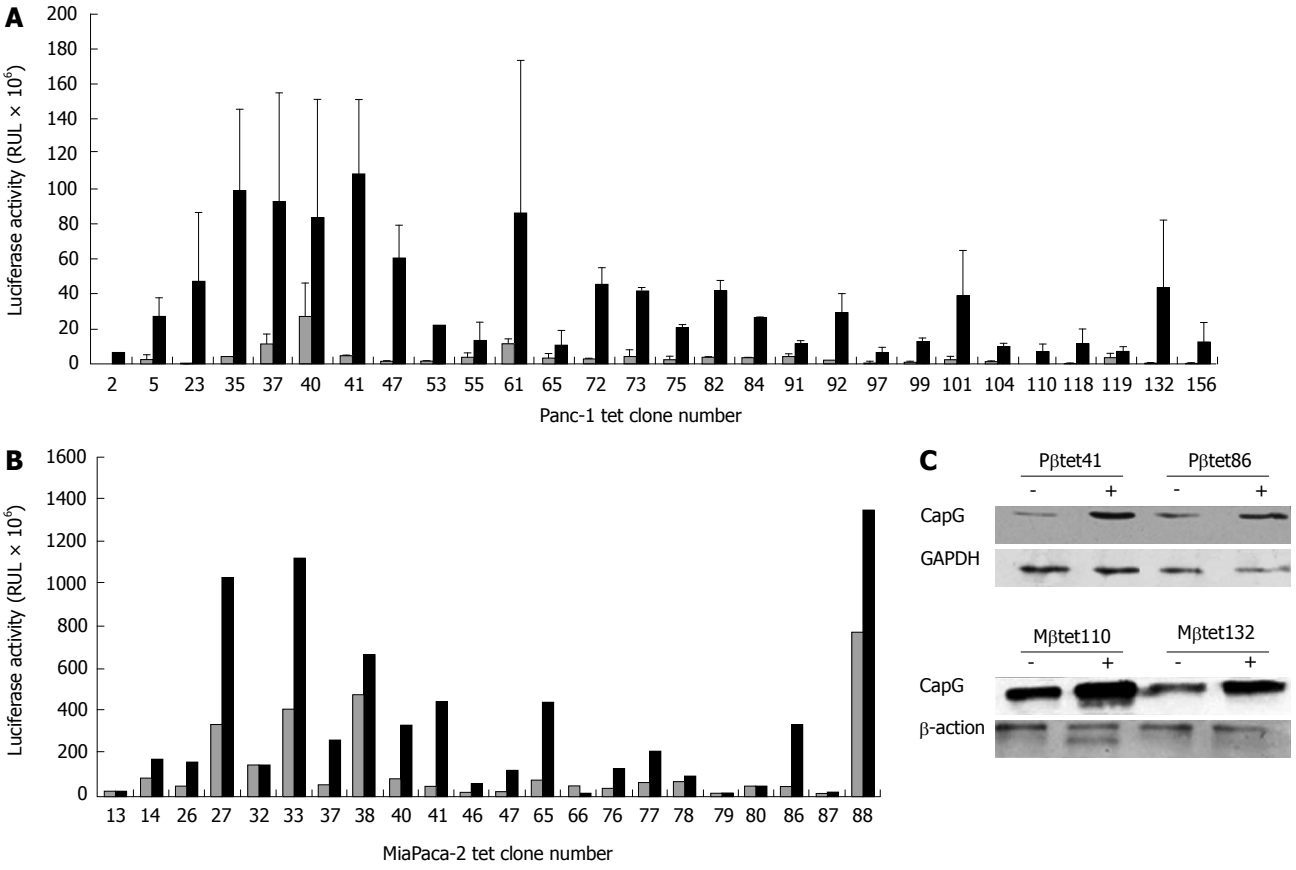


Figure 2 Identification of stable pN1βactin-rtTA2S-M2-IRES-EGFP- expressing Panc-1 (A) and MiaPaca (B) cell clones and their functionality (C). Panc-1 and MiaPaca cells were transfected with pN1βactin-rtTA2S-M2-IRES-EGFP and stable expressors selected using 300 μg/mL G418. Clones were isolated and transfected with pTRE2hygluc, used as an indirect measure of rtTA activity. Cells were treated for 24 h with 500 ng/mL doxycycline (black bars) or an equivalent volume of PBS (white bars) and luciferase activity measured in 50 μg protein (1 mg/mL protein; 50 μL was assayed per sample). A: 90 G418 resistant clones were isolated from Panc-1 cells, of which 25% showed > 5-fold increase in luciferase activity in the stimulated (black bars in Figure 1) compared to the unstimulated (white bars in Figure 1) condition; B: Of 170 G418-resistant cells identified in MiaPaCa-2 cells, 60% showed > 5-fold increase in luciferase expression; C: Two stable pN1βactin-rtTA2S-M2-IRES-EGFP Panc-1 (Pβtet41 and Pβtet86) and MiaPaCa-2 (Mβtet110and Mβtet132) cell lines were transiently transfected with the pTRE2hygCapG vector and treated for 24 h with or without 500 ng/mL doxycycline. Cell lysates was subjected to Western Blotting for the detection of CapG. β-actin and GAPDH were used as loading controls, respectively. Tet: Tetracycline.

a broader question of whether elevated CapG affected the expression of any cellular proteins. CapG overexpression was analysed by 2D-gel electrophoresis in two stable CapG inducible clones (Sβtet29Cap35 and Sβtet29Cap43), and, as the control, the rtTA protein expressing clone from which those two clones were derived (TetSβtet29). An increase in the intensity of a 38 kD protein (Figure 5A) was observed on 2-D gels following dox treatment of Sβtet29Cap35 and Sβtet29Cap43, but in not the control TetSβtet29, indicating that treatment with doxycycline alone is not sufficient to induce the expression of CapG, i.e. the cells must harbour a doxycycline-inducible CapG construct. Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) confirmed the identity of this protein as CapG (Figure 5B). This finding is consistent with our previously obtained Western blot data (Figure 3). A number of other proteins also differentially expressed in cells induced to express high levels of CapG were also identified by MALDI-MS (Figure 6). HNRPH1, T-complex protein 1 subunit alpha, Adenosylhomocysteinase, and Ornithine aminotransferase were upregulated in CapG overexpressing cells. Conversely, Enolase, P27BBP protein, eukaryotic translation elongation factor 1 beta 2, and human pre-mRNA splicing factor SF2p32 were downregulated in CapG overexpressing cells. As none of these proteins are associated with the cytoskeleton, they were not validated further.

Subcellular localization of capG

The localisation of CapG in the inducible clones Sβtet29Cap35, Sβtet29Cap43 and the control clone Sβtet29 was examined by immunohistochemistry. In untreated (-dox) cells, CapG was expressed homogeneously in the cytoplasm (Figure 7A), and nuclei were devoid of staining. After treatment of cells with doxycyline for 24 h, the intensity of CapG staining (brown colour) increased in the cytoplasm and around the nuclear membrane of Sβtet29Cap35 and Sβtet29Cap43 cells, suggesting CapG overexpression in the cytoplasmic compartment of these cells. As expected, the control clone Sβtet29 did not show any change in staining intensity upon treatment with dox, and no staining was observed in the nucleus. The relative levels of CapG in the cytoplasm and nucleus were quantitatively

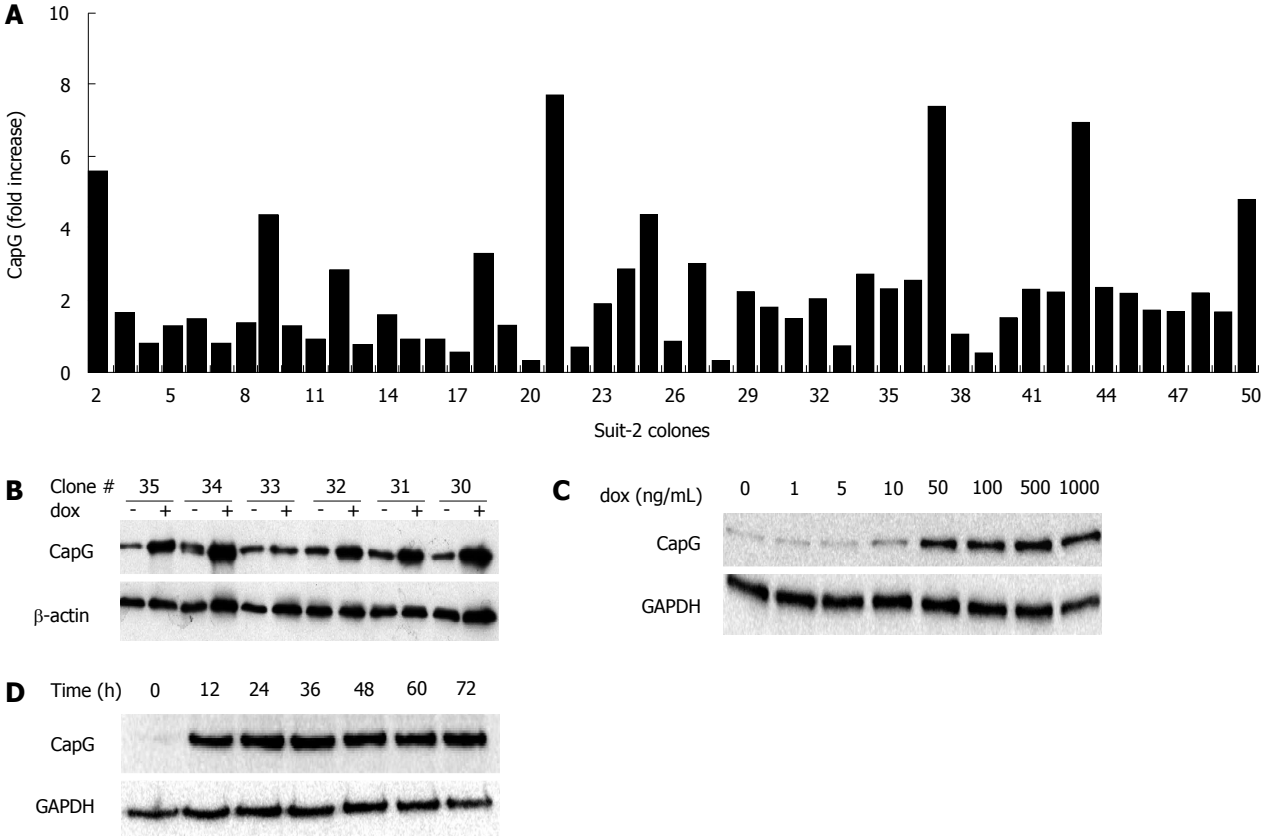


Figure 3 Selection (A, B) and inducibility (C, D) of stably-transfected Sβtet29Cap clones. The stable Sβtet29 clone was transfected with the pTRE2hygCapG full size vector and clones selected with hygromycin B (200 μg/mL). A: Individual clones (n = 49) were induced with 500 ng/mL doxycycline for 24 h and the protein lysate subjected to Western blotting. The CapG level was calculated for each individual clone in the non-induced and the induced state. The fold increase (normalised to actin) in CapG for each of the 49 clones is shown in the bar chart; B: A representative Western blotting for CapG is shown for pTRE2hygCapG clones 30 to 35. The indicates the basal CapG expression, + doxycycline (dox) induced; C: The inducibility of CapG protein expression was investigated by treating the Sβtet29Cap35 cells with 0, 1, 5, 10, 50, 100, 500, 1000 ng/mL dox for 24 h. The resulting Western blotting for CapG and GAPDH as a loading control is shown; D: The Western blotting represents the CapG protein level of Sβtet29Cap35 cells, induced with 500 ng/mL doxycycline for 0, 12, 24, 36 and 48 h. GAPDH was used as a loading control.

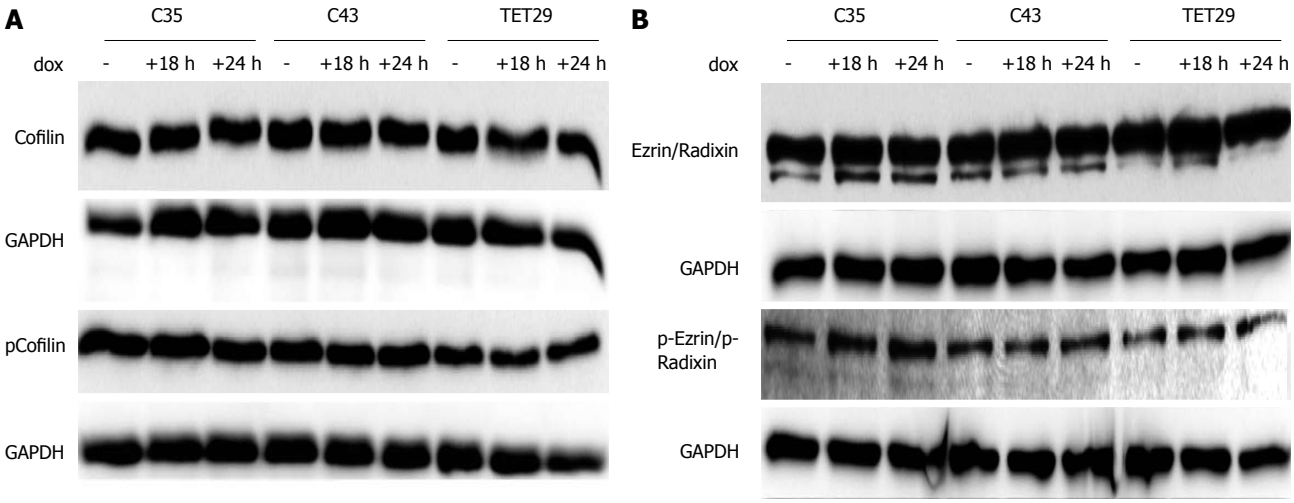
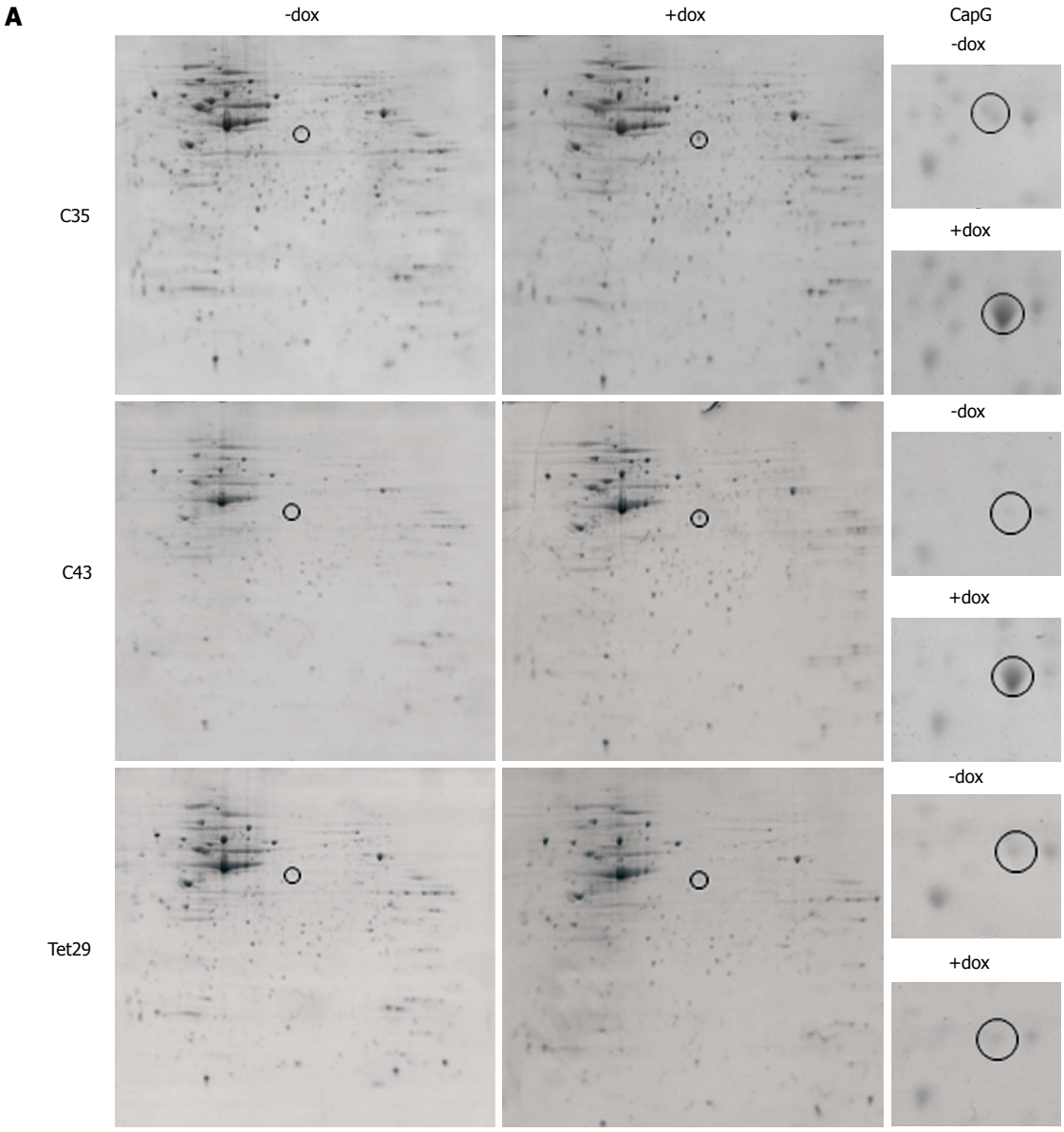


Figure 4 The effects of CapG overexpression on Cofilin and Ezrin/Radixin levels and phosphorylation status. Sβtet29Cap35 (C35), Sβtet29Cap43 (C43) and control Sβtet29 (TET29) cells were incubated with dox (500 ng/mL) for the indicated times and lysates assayed by Western blotting for changes in the levels of Cofilin (A) and Ezrin/Radixin (B) and phospho-Cofilin (A) and phosphor-Ezrin/Radixin (B).

evaluated. The nuclear and cytoplasmic fractions of Sβtet29Cap35 cells were enriched and Western blotting for CapG performed. The levels of CapG in non-fractionated extracts in the non-induced and induced state are shown Figure 7B, lanes 1 and 2 respectively. Lanes 3, 4 and 5, 6 show nuclear and cytoplasmic CapG levels in



B

MYTAIPQSGSPFPGSVQDPGLHVWRVEKLKPVPVAQENQGVFFSGDSYLVLHNGPEEVSHLHLWIGQQSSRDEQGACAVLAVHLNTLLGERPVQH**REVQG**
NESDLFMSYFPRGL**KYQEGGVESAFHK**TSTGAPAAIKKLYQVKGKKNIRATERALNWDSFNTGDCFILDLGQNIFAWCGGKSNILERNKARDLALAIRDS
ERQG**KAQVEIVTDGEEPAEMIQVLGPKPALKEG**NPEEDLTADKANAQAAALY**KVSDATGQMNLTKV**ADSSPFALELLISDDCFVLDNGLCGKIYIWKGRK
ANEKE**RQAALQVAEGFISRM**QYAPNTQVEILPQGHESPIFKQFFKDWK

Figure 5 Two-dimensional gel analysis of stable inducible CapG overexpressing cells. A: Colloidal Coomassie Blue stained gels displaying the proteome of Sβtet29Cap35 (C35), Sβtet29Cap43 (C43) and control Sβtet29 (TET29) cells. Cells were lysed in their uninduced state (- dox) and 18 h after treatment with 500 ng/mL doxycycline (+ dox). The area around the spot representing CapG (black circle) was magnified in the right hand column (CapG); B: The spot representing CapG was excised from the gels, trypsin digested, and analysed by Maldi-Tof. The sequence is shown and peptides identified by Maldi are bold and underlined.

the uninduced and induced states respectively. The enrichment of Lamin A is used as a marker of the nuclear fraction and GAPDH as a marker of the cytoplasmic fraction. A low level contamination of the nuclear fraction by the cytoplasmic fraction was observed. CapG protein was found exclusively in the cytoplasmic fraction in the non-induced state (lane 3). Following 24 h treatment with dox, the level of CapG was greatly increased in the cytoplasmic fraction (lane 5), with a very small proportion observed in the nuclear fraction (lane 6). The low level of CapG observed in the nuclear fraction may be due to contamination from the cytoplasmic fraction.

Stable pTRE2CapG cells show no difference in viability, proliferation or cell cycle

The viability of Sβtet29Cap35, Sβtet29Cap43, and Sβtet29 cells was measured 48 h after plating with or without 500 ng/mL dox. The MTS solution was added (t = 48 h) and the absorbance measured at 0, 1, 2, 3, and 4 h later. The kinetics for the turnover of the MTS solution was similar in all cell lines, as can be seen by the slope of the lines (0.3577 ± 0.031, Figure 8A). This indicates that 500 ng/mL dox was not toxic in any of the cell clones, including those in which dox caused induction of CapG (Sβtet29Cap35, Sβtet29Cap43).

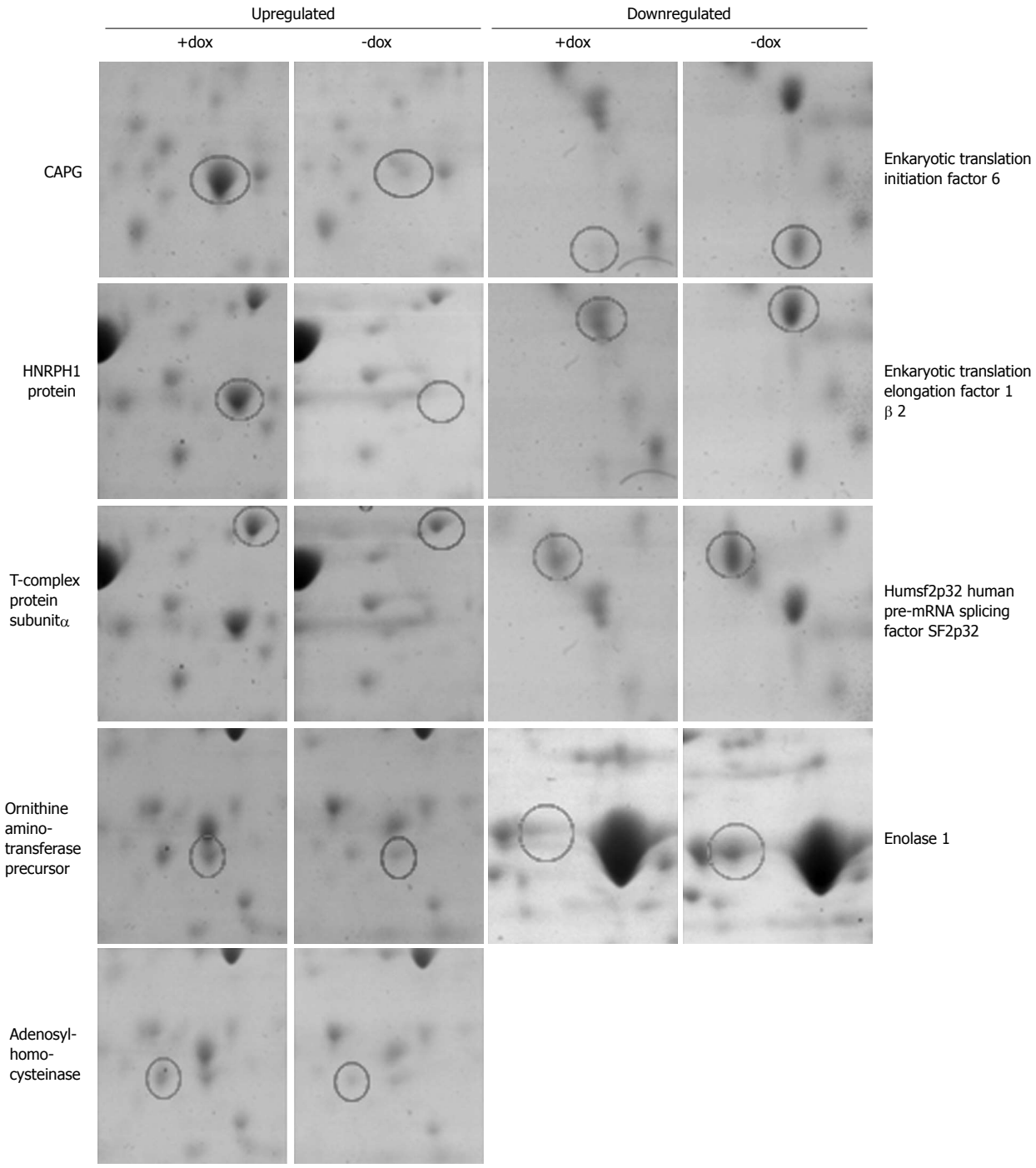


Figure 6 Two-dimensional gel analysis shows up- or downregulation of proteins associated with CapG overexpression. Colloidal Coomassie Blue stained gel insets displaying proteins found to be up- or downregulated in Sβtet29Cap35 (C35), Sβtet29Cap43 (C43) but not in control Sβtet29 (TET29) cells, following treatment for 18 h with 500 ng/mL doxycycline (+dox). Spots were excised from the gels, trypsin digested, and analysed by Maldi-Tof, leading to the identification of proteins.

Cell cycle analysis at 24 and 72 h after dox treatment did not result in any significant change in cell cycle profile. A representative figure showing the cell cycle profile of Sβtet29Cap35 (C35) and the parental cell line Sβtet29 (TET29) cultured for 24 or 72 h with or without 500 ng/mL dox is presented (Figure 8B). The percentage of cells in G1, G2 and S-Phase for all three experiments is summarized in Figure 8C and Table 1. There was a considerable change in cell cycle phases between the two time points (24 and 72 h), as cells are cultured for 48 h (24 h ± dox) or 96 h (72 h ± dox). There was no difference in the cell cycle phases between the groups cultured with or without dox. This shows that neither dox nor CapG overexpression affected the cell cycle of Suit-2 cells.

CapG overexpression increases Suit-2 wound healing capacity and motility

To investigate the effect of CapG overexpression on cell

Table 1 Cell cycle analysis (%)

	- doxycycline			+ 500 ng/mL doxycycline		
	G1-phase	G2-phase	S-phase	G1-phase	G2-phase	S-phase
24 h						
C35	34.8 ± 1.2	30.6 ± 5.2	34.7 ± 5.0	36.2 ± 3.2	27.3 ± 3.6	36.6 ± 1.1
C43	30.8 ± 0.9	29.8 ± 2.5	39.4 ± 3.1	30.7 ± 1.6	31.7 ± 0.9	37.6 ± 1.8
TET29	29.0 ± 1.7	38.9 ± 3.5	32.1 ± 2.4	30.2 ± 0.7	30.2 ± 1.5	39.6 ± 0.9
72 h						
C35	39.8 ± 1.1	24.7 ± 2.4	35.5 ± 2.8	41.1 ± 3.1	31.8 ± 3.6	27.1 ± 5.0
C43	42.6 ± 0.8	30.3 ± 3.5	27.1 ± 2.8	43.0 ± 4.1	24.0 ± 1.8	33.0 ± 3.0
TET29	40.4 ± 5.4	19.4 ± 3.7	40.2 ± 1.9	41.6 ± 4.3	25.9 ± 3.1	32.5 ± 5.6

10 000 cells were measured, and the percentage of cells in G1, G2, and S-phase are presented with their error values. The experiment was repeated thrice.

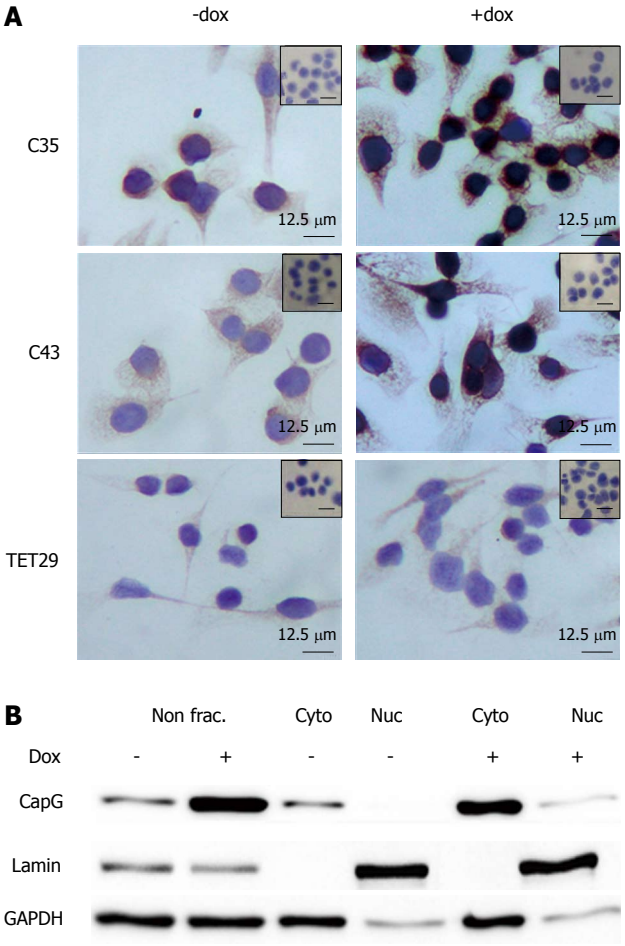


Figure 7 Subcellular localization of CapG in stable CapG inducible clones by immunohistochemistry (A) and subcellular fractionation (B). A: CapG protein expression and localization in Sβtet29Cap35 (C35), Sβtet29Cap43 (C43) and the control Sβtet29 (TET29) cells was analyzed 24 h after treatment with 500 ng/ml doxycycline (+ dox) or PBS as control (- dox). Specific antibody reaction (anti-CapG) was visualized by a peroxidase labelled secondary antibody (DAB detection, brown colour). Nuclei were counterstained with hematoxylin (blue colour). Bars = 12.5 μm. B. Whole protein lysate (non frac.) or enriched nuclear (Nuc) and cytoplasmic (Cyto) fractions of Sβtet29Cap35, Sβtet29Cap43 and the control Sβtet29 clones 24 h after treatment with 500 ng/mL doxycycline (+) or PBS as control (-) were analysed using Western blotting for the detection of CapG, Lamin (marker for nuclear fraction) and GAPDH (marker for cytoplasmic fraction). The experiment was performed three times. A representative Western blot of Sβtet29Cap35 is shown.

motility, wound healing and translocation assays were performed. When CapG was induced following dox treatment, we observed a significant increase in the distance travelled by the Sβtet29Cap35 and Sβtet29Cap43 cell clones. The distance travelled by Sβtet29Cap35 increased by 23% ± 5% and for Sβtet29Cap43 by 35% ± 7%. When treated with dox, the control clone Sβtet29 did not show any change in wound healing capacity (Figure 9A).

In Boyden chamber translocation assays, we observed a significant increase in cell migration by the Sβtet29Cap35 and Sβtet29Cap43 cell clones when CapG was induced following dox treatment (Figure 9B). The capability of Sβtet29Cap35 to cross through the membrane pores increased by 62% ± 10% and for Sβtet29Cap43 cells by 84% ± 16%. The control clone Sβtet29 did not show any change in cell migration capacity when treated with dox (Figure 9B).

DISCUSSION

In this study, we have described the generation and use of a tetracycline-inducible expression system in pancreatic cancer cell lines. Our use of both a CMV and a β-actin promoter-driven second generation reverse tetracycline transactivator protein (the rtTA2S-M2) led us to conclude that the latter was superior in terms of both production and maintenance of dox-inducible cell lines. Although we did not formally investigate mechanisms that might explain this, it is possible that the viral CMV promoter is silenced through methylation, whilst the chicken β-actin promoter remains active[6].

Recently, Zhang *et al*[10], successfully used a commercially available Tet-on system with a CMV-driven tetracycline transactivator protein, in Panc-1 cells. The authors reported dox-induced overexpression of the zinc finger transcription factor INSM1, although the fold increase in INSM1 protein level, as shown by Western blotting, was modest compared to the fold increases observed in the current study. This may reflect differences in the proteins under investigation in the respective studies, although it could be due to enhanced inducibility when using the β actin-rtTA2S-M2-IRES-EGFP vector.

The pancreatic cancer cell lines Suit-2, MiaPaca-2 and

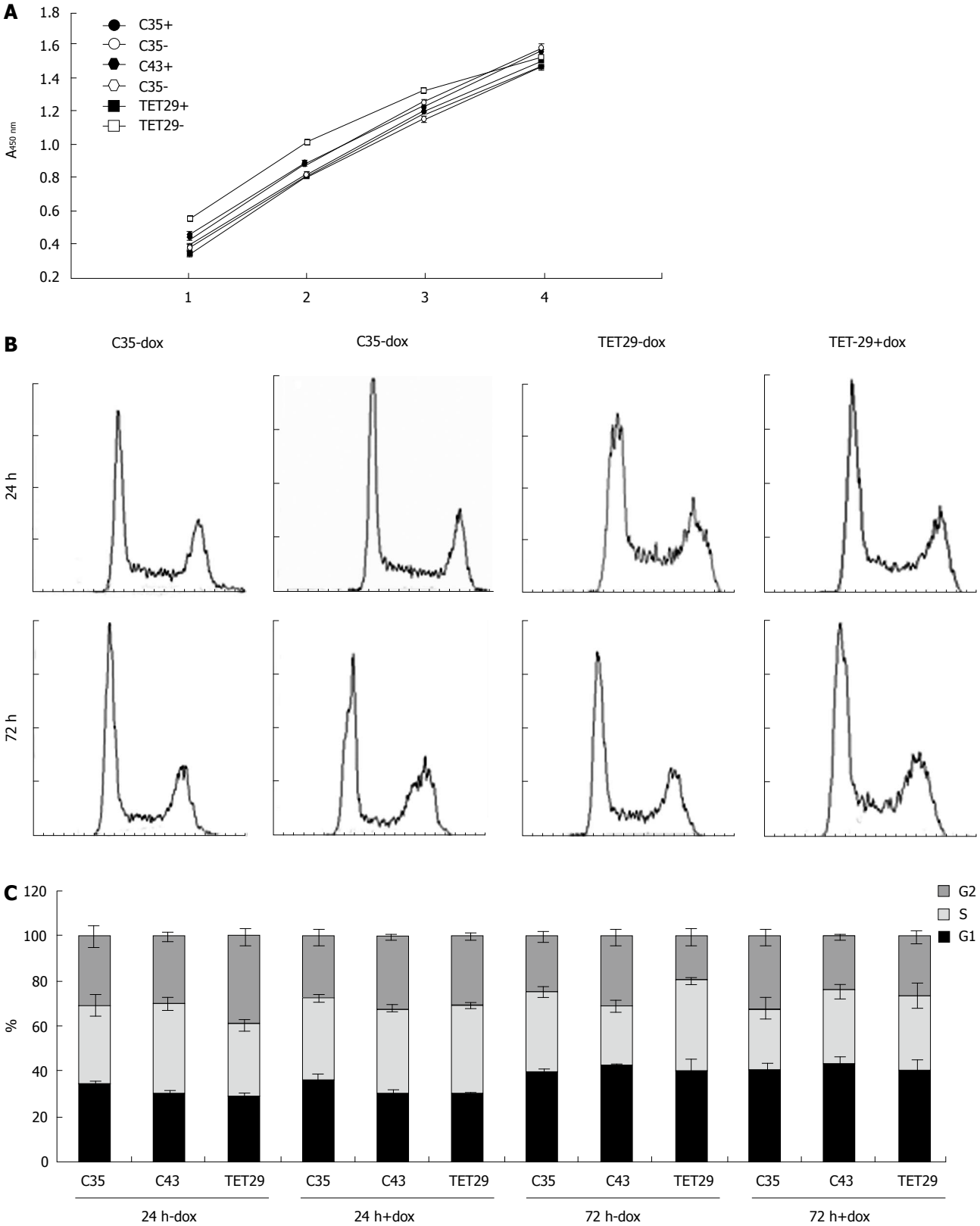


Figure 8 Cell proliferation (MTS-assay) (A) and cell cycle analysis (B) of doxycycline induced CapG overexpressing cells. A: Sβtet29Cap35 (C35), Sβtet29Cap43 (C43) and the control Sβtet29 (TET29) cells were treated with 500 ng/mL doxycycline (+) or PBS as control (-) for 48 h. Mitochondrial activity was measured with the EZ4U assay and absorbance measured 1 h, 2 h, 3 h, and 4 h after substrate incubation. The experiment was performed three times with at least ten replicates; B: Sβtet29Cap35 (C35), Sβtet29Cap43 (C43) and the control Sβtet29 (TET29) cells were treated with 500 ng/ml doxycycline (+) or PBS as control (-) for 24 h and 72 h. The cells were propidium iodide stained and FACS analyzed. 10 000 cells were measured and the experiment was performed in triplicate with three replicates each. A representative chart for the cell cycle of C35 and TET29 cells is presented; C: The graphics represent the average percentage (± SE) of cells in G1, G2, and S phase in the different experimental conditions.

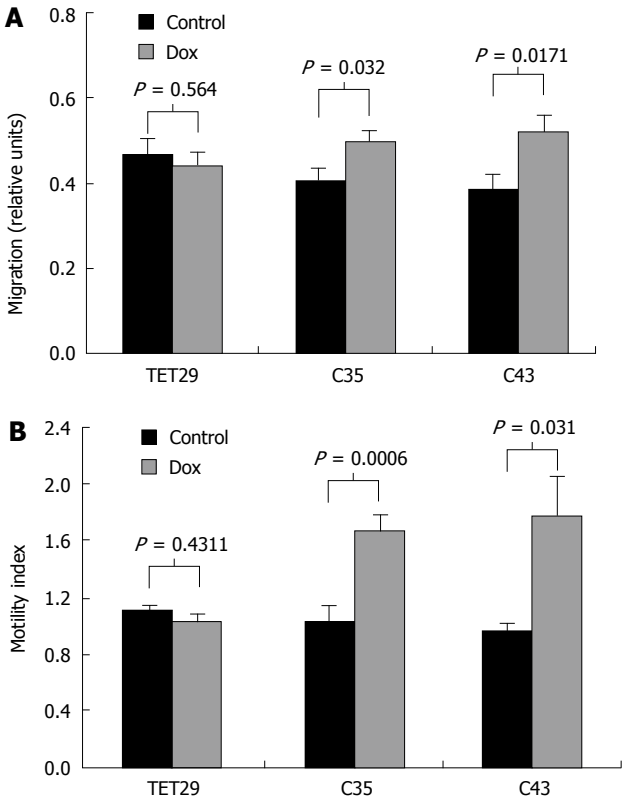


Figure 9 Wound healing capacity (A) and motility (B) of CapG-overexpressing cells. A: Bars represent the migration in relative units for Sβtet29Cap35 (C35), Sβtet29Cap43 (C43) and the control Sβtet29 (TET29) cells treated with doxycycline (dox, grey bar) or PBS (Control, black bar). Error bars present the SE for three experiments carried out in triplicate; B: The motility investigated by a Boyden Chamber assay of Sβtet29Cap35 (C35), Sβtet29Cap43 (C43) and the control Sβtet29 (TET29) cells treated with doxycycline (dox, gray bar) or PBS (Control, black bar) is shown. Each experiment was performed in triplicate with three replicates.

Panc-1 were all capable of stably harbouring the pN1βactin-rtTA2S-M2-IRES-EGFP vector. Moreover, inducible gene overexpression was observed for GST-P, CYP2E1, and S100A6 in Suit-2 and for CapG in all three investigated cell lines. This indicates that the system functions for a variety of different proteins. Equally, the method was efficient with 80% of pN1βactin-rtTA2S-M2-IRES-EGFP transfected Suit-2 clones and 60% of MiaPaca-2 clones showing greater than five-fold luciferase induction. Only Panc-1 cells showed relatively low efficiency, with only 14.4% of the selected cell clones showing a greater than five-fold luciferase increase. A clear advantage of the Tet-on system is the time and dose-dependent activation of expression of the gene of interest. Investigation of stable CapG overexpressing Suit-2 cell clones showed that CapG protein expression was dox-inducible in a time dependent manner. The level of CapG increased from 12 h after induction, and was maintained up to 72 h after induction. A similar time-dependent induction of gene expression in the liver carcinoma cells, HepG2 has been described previously[14]. However, we did not observe a linear correlation between dox concentration and protein expression, as was reported in the HepG2 cells[14]. Instead, we detected little increase in CapG protein concentration between 0-10 ng/mL dox, and overexpression of CapG with concentrations of 50 ng/mL and greater (up to 1000 ng/mL). Possible reasons for these different results could be related to the particular protein overexpressed or the different promoter used to drive the rtTA2S-M2 in both studies. The HepG2 Tet-on clones harboured CMV driven rtTA2S-M2, unlike the pancreas clones in this study.

Concerns have been raised about the toxicity of dox, the agent used to activate gene expression in the Tet-on vector system[18]. The cell growth of PC-12 cells was reduced after 96h incubation with concentrations ranging from as low as 0.2 to 100 μg/mL. Growth perturbations were observed with WI-38 VA-13 cells, but only with concentrations of dox greater than 2 μg/mL[18]. It appears that the concentration of dox which mediates a toxic effect is cell-line-dependent. At the concentrations of dox used in this study, no loss of proliferation was observed 48 h after dox treatment. These data were supported by cell cycle analyses profiles, which showed no change in cell cycle following dox treatment for 24 h and 72 h.

The function of CapG in the cytoplasm is well described. CapG caps and therefore blocks rapidly growing actin filaments, promoting the elongation of shorter actin filaments. It thus contributes to cell motility[19] and membrane ruffling[20]. Increased levels of CapG protein have been described in several tumors, including pancreatic ductal adenocarcinoma[12,21] and glioblastomas[22]. Transient CapG knockdown experiments in pancreatic cancer cell lines Suit-2, MiaPaca-2, and Panc-1 cell lines led to a significant decrease in wound healing capacity and cell motility[12]. Furthermore, Van den Abbeele *et al*[23] showed that downregulation of CapG in the breast cancer cells MDA-MB 231 and the prostate cancer cells PC-3 also decreased invasion and motility. Here, we show for the first time that overexpression of CapG in a pancreatic cancer cell line caused a significant increase in motility and a modest, but significant, increase in wound healing capacity. This is consistent with a previous report of an increase in invasion after CapG overexpression in Madin-Darby Canine Kidney Epithelial Cells MDCK cells[24]. Interestingly, it has been reported that active nuclear import of CapG is necessary for CapG to promote invasion[24]. Prevention of nuclear accumulation of CapG in MDCK cells abolished collagen invasion, whereas restoring the nuclear import also restored collagen invasion of these cells. We found no evidence for elevated nuclear levels of CapG following overexpression in Suit-2 derived cells. Immunohistochemical staining localized CapG to the cytoplasm in Suit-2 cells. Dox treatment of stable inducible CapG expressors led to an increase in cytoplasmic staining, with increased CapG around the nuclear membrane, but CapG did not accumulate in the nucleus. Subcellular fractionation further validated this finding, showing increased CapG protein levels in the cytoplasmic fraction after dox treatment. We observed increased motility, suggesting that elevated cytoplasmic CapG may be sufficient for increased cancer cell motility.

CapG has been shown to interact with microtubule-dependent organelles during the cell cycle[25], possibly mediating cross-talk between the actin cytoskeleton and

microtubule-based organelles involved in mitosis. CapG is imported into the nucleus by NTF2, Ran GTPase and nucleoporin Nuc62[26], a phenomenon that was observed in HEK293T, HeLa, and MDCK-AZ cells. We found no changes in the cell cycle following induction of CapG overexpression. This suggests that an excess of CapG is not sufficient to alter cell cycle dynamics, but it does not exclude a role for CapG in cell cycle processes.

In summary, we have shown that the pN1βactin-rtTA2S-M2-IRES-EGFP Tet-on vector system can be stably transfected in a variety of pancreatic cancer cell lines, permitting the investigation of dox inducible gene overexpression in a time and dose-dependent manner. Our use of this system to overexpress CapG in Suit-2 cells resulted in accumulation of CapG protein in the cytoplasmic cellular compartment with associated increases in wound healing and cell motility, but no alterations to the cell cycle or to cellular proliferation.

ACKNOWLEDGMENTS

We thank W Hillen for the kind gift of the rtTA2S-M2 plasmid and Dr. A Welman for the pN1βactin-rtTA2S-M2-IRES-EGFP plasmid.

COMMENTS

Background

Pancreatic cancer cells have traditionally been recalcitrant to the tetracycline gene regulatory system, a method that allows precise control of the level of expression of specific proteins, enabling functional analysis of those proteins. In this study, the authors compare the performance, in pancreatic cancer cells, of a stable Tet-on system employing a *cytomegalovirus* (CMV) promoter with a system employing a chicken β-actin promoter. In doing so, the authors generated cells exhibiting stable inducible expression of the actin capping protein CapG, which plays a role in cell migration. This protein was recently shown to be overexpressed in pancreatic cancer tissue, and its downregulation is associated with loss of motility.

Research frontiers

Use of the chicken (β-actin) promoter proved superior to the CMV promoter for both the production and maintenance of tetracycline-inducible pancreatic cancer cell lines. The authors observed that the system was versatile, enabling transient inducible expression of a variety of genes. Moreover, with the newly developed Tet-on system, the authors showed that CapG overexpression in pancreatic cancer cells led to increased cell motility, but did not alter the cell cycle, or cellular proliferation.

Innovations and breakthroughs

The Tet-on system using a chicken (β-actin) promoter allowed the generation of stable inducible pancreatic cancer cell lines, providing an important tool for investigating doxycycline induced overexpression of proteins associated with pancreatic cancer. Furthermore, the study illustrated that cytoplasmic CapG overexpression in pancreatic cancer cells increases motility of these cells.

Applications

The stable Tet-on pancreatic cancer cell lines developed are an excellent system that will provide quantitative and temporal information on the role of given proteins in these cells.

Terminology

Panc-1, Suit-2 and MiaPaca-2 are pancreatic cancer cell lines. CapG belongs to the family of actin regulatory proteins. CapG caps the ends of barbed actin filaments and therefore contributes of the actin associated motility of cells. Nuclear CapG is involved in the cell cycle regulation.

Peer review

There are precious few published papers describing the use of stable tetracycline-inducible pancreatic cancer cells for the overexpression of proteins. This reflects the difficulty that scientists in this field have faced with the available systems. The authors describe the successful generation of stable tetracycline-inducible pancreatic cancer cells, using a chicken β-actin promoter to express the reverse tetracycline-controlled transactivator protein. This is an important advance, illustrating an improvement to the system which other pancreatic cancer researchers could also benefit from.

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S- Editor Sun H **L- Editor** Stewart GJ **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.1961

World J Gastroenterol 2011 April 21; 17(15): 1961-1970
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Unusual histopathological findings in appendectomy specimens: A retrospective analysis and literature review

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Received: November 27, 2010 Revised: December 23, 2010
Accepted: December 30, 2010
Published online: April 21, 2011

Abstract

AIM: To document unusual findings in appendectomy specimens.

METHODS: The clinicopathological data of 5262 patients who underwent appendectomies for presumed acute appendicitis from January 2006 to October 2010 were reviewed retrospectively. Appendectomies performed as incidental procedures during some other operation were excluded. We focused on 54 patients who had unusual findings in their appendectomy specimens. We conducted a literature review *via* the PubMed and Google Scholar databases of English language studies published between 2000 and 2010 on unusual findings in appendectomy specimens.

RESULTS: Unusual findings were determined in 54 (1%) cases by histopathology. Thirty were male and 24 were female with ages ranging from 15 to 84 years (median, 32.2 ± 15.1 years). Final pathology revealed 37 cases of enterobiasis, five cases of carcinoids, four mucinous cystadenomas, two eosinophilic infiltrations, two mucoceles, two tuberculosis, one goblet-cell carcinoid, and one neurogenic hyperplasia. While 52 patients underwent a standard appendectomy, two patients who were diagnosed with tuberculous appendicitis underwent a right hemicolectomy. All tumors were located at the distal part of the appendix with a mean diameter of 6.8 mm (range, 4-10 mm). All patients with tumors were alive and disease-free during a mean follow-up of 17.8 mo. A review of 1366 cases reported in the English literature is also discussed.

CONCLUSION: Although unusual pathological findings are seldom seen during an appendectomy, all appendectomy specimens should be sent for routine histopathological examination.

Key words: Appendicitis; Carcinoid; Unusual findings; Goblet cell carcinoid; Enterobius vermicularis; Mucocele

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Akbulut S, Tas M, Sogutcu N, Arikanoglu Z, Basbug M, Ulku A, Semur H, Yagmur Y. Unusual histopathological findings in appendectomy specimens: A retrospective analysis and literature review. *World J Gastroenterol* 2011; 17(15): 1961-1970 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/1961.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.1961

INTRODUCTION

Appendicitis is one of most common acute surgical condi-

tions of the abdomen, and an appendectomy is one of the most frequently performed operations worldwide. The incidence of acute appendicitis roughly parallels that of lymphoid development, with peak incidence in the late teens and twenties. Obstruction of the lumen is the dominant factor in acute appendicitis, and although fecoliths and lymphoid hyperplasia are the usual cause of obstructions, some unusual factors could also be involved[1-128]. Obstruction may be due to enterobiasis[1,4,7,29], ascariasis[57,92-94], balantidiasis[2,92], taeniasis[14,18], actinomycosis[32-38], schistosomiasis[2,8,42-51,57], amebiasis[7,84-86,90], trichuriasis[52,57], *Blastocystis hominis*[20], tuberculosis (TB)[8,23,53-55,57], carcinoid tumor[1-3,5,9,12,26,28,31,95], goblet-cell carcinoid (GCC)[5,12,21,25], primary or secondary adenocarcinoma[16,31], cystadenocarcinoma[31], lymphoma[2], dysplastic changes[2], endometriosis[1,16,58-69], granulomatous diseases[31,32], gastrointestinal stromal tumor (GIST)[71,72,103], mucocele[1-3,52], villous adenoma[24,39,56], tubulovillous adenoma[24], tubular adenoma[24,31], leiomyoma[2], eosinophilic granuloma[32,52], or neurogenic appendicopathy[30].

MATERIALS AND METHODS

Between January 2006 and October 2010, 5262 patients with presumed acute appendicitis underwent surgical treatment at Diyarbakir Education and Research Hospital, Turkey. Appendectomies performed as an incidental procedure during some other operation were excluded. The data of 54 (1%) patients who were pathologically reported to have unusual appendix findings were retrospectively collected. The original pathology specimens with unusual findings were evaluated again by an experienced pathologist. The records analysis was composed of the patient's age, gender, clinical presentation, operative reports, radiological tools, pathological report, and follow-up. The length of follow-up was calculated by months from the date of diagnosis until the last clinical information available on the patient up to November 2010.

English medical language PubMed and Google Scholar database searches were conducted for case reports, retrospective and prospective studies, and literature reviews relating to "unusual causes of appendicitis". Keywords used were parasites, enterobiasis, schistosomiasis, amebiasis, yersiniosis, strongyloidiasis, actinomycosis, TB, idiopathic granulomatous appendicitis, Crohn's disease, endometriosis, appendicular adenocarcinoma, carcinoid, GCC, mucocele, mucinous cystadenoma, lymphoma, polypoid lesion, appendectomy, and appendicitis. The search included all articles from 2000 until November 2010. Patients who had undergone an operation for presumed acute appendicitis and had "unusual findings" pathology were included in the study, whereas articles that provided inconclusive information about patients and those in which the patients could not be reached were excluded. Additionally, appendicitis cases that developed due to foreign bodies were also excluded[1-128].

RESULTS

In total, 5262 appendectomies were performed with a diagnosis of acute appendicitis at Diyarbakir Education and Research Hospital from January 2006 through October 2010. All patients were diagnosed clinically with acute appendicitis on the basis of physical and laboratory examinations. Of all appendectomies performed, 54 (1%) specimens revealed incidental abnormal histopathological diagnoses. The general characteristics of these 54 patients are summarized in Table 1. Thirty of the patients were male and 24 were female with ages ranging from 15 to 84 years (median, 32.2 ± 15.1 years). Thirty-seven of the 54 patients revealed *Enterobius vermicularis*, five a carcinoid tumor, six a mucinous cystadenoma (two were mucoceles), two TB, and two eosinophilic infiltration, and two each were diagnosed with GCCs and neurogenic hyperplasia (Figure 1). While 52 patients underwent a standard appendectomy, two patients, who were preoperatively diagnosed with tuberculous appendicitis, had a right hemicolectomy. All patients with malignant tumors were diagnosed clinically with acute appendicitis, and none of them had symptoms of carcinoid syndrome or were preoperatively diagnosed with an appendicular tumor. After pathological confirmation of the diagnosis, the patients were referred to our clinic for staging. Staging included abdominal ultrasonography (US), computed tomography (CT), and 24-h urinary 5-hydroxyindoleacetic acid levels. After staging, all patients were followed up at the outpatient clinic every 3 mo for the first year. All patients with tumors were alive and disease-free during a mean follow-up of 17.8 mo. The clinicopathological characteristics of six patients with tumors are summarized in Table 2.

A histopathological examination of patients with *E. vermicularis* revealed 12 with acute inflammation and 25 with no evidence of any pathological change. After obtaining the pathology reports, the patients with oxyuris were prescribed a single oral dose of 100 mg mebendazole, which was repeated 7-10 d later. All patients with oxyuris were asymptomatic on follow-up (mean, 7.2 mo;

Table 1 General characteristics of the 54 patients with abnormal pathological findings

Patients' characteristics	Results	Rate (%)
Age (yr) (range)	32.2 ± 15.1 (15-84)	
Sex		
Male	30	55.50
Female	24	44.50
WBC (K/UL) (range)	11.7 ± 4.9 (4.5-26.7)	
Histopathologic findings	54	
E.vermicularis	37	68.50
Tuberculosis	2	3.70
Carcinoid	5	9.20
Goblet-cell carcinoid	1	
Mucocele	2	3.70
Mucinous cystadenoma	4	7.40
Eosinophilic infiltration	2	3.70
Neurogenic hyperplasia	1	
Follow-up (mo) (range)	10.4 ± 12.4 (1-54)	
Surgical Approach		
Appendectomy	52	96.30
Right hemicolectomy	2	3.70
Recurrence	0	

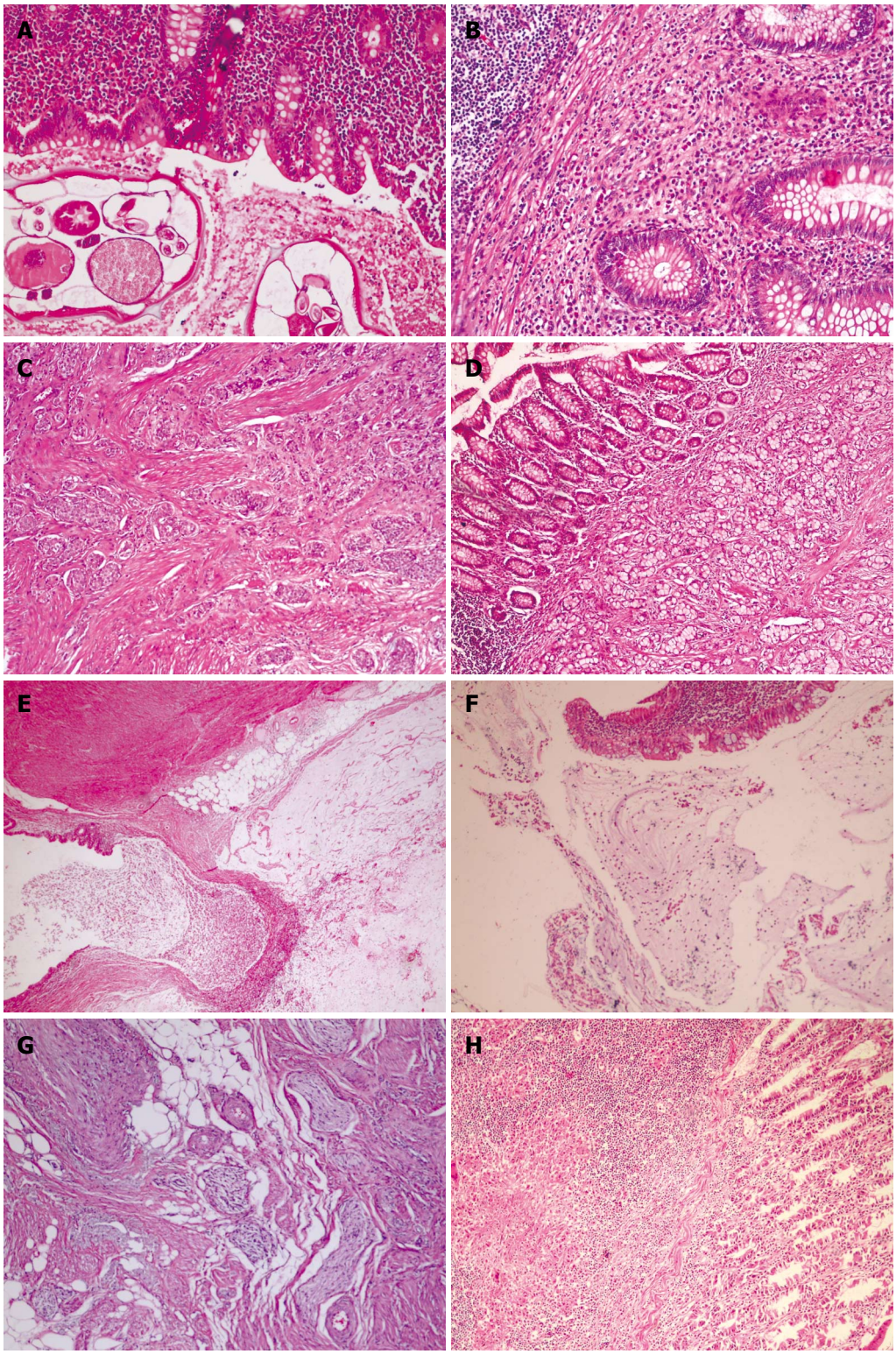

Figure 1 Unusual histopathologic findings. A: Adult of *E.Vermicularis* in appendices (HE, × 200); B: Eosinophilic appendicitis: diffuse eosinophilic infiltrate in lamina propria (HE, × 200); C: Carcinoid tumor of classic type is formed by solid nest of small monotonous cells with occasional acinar formation (HE, × 100); D: Microglandular goblet cell carcinoma. Acute appendicitis with a diffusely infiltrating goblet cell neoplasm. tumor cells infiltrated muscularis propria (HE, × 200); E: Mucosel. Dilatation of lumen by mucinous secretion, thin appendiceal wall. Mucin is protruding into surrounding fatty tissue (HE, × 40); F: Mucinous cystadenoma of appendix. Typical epithelium of a cystadenoma with pseudostratified, columnar cells containing elonged, crowded, hyperchromatic nuclei and scattered goblet cells with mucus in cavity (HE, × 100); G: Neurogenous hyperplasia of appendix. The proliferating spindle cells shown in this photography (HE, × 200); H: Tuberculous appendicitis. Granuloma which contain a caseating center surrounded by epithelioid cells, lymphocytes and histiocytes. A giant cell is present in the granuloma (HE, × 20).

range, 1-54 mo).

Two female patients (18 and 48 years old, respectively) with tuberculous appendicitis received antitubercular therapy during the preoperative period. A right hemicolectomy was

Table 2 Clinicopathological characteristics of the six patients with primary appendicular tumors

Age	Sex	Tumor size (mm)	Location	Treatment	Pathology	Parietal spread	Follow-up (mo)
43	F	5	Distal	Appendectomy	Carcinoid	Serosa	54
42	F	10	Distal	Appendectomy	Carcinoid	Serosa	33
23	F	6	Distal	Appendectomy	Carcinoid	Subserosa	15
39	M	4	Distal	Appendectomy	Carcinoid	Submucosa	1
36	M	10	Distal	Appendectomy	Goblet cell	M.Propria	3
26	M	6	Distal	Appendectomy	Carcinoid	Subserosa	1

Table 3 Distribution of the 1366 cases defined as "unusual findings" according to etiological causes

Total patients	1366 (1366/80 698 = 1.7%)	
Unusual findings	1366	1.7%
Enterobius vermicularis	389	28.4%
Carcinoid	287	21.0%
Schistosomiasis	174	12.7%
Amoebic appendicitis	118	8.6%
Mucinous cystadenoma (+mucocele)	72	5.2%
Ascaris lumbricoides	39	2.8%
Tuberculous appendicitis	34	2.5%
Endometriosis	41	3.0%
Goblet-cell carcinoid	28	2.0%
Trichuris trichiura	22	1.6%
Idiopathic granulomatous appendicitis	35	2.5%
Crohn disease	18	
Lymphoma	14	
Primary adenocarcinoma	11	
Mucinous cystadenocarcinoma	9	
Actinomycosis	8	
Melanosis	8	
Secondary adenocarcinoma	7	
Dysplastic change	7	
Villous adenoma	6	
Hyperplastic polyp	5	
Taeniasis	8	
GIST (+leiomyoma)	5	
Balantidium coli	3	
Tubulovillous adenoma	3	
Eosinophilic granuloma	3	
Neurogenic hyperplasia	2	
Tubular adenoma	2	
Leukaemia	4	
Blastocystis hominis	1	
Adenovirus	1	
Strongyloides stercoralis	1	
Yersinia enterocolitica	1	

performed in patients with an acute abdomen in the follow-up, considering the intraoperative findings. We have presented the details of these two cases in a previous article[53].

Results of the literature review

Using the PubMed and Google Scholar databases, 128 studies published between January 2000 and November 2010 were compatible with our criteria. Fifty-one of these were written as original articles (50 retrospective and 1 prospective), 67 as case reports, eight as letters to the editor, and two as case series. When we looked at the countries in which the articles were prepared, 59 were from Europe, 40 from Asia, 19 from the Americas, six from Africa, and four were from Australia. In total, 80 698 cases

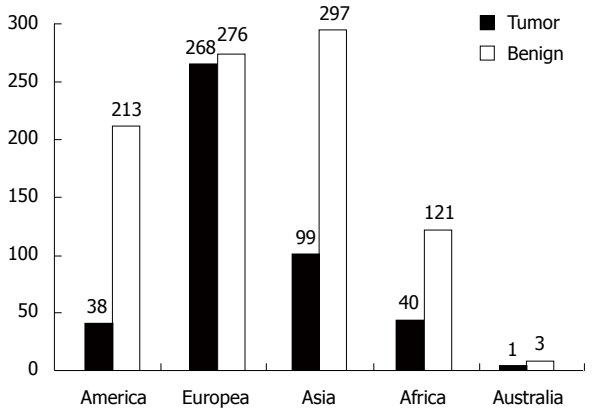

Figure 2 Worldwide distribution of the 1366 cases defined as "unusual findings". Tumor: Carcinoid, goblet cell carcinoid, mucocele, appendix adenocarcinoma, lymphoma, mucinous cystadenoma and adenocarcinoma, polypoid lesions, leukemia, gastrointestinal stromal tumor, dysplastic change; Benign: Non-tumoral causes.

were discussed in these articles, and all patients who were operated on had presumed acute appendicitis. Unusual findings were detected in 1366 (1.7%) of the cases with or without histopathologically acute appendicitis in their appendectomy specimens. We have summarized the causes that we qualified as "unusual findings in appendectomy specimens" in Table 3. As shown in the table, causes such as enterobiasis, schistosomiasis, amebiasis, and carcinoid tumor comprised 75.7% of all cases. The etiological (tumoral and non-tumoral causes) distribution of the 1366 patients by continent is summarized in Figure 2 to demonstrate the effects of geographic and sociocultural differences.

DISCUSSION

Acute appendicitis is the most common general surgical emergency, and obstruction of the appendiceal lumen seems to be essential for developing an appendiceal infection. Although fecaliths and lymphoid hyperplasia are the usual causes of the obstruction, some unusual factors could also be involved[2,8,16,57].

Appendiceal tumors, occurring in less than 3% of all appendectomies, are rarely associated with clinical manifestations; they are frequently recognized either during an operation or the pathological examination. Malignant tumors of the appendix include carcinoids, GCCs, lymphomas, mucoceles, primary adenocarcinomas, and mucinous cystadenocarcinomas. Benign tumors of the ap-

pendix consist of tubular adenomas, villous adenomas, leiomyomas, neuromas, and lipomas[2,5,31].

An appendiceal carcinoid tumor is considered the most common type of appendiceal primary malignant lesion and accounts for almost 60% of all appendiceal tumors[28]. An appendiceal carcinoid tumor is found in 0.3%-2.27% of patients undergoing an appendectomy. Characteristics of all appendiceal carcinoids predicting aggressive behavior include tumor size, histological subtype, and mesoappendiceal involvement. The tumors are smaller than 1 cm in 70%-95% of cases[26-28]. The calculated risk of metastasis from tumors 1 cm or smaller is nearly zero and therefore may be managed with a simple appendectomy. An increase in metastasis risk of up to 85% occurs with a tumor of 2 cm or larger. An appendiceal carcinoid tumor larger than 2 cm should be managed with a formal right hemicolectomy[1,3,5,6,9-13,16,26,28].

GCCs, also known as adenocarcinomas and first described by Gagne in 1969, are uncommon primary tumors of the vermiform appendix characterized by dual endocrine and glandular differentiation[129]. Whether GCCs represent a morphological variant of appendiceal classical carcinoid or a mucin-producing adenocarcinoma is a matter of conjecture[12]. GCCs account for 2% of primary appendiceal malignancies. Most tumors are less than 2 cm in diameter and 20% metastasize to the ovaries. Recent studies suggest that GCCs have biological and immunohistochemical profiles more similar to adenocarcinomas than to classical carcinoids, which may explain their aggressive behavior and therefore requirement for more extensive treatment[12]. A right hemicolectomy is generally advised if any of the following features are present: tumors greater than 2 cm, involvement of resected margins greater than 2 mitoses/10 high-power fields, extension of the tumor beyond the serosa, lymphovascular invasion, or lymph node metastases[5,12,21,25]. In our series, one patient had a GCC tumor located distally in the appendix that measured 1 cm in diameter. The patient was advised to undergo a right hemicolectomy, but he refused the procedure.

Mucinous cystadenoma is a rare tumor of the appendix associated with cystic dilatation, to which the more general term of mucocele has been applied. A mucocele of the appendix denotes an obstructive dilatation of the appendiceal lumen due to abnormal accumulation of mucus, which may be caused by a retention cyst, endometriosis, mucosal hyperplasia, cystadenoma, or a cystadenocarcinoma. The incidence of mucocele ranges from 0.2% to 0.3% of all appendectomy specimens. Mucoceles are often asymptomatic and discovered as incidental findings at appendicectomy, or during laparotomy for another indication or at histological examination of an operative specimen. However, mucoceles may be diagnosed clinically from features of acute appendicitis. Appendectomy is the standard of care for mucinous cystadenoma, whereas a cystadenocarcinoma requires a right hemicolectomy. Because of the high association of mucinous cystadenoma with colon and ovarian malignancy, follow-up CT, US, and colonoscopy examinations must be performed during the postoperative period[1-3,5,16,25,27,31,52,106,107].

Mucinous cystadenocarcinoma of the appendix**,** also known as a mucinous adenocarcinoma or malignant mucocele, constitutes a rare malignancy of the appendix and is often associated with a second malignancy of the gastrointestinal (GI) tract. The most common type of presentation is that of acute appendicitis. The diagnosis of mucinous adenocarcinoma of the appendix is usually given after an appendectomy, or other explorative surgical procedure, and consequent pathological evaluation of the appendiceal specimen[5,25,31,108].

Primary adenocarcinoma of the appendix is an extraordinarily rare tumor, and its incidence was 0.01% (11 of 80 698 cases) in our literature review. Adenocarcinomas behave aggressively and in a fashion similar to that of colonic adenocarcinomas, so in the case of an appendicular adenocarcinoma, oncologic resection with right hemicolectomy is the treatment of choice[2,16,23,31,105].

The GI tract is the most common site for extranodal lymphomas and accounts for 30%-45% of all extranodal cases. The stomach is the most commonly involved organ followed by the small intestine, colon, and esophagus. The incidence of primary appendiceal lymphoma has been estimated at 0.015%-0.022% of all appendiceal specimens. An appendiceal lymphoma usually presents in the second and third decades of life, usually manifests as acute appendicitis, and is often diagnosed postoperatively by histopathology. Therapy guidelines for primary appendiceal lymphomas are unclear because of their rarity. Our literature review revealed 14 lymphoma cases with clinical evidence of acute appendicitis; 12 of these were of B-cell origin, whereas two were of T-cell origin[2,8,97-101,109-112].

Leukemia can involve the GI tract but rarely involves the appendix. Although appendicitis is a known complication in patients with leukemia, leukemic cell involvement in the appendix is extremely rare. When the leukemia involves soft tissue including the appendix, it is called granulocytic sarcoma. The incidence of leukemic appendicitis was 0.005% (4 of 80 698 cases) in our literature review. Surgical management of patients with leukemia and acute abdomen has not been advocated because of the high rate of operative mortality. However, some support exists for surgically managing appendicitis as the most effective method of therapy in acute leukemia cases. Systemic chemotherapy is necessary prior to additional surgery in patients with leukemia[113-115].

GISTs, which occur most commonly in the stomach (60%) and the small bowel (30%), are the most common primary mesenchymal neoplasms of the GI tract. GISTs, known as leiomyoma or leiomyosarcoma before 1983, primary to the vermiform appendix are exceptionally rare, with only eight cases reported so far[2,71,72,103]. Five out of eight patients were operated due to acute appendicitis symptoms. The size of the mass and degree of mitotic activity play a crucial role in tumor behavior and recurrence development. Therefore, when approaching the appendix for GIST tumors, tumor location should be evaluated along with tumor size and mitotic activity.

Enterobius vermicularis, also known as pinworm or oxyuris, is a widespread parasitic infection estimated to

affect up to 200 million people worldwide. The association of oxyuris and appendicitis was first made in the late 19 century, when Still initially documented this organism in the appendix lumen. While the reported incidence of pinworm in appendectomy specimens of patients with presumed appendicitis ranged from 0.2% to 41.8%, the reported rates of inflammation in specimens from appendices infested with pinworm ranged from 13% to 37%[4,7,14,29]. Patients must receive antihelminthic treatment because the appendectomy treats only the consequence and not the cause of the disease. An *E. vermicularis* infestation is treated with an oral dose of mebendazole, which is repeated in 1-2 wk[1,2,4,7,11,14,16-20,22,29,52,57,92,93].

TB may affect all tissues and organs in the body, but it most frequently involves the lungs. The GI system is ranked sixth among all extrapulmonary involvements. TB may affect all of the segments of the GI system, from the mouth to anus. However, the ileum and ileocecal region are the sites most commonly involved, followed by the colon and vermiform appendix. The appendix may be affected secondarily to ileocecal TB, but appendicular TB may occur in an even rarer primary form without any evidence of the disease elsewhere. The reported incidence of appendicular TB varies from 0.1% to 3.0% among all appendectomies performed. An accurate diagnosis is usually established after histopathological examination of a specimen. Classic histopathological analysis of an appendectomy specimen usually reveals the presence of caseating granulomas and Langhans giant cells, suggesting TB of the appendix. Although some studies have reported that treatment is not necessary for the primary disease and that appendectomy alone is sufficient, no consensus has been reached. When we reviewed the literature, 34 cases of patients undergoing an appendectomy with presumed appendicitis have been published in the last decade, including our own two cases[23,32,53-55,57].

Actinomycosis is an uncommon chronic infectious disease. Common sites of involvement include the cervicofacial, thoracic, and abdominopelvic regions. In abdominal actinomycosis, the ileocecal region including the appendix is the most commonly involved site. A correct diagnosis can be made by culture or histopathological examination, although a definitive diagnosis of actinomycosis requires microscopic proof of either the pathogen itself or the presence of specific sulfur granules. After the diagnosis has been confirmed, the general therapeutic recommendation is to initiate treatment with intravenous antibiotic therapy for 2-12 mo. Eight cases of patients undergoing an appendectomy with presumed appendicitis have been published in the last decade[32-38].

Taeniasis, a well-known worm infection, is characterized by the presence of the helminth in the intestine. Infection is generally recognized when a segment of the parasite appears in the stool. The occurrence of *Taenia spp.* in the appendix is so rare that the situation invites a case report. In our literature review, *Taenia* was found in only five of the cases operated on for presumed acute appendicitis. In cases of taeniasis, specific species identification is not required for treatment, as patients are treated with a single dose of praziquantel[14,18,93,127,128].

Amebiasis is an infection of the large intestine caused by *Entamoeba histolytica*, which affects 10% of the world population and has a worldwide distribution. This parasite is occasionally found in the appendix, usually in the lumen without accompanying inflammation, but is rarely associated with acute appendicitis. A preoperative diagnosis of amebic appendicitis is almost impossible because no clinical features or diagnostic laboratory tests distinguish amebic from bacterial appendicitis, other than a stool examination. The clinical picture presented in this report represents a typical case of amebic appendicitis with a good outcome after surgical resection and treatment with metronidazole[84,85,87-93].

Schistosomiasis, also known as bilharziasis and most commonly caused by *Schistosoma haematobium*, only rarely leads to appendicitis, even in nations in which schistosomiasis is endemic. The pathogenesis is most probably due to a periappendicular granulomatous reaction of the host against the schistosome. Inflammation and repair causes scarring and strictural deformation of the appendiceal wall, leading to luminal obstruction and acute appendicitis. Histologically, appendices may show transmural inflammation rich in eosinophils, with a granulomatous reaction to ova. Treatment for schistosomal appendicitis consists of an appendectomy and administration of praziquantel[2,8,13,16,32,40-52,57,93,125,126].

Ascaris lumbricoides, also known as roundworm, is one of the most common human helminthic diseases worldwide. The highest prevalence of ascariasis occurs in tropical and semitropical countries. The domain of the worm extends from the stomach to the ileocecal valve; 99% of worms inhabit the jejunum and proximal ileum, and it is rarely seen in the appendix. Appendicitis due to migration of roundworm into the appendix is still debatable because the symptoms of this migration may simulate appendicitis but rarely cause it[57,92,94].

Because parasites such as *Balantidium coli*[2,92], *Blastocystis hominis*[20], *Trichuris trichiura*[52,57,92], and *Strongyloides stercoralis*[121] have few causative roles, interpreting their pathogenesis is difficult. A final diagnosis should be established with a histopathological evaluation of all three parasites, and antihelminthic treatment should be administered after the appendectomy.

Endometriosis is defined as the presence of ectopic endometrial tissue outside the lining of the uterine cavity. Many women of reproductive age suffer from this disease, but its occurrence in the GI tract is rare. Intestinal endometriosis is classified as external endometriosis and occurs in only about 10% of women with endometriosis. Most intestinal endometriosis occurs in the rectum and sigmoid colon but rarely in the appendix. Appendiceal endometriosis is usually asymptomatic, but it occasionally causes appendicitis, perforation, and intussusception. The diagnosis of appendiceal endometriosis is based on the histological presence of endometrial tissue in the specimen. The treatment strategy consists mainly of surgery and hormone therapy[1,16,27,57-69,102].

The incidence of granulomatous appendicitis (GA),

a rare condition that may be discovered incidentally in a patient with a clinical presentation of acute appendicitis, ranges from 0.31% to 0.95%. Various infectious and non-infectious factors cause GA. Systemic conditions, such as Crohn's disease and sarcoidosis, may also be associated with granulomatous inflammation of the appendix. The initial belief that it represented a manifestation of Crohn's disease is incorrect in the great majority of cases, as only 5%-10% of patients with GA develop Crohn's disease elsewhere in their GI tract. Distinguishing idiopathic granulomatous appendicitis from early Crohn's disease, which affects only the appendix, is difficult. A definitive diagnosis can only be made after long-term follow-up, and sometimes further investigations are required[31,52,116-120,122,123].

Crohn's disease is a chronic transmural inflammation characterized by epithelioid granulation formation in the intestinal wall. The clinical presentation is always variable, and patients often present with findings consistent with acute appendicitis such as right-lower quadrant pain, fever, nausea, and anorexia. The diagnosis of appendiceal Crohn's disease requires exclusion of multiple entities. Infectious causes of granulomatous appendicitis include *Yersinia*, *Mycobacterium tuberculosis*, blastomycosis, *Schistosoma*, *Actinomyces*, *Campylobacter*, *Histoplasma capsulatum*, and some parasites. An appendectomy is a routine surgical procedure when the Crohn's disease is limited to the appendix with no postoperative or intraoperative mortality and a low rate of fistula formation[16,32,123].

In summary, although fecaliths and lymphoid hyperplasia are the usual causes of acute appendicitis, some unusual factors may also cause appendicitis. The most common unusual findings in appendectomy specimens are parasites and benign or malignant tumors. A simple appendectomy or right hemicolectomy can be performed depending on the localization, size, and histopathological structure of the tumor in the primary malignant appendiceal tumor, whereas an appendectomy alone is sufficient for benign tumors. Administering the appropriate antibacterial or antiparasitic treatment after the appendectomy is the proper approach for parasitic and bacterial infections that cause chronic inflammation. We emphasize and strongly recommend that all appendectomy specimens be examined histopathologically regardless of whether the specimens are macroscopically normal.

COMMENTS

Background

Appendicitis is one of most common acute surgical conditions of the abdomen, and an appendectomy is one of the most frequently performed operations worldwide. Obstruction of the lumen is the dominant factor in acute appendicitis, and although fecoliths and lymphoid hyperplasia are the usual causes of obstructions, some unusual factors could also be involved.

Research frontiers

The authors conducted a literature review *via* the PubMed and Google Scholar databases of English language studies published between 2000 and 2010 on unusual findings in appendectomy specimens. Also, we presented 54 patients who had unusual findings in their appendectomy specimens.

Innovations and breakthroughs

The authors emphasize and strongly recommend that all appendectomy specimens be examined histopathologically regardless of whether the specimens are macroscopically normal.

Peer review

This is a very interesting paper. It will be cited many times in the future and this is good for our journal.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.1971

World J Gastroenterol 2011 April 21; 17(15): 1971-1975
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Assay of ghrelin concentration in infant formulas and breast milk

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Supported by a MIUR Grant for Project of Research (ex 60%-2008) Italy
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Received: August 9, 2010 Revised: November 12, 2010
Accepted: November 19, 2010
Published online: April 21, 2011

Abstract

AIM: To test if total ghrelin is present in infant formulas.

METHODS: Using a radioimmunoassay, we measured total ghrelin concentrations in 19 samples of commercial infant formulas and in 20 samples of human milk. We also determined ghrelin concentration in the serum of infants and lactating mothers.

RESULTS: Ghrelin concentrations were significantly higher in artificial milk (2007.1 ± 1725.36 pg/mL) than in human milk (828.17 ± 323.32 pg/mL) (P = 0.005). The mean ghrelin concentration in infant serum (n = 56) was 1115.86 ± 42.89 pg/mL, and was significantly higher (P = 0.023) in formula-fed infants (1247.93 ± 328.07 pg/mL) than in breast-fed infants (1045.7 ± 263.38 pg/mL). The mean serum ghrelin concentration (mean ± SD) in lactating mothers (n = 20) was 1319.18 ± 140.18 pg/mL.

CONCLUSION: This study provides evidence that total ghrelin is present in infant formulas. This finding raises diverse questions regarding the uptake, absorption and metabolic effects of this hormone.

Key words: Breast feeding; Ghrelin; Human milk; Infants; Infant formulas

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Savino F, Petrucci E, Lupica MM, Nanni GE, Oggero R. Assay of ghrelin concentration in infant formulas and breast milk. *World J Gastroenterol* 2011; 17(15): 1971-1975 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/1971.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.1971

INTRODUCTION

It is now well established that early life nutrition plays an important role in long-term appetite control[1]. Indeed, neonatal nutrition is involved in the programming of feeding regulatory mechanisms in the central nervous system, and in those mediated by factors secreted from peripheral tissues. Besides peripheral circulating factors that regulate energy balance and adiposity, gastrointestinal peptides have been demonstrated to act as hunger signals. Among the known orexigenic peptides, ghrelin has been found to be the most powerful[2].

Ghrelin is involved in the short-term regulation of food intake, by stimulating appetite, and in the long-term regulation of weight and energy metabolism, by inducing adiposity[3]. It is released in a pulsatile manner, with a nocturnal peak. Ghrelin responds to meals, increasing 1-2 h before eating and returning to trough levels 1-2 h after a meal[4,5]. Ghrelin secretion increases under negative energy-balance

conditions, and decreases under positive energy-balance conditions, such as food intake and obesity[6,7]. It is one of the most powerful orexigenic and lipogenic hormones and represents an interface between energy balance regulation, glucose homeostasis and hypothalamic neuropeptides[8]. The amino acid sequence of ghrelin is highly conserved throughout mammalian species[9]. Ghrelin has been found in human milk, but the source of the hormone is unclear. Aydin *et al*[10] reported that its levels in colostrum, transitional and mature milk were lower than those found in plasma and they assumed that ghrelin present in milk probably comes from the plasma of lactating mothers. In contrast, Kierson *et al*[11] showed that ghrelin levels in breast milk are higher than plasma levels and identified ghrelin mRNA from human mammary epithelial cells and mammary gland. Based on these findings these authors suggested that ghrelin in breast milk is probably synthesized and secreted from the breast. The identification of ghrelin in breast milk suggests that breast milk is a source of compounds critical for the metabolic development of infants[12,13].

Early feeding mode affects growth and body composition[14]. Breast-fed (BF) and formula-fed (FF) infants have similar weight gains in the first three months of life, however, BF infants gain weight less rapidly during the following months of the first year[15]. BF and FF infants have different feeding behaviors: FF infants eat less frequently and consume higher amounts of food than BF infants[16,17]. We previously reported that serum ghrelin concentrations were higher in infants exclusively FF than in infants exclusively BF[18]. Recently, serum ghrelin values were positively correlated with fasting times only in FF infants[19].

The aim of this study was to investigate whether infant formulas contain ghrelin.

MATERIALS AND METHODS

In this study, we enrolled 56 infants aged from 11 d to 5 mo (mean ± SD: 81 ± 46 d) born from a normal spontaneous vaginal delivery, consecutively referred to the Department of Paediatrics of the University of Turin, Regina Margherita Children's Hospital. The inclusion criteria for infants were gestational age between 37 and 42 wk, birth weight appropriate for gestational age (between 2500 and 4000 g), Apgar score higher than 7 at 5 min, no fetal anomaly, absence of acute or chronic gastroenteric diseases or other growth-affecting pathologies.

We collected milk samples from 20 lactating mothers of the infants enrolled. Eligibility criteria for mothers were: no maternal medical complications, non-smoking mothers, normal response to a glucose tolerance test, no mastitis, no prescribed medication, no digestive disorders.

Appropriate Ethics Committee permission was obtained and each parent signed a written informed consent. Milk samples (2 mL) were collected from the lactating women (n = 20) before breakfast at around 09:00 h. Samples of 19 infant formulas were collected: 9 starting formulas, 6 follow-on formulas and 4 special formulas (anti-regurgitation and hydrolyzed casein protein formulas). We also obtained unpasteurized milk samples (2 mL) from 5 dairy cows. Breast, cow and artificial milk were separated by centrifuging the samples twice at 2000 r/min and 4℃ for 20 min. After the first centrifugation, the thick fat layer at the top of the tube, which could interfere with detection of the hormone, was removed with a sterile toothpick. We collected a venous blood sample from 56 infants (37 BF infants and 19 FF infants) after a fast of 3 h. We also collected venous blood samples from 20 lactating mothers. Blood samples were immediately centrifuged at 4000 r/min and 4℃ for 10 min and each sample of the resulting serum was divided in 3 tubes and these were stored at -30℃ until analysed.

Hormone assays

Serum and milk total ghrelin were assayed by radioimmunoassay using a commercial kit (Ghrelin (total) RIA 3967, DRG Diagnostic, New Jersey, USA) according to the manufacturer's instructions (using a polyclonal antibody that recognizes octanoylated and non-octanoylated ghrelin with I^{125} ghrelin as a tracer molecule). Measurements of total ghrelin in mature milk and serum samples have been validated as reported elsewhere[10]. The intra-assay and inter-assay coefficients of variation of ghrelin were 5% and 7.6%, respectively. The lowest level of ghrelin that can be detected by this assay is 93 pg/mL with a 100-μL sample size. The specificity for human ghrelin is 100%. The limit of linearity for the ghrelin assay is 6000 pg/mL (any result greater than 6000 pg/mL was repeated on dilution using Assay Buffer as a diluent). Spike and Recovery of ghrelin in human plasma are shown in Table 1. As the kit is designed for human samples, a validation process was undertaken for use with bovine milk. We obtained 3 measurements of the hormone for each blood sample and milk sample. The manufacturer's protocol was followed and standard validations, including parallelism and recovery, conducted.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

A normal distribution was verified with the Shapiro-Wilk test ($P > 0.05$). The data are expressed as arithmetic means ± SD; $P < 0.05$ was considered statistically significant. Differences in ghrelin concentrations between human milk and formula milk were determined by the Student's t test.

RESULTS

As shown in Table 2, the mean ± SD of ghrelin concentration in human milk was 828 ± 323 pg/mL. Ghrelin levels varied widely in infant formulas (from 300 to 6110 pg/mL; mean: 2007 ± 1725 pg/mL). The mean ghrelin concentration in starting formulas and in follow-on formulas was 2699 ± 233.5 pg/mL and 2561 ± 215.2 pg/mL, respectively, whereas the mean ghrelin concentration was 1160 ± 340 pg/mL in special formulas (Table 3). Ghrelin

Table 1 Spike and recovery of ghrelin in human plasma (mean of the observed levels from 3 duplicate determinations in 3 separate assays)

Sample No.	Ghrelin added[1] (pg/mL)	Recovery (%)
1	500	96
2	1000	90
3	2000	91

[1]Different concentrations of human ghrelin were added to 3 different human plasma samples and the ghrelin content was determined by RIA. Percent recovery was calculated on the observed *vs* expected.

Table 2 Ghrelin concentrations in infant formulas, in mother's milk, in cow's milk and in serum of lactating mothers, breast-fed infants and formula-fed infants

	Ghrelin concentration (pg/mL ± SD)
Milk	
Different kinds of artificial infant formula (n = 19)	2007.1 ± 1725.36[a]
Breast milk (n = 20)	828.17 ± 323.32[a]
Non pasteurized cow milk (n = 5)	2816.00 ± 219.00
Serum	
Lactating mothers (n = 20)	1319.18 ± 140.18
Breast-fed infants (n = 37)	1045.7 ± 263.38[b]
Formula-fed infants (n = 19)	1247.93 ± 328.07[b]

[a]P = 0.005, infant formulas *vs* human milk, student's t test; [b]P = 0.023, breast-fed infants *vs* formula-fed infants, student's t test.

levels were significantly higher in infant formulas than in human milk (P = 0.005). The mean ghrelin concentration in unpasteurized cow milk was 2816 ± 219 pg/mL. The mean ghrelin concentration in infant serum was 1115.86 ± 42.89 pg/mL, and was significantly higher (P = 0.023) in FF infants (1247.93 ± 328.07 pg/mL) than in BF infants (1045.7 ± 263.38 pg/mL). The mean serum ghrelin concentration (mean ± SD) in lactating mothers was 1319.18 ± 140.18 pg/mL.

DISCUSSION

In this study, we showed that artificial milk contains the orexigenic hormone, ghrelin, and that its concentration was higher in infant formulas than in human milk. This finding might explain the higher serum levels we previously observed in FF infants *vs* BF infants, which was also confirmed in the present research[18,19].

If FF infants receive a higher amount of ghrelin, it is conceivable that they have a greater feeding stimulus than BF infants, and a consequent increase in weight and growth rate. Our observations could explain the more appropriate growth curves of BF infants, who physiologically receive less ghrelin[20]. Therefore, breast-feeding may protect against the development of obesity in childhood and adulthood, not only because of its nutrient composition, but also because of the presence of bioactive factors such as ghrelin, leptin and adiponectin[12,21].

Table 3 Ghrelin concentrations in three different types of artificial infant formula

Type of infant formula	Ghrelin concentration (pg/mL ± SD)
Starting infant formulas (n = 9)	2699 ± 233.5
Follow-on infant formulas (n = 6)	2561 ± 215.2
Special infant formulas (anti-regurgitation and hydrolyzed casein protein formulas) (n = 4)	1160 ± 340

Several assays are available to measure human serum ghrelin, whereas there is no commercial milk ghrelin assay kit. Consequently, we carried out a validation process using a basic clinical chemistry method (linearity)[10]. Similarly, there is no assay kit specific for bovine ghrelin; however, given the high structural homology between human ghrelin and mammalian ghrelin, the kit used in our study to detect ghrelin in infant formulas can be considered reliable[22].

The ghrelin concentrations reported in our study refer to the final volume after centrifugation, because the RIA kit instructions indicate that samples with high lipidemia be avoided. After centrifugation, we removed the supernatant and the fat layer that could interfere with detection of the hormone. This method has been used in previous studies[10]. Kierson *et al*[11] reported higher ghrelin levels in whole milk than skimmed milk, with a direct relationship between estimated milk fat content and ghrelin levels. Therefore, it is possible that ghrelin levels detected in milk after centrifugation are lower than those present in whole milk and consumed by the infants.

The mechanism by which ghrelin influences growth in early infancy is not yet completely known. Ghrelin levels are higher in small-for-gestational-age (SGA) newborns than in adequate-for-gestational age (AGA) newborns[23]. Reduced ghrelin suppression and higher postprandial ghrelin concentrations in SGA infants could cause a sustained orexigenic drive and may contribute to catch-up growth in these infants. Kitamura *et al*[24]. reported high ghrelin levels during the early neonatal period. Onal *et al*[25] observed that plasma ghrelin concentration was inversely associated with birth weight and body length in term newborns. We previously observed a negative correlation between ghrelin concentration and weight gain in BF infants in the first months of life, which suggests that ghrelin may play a role in body weight regulation in healthy infants[26,27]. More recently, we found a positive correlation between circulating ghrelin concentration and fasting time in FF infants; these infants have a higher serum ghrelin concentration, longer fasting time and fewer meals than BF infants[19]. Clearly, we need to learn more about early feeding and the mechanisms regulating satiety and feeding behavior.

Finally, it should be noted that, similar to previous studies[11,28], we measured total milk ghrelin level. Deacyl ghrelin influences food intake, gut motility, insulin secretion and resistance and adipogenesis, whereas the acylated form of ghrelin, known as active ghrelin, is essential for binding to the growth hormone secretagogue receptor 1a[29]. Additional studies are needed to evaluate active ghre-

lin in infant formulas.

There have been some studies conducted on the effects of ghrelin administration (i.e. in anorexia, cachexia), however, the hormone was administered intravenously and not orally[30]. More recently, animal studies have investigated the effects of oral administration of ghrelin receptor agonist, and found that it survives the acid environment of the stomach and could exert some of its biological functions[31,32].

In conclusion, specific research is needed to understand the origin of ghrelin found in infant formulas. Furthermore, it would be interesting to determine whether ghrelin present in infant formulas survives the acid environment of the stomach in humans, exerts biological activity through receptors present in the gastrointestinal tract of newborns and whether the higher ghrelin concentration in infant formulas result in a higher serum concentration in FF infants. Lastly, investigations are required to determine whether these higher levels of ghrelin affect feeding habits and thus obesity in later life.

ACKNOWLEDGMENTS

We are grateful to Drs. G Brossa and F Pluviano of the Nuclear Medicine Hospital Molinette, Turin, Italy who evaluated the RIA data. We thank Jean Ann Gilder (Scientific Communication srl) for text editing.

COMMENTS

Background

Current research highlights the importance of early life nutrition in long-term appetite control, with consequent programming of regulatory mechanisms. Ghrelin is a recently discovered hormone involved both in the short-term regulation of food intake, by stimulating appetite, and in the long-term regulation of weight and energy metabolism, by inducing adiposity.

Research frontiers

Ghrelin has recently been detected in breast milk, but data on ghrelin in infant formula are lacking. Using a radioimmunoassay, the authors measured ghrelin concentrations in commercial infant formulas *versus* concentrations in human milk. Surprisingly, ghrelin was significantly higher in artificial formulas. This finding raises diverse questions.

Innovations and breakthroughs

Little is known about ghrelin regulation, especially in early infancy. Breast milk contains ghrelin, but it was not known whether infant formulas contain ghrelin. The finding that infant formulas do indeed contain ghrelin, and at levels higher than those found in breast milk, raises questions about the uptake, absorption and metabolic effects of this feeding stimulus and growth rate of artificially fed infants. Further research is needed to determine whether the higher levels of ghrelin in formulas could affect infant feeding habits and thus obesity in later life.

Applications

The higher ghrelin levels found in artificial milk in the present study might explain the higher serum values the authors recently observed in formula-fed infants compared with breast-fed infants. Thus, if artificially fed infants receive a higher amount of ghrelin together with a higher intake of protein, it is conceivable that formula-fed infants have a greater feeding stimulus with a consequent increase in weight and growth rate.

Terminology

Ghrelin is involved in the short-term regulation of food intake, with an orexigenic action, and in the long-term regulation of weight and energy metabolism, by inducing adiposity.

Peer review

The manuscript by Savino *et al* provides original data on the presence of the orexigenic hormone ghrelin cow's milk formulas. The authors suggest that the high levels of hormone present in formulas can influence the feeding habits of formula feed subjects in infancy and also later and can be responsible for the high risk of obesity observed in formula fed infants as compared to breast fed ones. The article could be of some general interest, both for pediatricians and for nutritionists.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.1976

World J Gastroenterol 2011 April 21; 17(15): 1976-1981
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Factors associated with irritable bowel syndrome symptoms in hemodialysis patients

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Received: August 19, 2010 Revised: October 5, 2010
Accepted: October 12, 2010
Published online: April 21, 2011

Abstract

AIM: To investigate clinical characteristics associated with the presence of irritable bowel syndrome (IBS) symptoms in hemodialysis (HD) patients.

METHODS: This was a cross-sectional study. A questionnaire based on the Bowel Disease Questionnaire that records gastrointestinal symptoms was given to 294 patients in 4 dialysis centers. A total of 196 (67%) subjects returned the survey. A multivariable logistic regression model was used to identify factors significantly associated with IBS symptoms.

RESULTS: Symptoms compatible with IBS were present in 27 (13.8%) subjects and independently associated with low post-dialysis serum potassium [OR = 0.258, 95% CI (0.075-0.891), P = 0.032], paracetamol use [OR = 3.159, 95% CI (1.214-8.220), P = 0.018], and Kidney Disease Quality of Life (KDQOL) cognitive function score [OR = 0.977, 95% CI (0.956-0.999), P = 0.042]. Univariate regressions were also performed and the reported significance is for multivariate analysis. No association was detected for age, gender, depressed mood, smoking (present or past), body mass index, albumin level, Kt/V, sodium pre- or post-dialysis level, change in potassium level during HD, proton pump inhibitor or H2 blocker use, aspirin use, residual diuresis, hepatitis B or C infection, diabetes mellitus, marital status and education level.

CONCLUSION: This study examined potential risk factors for symptoms compatible with IBS in HD patients and identified an association with paracetamol use, post-dialysis potassium level and KDQOL-cognitive function score.

Key words: Hemodialysis; Irritable bowel syndrome; Risk factors

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Fiderkiewicz B, Rydzewska-Rosołowska A, Myśliwiec M, Birecka M, Kaczanowska B, Rydzewska G, Rydzewski A. Factors associated with irritable bowel syndrome symptoms in hemodialysis patients. *World J Gastroenterol* 2011; 17(15): 1976-1981 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/1976.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.1976

INTRODUCTION

Chronic gastrointestinal symptoms are common in patients with chronic kidney disease (CKD). The prevalence rate is reportedly as high as 70%[1-4], and there is an association with impaired psychological well-being[5]. Among these gastrointestinal symptoms, irritable bowel syndrome (IBS) is also more frequent than in the general population, and is present in 11%-44% of hemodialysis (HD) patients[2-4]. Although the pathophysiology of IBS is uncertain, altered gut reactivity (motility, secretion), visceral hypersensitivity and dysregulation of the brain-gut axis are believed to play an important role[6]. The risk factors associated with IBS in HD patients are not known. The aim of this study was to determine the possible relationship between IBS symptoms in HD patients and their clinical characteristics.

MATERIALS AND METHODS

Patients

This was a cross-sectional study. All patients in 4 HD centers (2 state financed and 2 privately owned) were asked to complete a questionnaire. The questionnaires were given to patients during a planned hemodialysis procedure. The study was approved by the local Ethics Committee.

All of the subjects were Caucasian. They were dialyzed 3 times a week for 180 to 300 min using either polysulfone or cellulose acetate dialyzers and bicarbonate dialysis fluid containing 2 mEq/L of potassium in 3 centers and 2 or 3 mEq/L (adjusted on the basis of the knowledge of prevailing pre-dialysis serum potassium levels in a given individual) in 1center.

Causes of ESRD were as follows: glomerulonephritis, $n = 60$ (30.6%); diabetic nephropathy, $n = 32$ (16.3%); amyloidosis, $n = 11$ (5.6%); polycystic kidneys, $n = 22$ (11.2%); hypertension/atherosclerosis, $n = 16$ (8.2%); tubulointerstitial disease, $n = 39$ (19.9%); unknown/uncertain, $n = 15$ (7.7%); nephrectomy, $n = 1$ (0.5%).

Questionnaire

A questionnaire based on the Bowel Disease Questionnaire was used[7]. It was translated to the Polish language by 2 of the authors (BF, AR). Translations were compared and discrepancies reconciled. The resulting translation was then tested in 20 randomly selected dialysis patients, and as a result some of the expressions in the translation were altered to make them easier to understand. The questionnaire was then checked by a person who was not involved in translation (ARR), and finally evaluated by a certified gastroenterologist (GR).

IBS was defined using Manning criteria[8] as described by Talley *et al*[9], as an ache or pain that occurred more than 6 times per year which was either often made better by a bowel movement or often associated with more frequent or looser bowel movements when the pain began. In addition, 2 or more of the following symptoms had to be present: fewer than 3 bowel movements per week or more than 3 bowel movements per day; loose, watery stools or hard stools; straining to have bowel movements; feelings of incomplete rectal evacuation; urgency; mucus; or bloating with distention.

Additionally, we included questions taken from the validated Polish translation of Kidney Disease Quality of Life (KDQOL) questionnaire, related to depressed mood and cognitive function[10]. Depressed mood was measured by the following KDQOL items: How much of the time during the last 30 d have you felt so down in the dumps that nothing could cheer you up? and How much of the time during the last 30 d have you felt downhearted and blue? The six possible responses to these questions were (1) none of the time; (2) a little of the time; (3) some of the time; (4) a good bit of the time; (5) most of the time; and (6) all of the time. Patients were classified as reporting depressed mood when they indicated that they had felt down in the dumps or felt downhearted and blue a good bit of the time or more often[11].

Cognitive function was measured by the KDQOL-CF score. Patients had to answer the following questions: During the past 4 wk, did you react slowly to things that were said or done? Did you have difficulty concentrating or thinking? Did you become confused? Responses on a six-point scale were weighted and transformed to a score ranging from 0 to 100, with higher scores indicating better self-assessed cognitive function[12].

Relevant laboratory and clinical data were extracted from medical records. Data corresponding closest to the date of the HD session during which the questionnaire was distributed, were used. We allowed for a time span of 14 d before and after HD.

Statistical analysis

Results are expressed as means ± SD or frequency. Variables were tested for normality of distribution using the Wilk-Shapiro test. The Fisher's exact test and χ^2 test were used for comparing categorical variables, as appropriate.

Univariate and multivariable logistic regression was used to identify patient characteristics associated with IBS compatible symptoms. Risk factors considered in this analysis included age, sex, education level, marital status, presence of diabetes mellitus, procedure, hemoglobin level, pre- and post-HD potassium level, change in potassium level during HD, use of paracetamol in the last year, KDQOL-CF score, depressed mood, smoking

Table 1 Patients' demographic and clinical data expressed as (mean ± SD) *n* (%)

Group	IBS symptoms		All	*P* value
	(+)	(-)		
n	27	169	196	
Gender (M/F)	13/14	105/64	118 / 78	0.168
Age (yr)	68.1 ± 11.5	63.2 ± 13.4	63.9 ± 13.2	0.073
Dialysis duration (min)	40.1 ± 36.9	38.6 ± 45.8	38.8 ± 44.6	0.874
BMI (kg//m^2)	25.0 ± 3.7	24.9 ± 4.9	24.9 ± 4.7	0.897
Residual diuresis (mL/24 h)	301 ± 559	401 ± 592	388 ± 42	0.411
Kt/V	1.26 ± 0.31	1.21 ± 0.27	1.22 ± 0.28	0.355
Hepatitis C or B infection (*n*)	7 (25.9%)	31 (18.3%)	38 (19.4%)	0.430
Hemoglobin (g/dL)	11.4 ± 1.4	10.7 ± 1.5	10.8 ± 1.5	0.026[a]
Albumin (g/dL)	3.72 ± 0.40	3.71 ± 0.44	3.71 ± 0.44	0.857
Smoking (*n*)	7 (25.9%)	23 (13.6%)	30 (15,3%)	0.144

IBS: Irritable bowel syndrome; BMI: Body mass index. [a]$P < 0.05$.

(present or past), body mass index (BMI), albumin level, Kt/V, sodium pre- and post-dialysis level, proton pump inhibitor (PPI) or H2 blocker use, aspirin and paracetamol use, residual diuresis, hepatitis B or C infection. Variables were included in the multivariable logistic model if $P < 0.10$ in the univariate analysis. A P value less than 0.05 was considered statistically significant. The software, used for statistical computations was Stata 9.2 (StataCorp, College Station, TX, USA).

RESULTS

Patients

A total of 294 HD patients were asked to complete the questionnaire, of which 196 were returned giving a 67% response rate. All the responders completed the questionnaires by themselves. Their clinical characteristics are given in Table 1.

IBS symptoms

Symptoms compatible with IBS were present in 27 (13.8%) subjects. They were more common in women (18.0%) than in men (11.0%), but the difference was not statistically significant ($P = 0.168$). Symptoms of IBS were more frequent in patients with a post-hemodialysis potassium level ⩽ 3.5 mEq/L than in subjects with potassium > 3.5 mEq/L. Also pre-dialysis potassium level was related to the frequency of IBS symptoms (Figure 1).

In univariate logistic regression, pre-dialysis serum potassium [OR = 0.462, 95% CI (0.222-0.965), $P = 0.040$], post-dialysis serum potassium [OR = 0.237, 95% CI (0.084-0.666), $P = 0.006$], hemoglobin level [OR = 1.403, 95% CI (1.038-1.897), $P = 0.028$], use of paracetamol in the last year [OR = 3.541, 95% CI (1.499-8.364), $P = 0.004$], and KDQOL-CF score [OR = 0.972, 95% CI (0.954-0.991), $P = 0.004$] were associated with IBS symptoms. Age ($P = 0.076$), gender ($P = 0.172$), depressed mood ($P = 0.118$), smoking (present or past) ($P = 0.105$), BMI ($P = 0.896$), albumin level ($P = 0.856$), Kt/V ($P = 0.353$), sodium pre- ($P = 0.961$) or post-dialysis level ($P = 0.176$), change in potassium level during HD ($P = 0.556$), PPI or H2 blocker use ($P = 0.857$), aspirin use ($P = 0.172$), residual diuresis ($P = 0.411$), hepatitis B or C infection ($P = 0.358$), diabetes mellitus ($P = 0.822$), marital status ($P = 0.941$) and education level ($P = 0.377$) were not associated with IBS symptoms.

Table 2 Multiple logistic regression analysis to identify independent predictors of irritable bowel syndrome symptoms in hemodialysis patients

Variable	*b* coefficient (SE)	*P* value	OR (95% CI)
Potassium level pre-HD	-0.325 ± 0.437	0.457	0.723 (0.307-1.703)
Potassium level post-HD	-1.356 ± 0.633	0.032[a]	0.258 (0.075-0.891)
Paracetamol use	1.150 ± 0.488	0.018[a]	3.159 (1.214-8.220)
Cognitive function	-0.023 ± 0.011	0.042[a]	0.977 (0.956-0.999)
Hemoglobin	0.327 ± 0.168	0.052	1.387 (0.998-1.928)
Age	0.026 ± 0.021	0.213	1.027 (0.985-1.070)

HD: Hemodialysis; OR : Odds ratio. [a]$P < 0.05$.

When the risk factors for symptoms of IBS were assessed by multiple logistic regression analysis, independent predictors of IBS symptoms included: paracetamol use, post-dialysis serum potassium and KDQOL-CF score (Table 2).

DISCUSSION

The frequency of symptoms compatible with IBS in our study is somewhat higher than that reported (11%) among 105 Austrian HD patients[3] using similar criteria, and lower than that among 148 English HD patients (21%) using Rome Ⅱ criteria[2]. In the latter study, IBS was significantly more common in HD subjects than in both hospital outpatients and community controls[2]. In a study from Turkey, the prevalence of IBS, using Rome Ⅱ criteria, was 44% among 93 HD patients, significantly more common than in healthy volunteers (21%)[4].

The overall IBS prevalence in Europe is 11.5%[13]. It varies, however, widely among countries, being highest in the UK and Italy[13], depending to a large extent on the diagnostic criteria used[14]. There is even more variability between continents[15]. Unfortunately, there is no data on IBS prevalence in the general population in Poland.

As there is no biologic marker of the disease, the diagnosis of IBS relies heavily on symptom-based criteria. The most widely used is a consensus definition called the Rome criteria[16,17], where IBS is defined chiefly by abdominal pain associated with defecation or a change in bowel habit and with features of disordered defecation. Some researchers have suggested that these criteria overemphasize abdominal pain and fail to emphasize postprandial urgency, abdominal pain, and/or diarrhea[18,19]. We thought that for the HD population, with frequent comorbidities, the use of supportive symptoms that are not part of the Rome criteria would be more appropriate. The Kruis scoring system[20], which in addition to self-reported symptoms includes: erythrocyte sedimentation

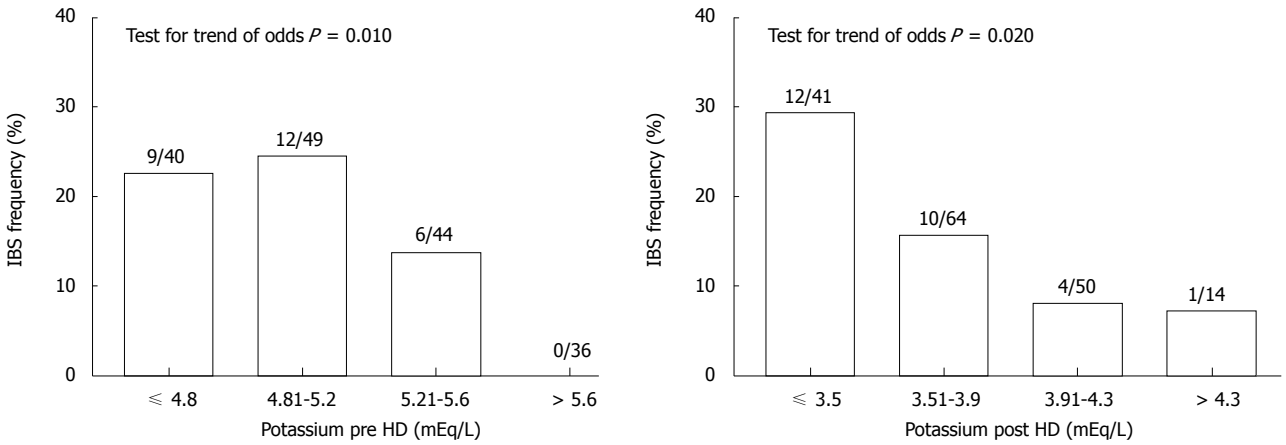

Figure 1 Frequency of irritable bowel syndrome symptoms in hemodialysis subjects stratified by pre- or post-hemodialysis potassium level. Numerator corresponds to number of irritable bowel syndrome (IBS) cases and denumerator to number of all patients with given potassium level. HD: Hemodialysis.

rate, leukocytosis and anemia could not be used in HD patients for obvious reasons.

Fulfillment of IBS criteria is not unequivocally diagnostic of IBS, however, to make a positive diagnosis of IBS the use of diagnostic criteria is the recommended method, rather than exhaustive investigations to exclude an underlying organic cause[21]. Surprisingly, however, very few studies have examined the utility of the various diagnostic criteria in differentiating IBS from organic disease. The authors of a recent systematic review were able to find only 4 studies that reported on the accuracy of the Manning criteria, 1 study that reported on the Rome I criteria, and no studies on the Rome Ⅱ or Rome Ⅲ criteria[21].

In clinic-based studies, IBS has been associated with female gender, psychological distress, physical and sexual abuse, food allergies, enteric infections, and previous abdominal surgeries[22]. In a community-based study Locke *et al*[23]found associations with somatic symptoms, analgesic use, and food allergies and sensitivities[23].

The association between acetaminophen use and IBS has been previously reported, and is difficult to explain[23]. A possibility exists that most people take paracetamol for their IBS. It is interesting that there was no association between aspirin use and IBS symptoms, similar to Locke *et al*[23] when the use of these drugs was reported independently. Additionally, in this country aspirin is most frequently used for cardiovascular prophylaxis.

Cognitive deficits often accompany chronic illnesses, although the underlying mechanisms are not fully understood and may differ between diseases. Cognitive impairment is common in HD patients[24]. Risk factors include: age, race, stroke, diabetes, low education status, anemia, measures of malnutrition and an equilibrated Kt/V $\geqslant$ 1.2[25,26]. Also, IBS in the general population is associated with cognitive impairment[27,28]. An observed association between IBS symptoms and KDQOL-CF score might suggest that IBS worsens cognitive deficits in HD patients. We used the KDQOL-CF self reported score for the assessment of cognitive function. Although the KDQOL-CF provides estimates rather than a definitive assessment of cognitive function, it was shown that the KDQOL-CF scale scores correlate with the Modified Mini-Mental State Examination, and are acceptable for estimating cognitive function in dialysis subjects[12,29].

In the general population, IBS is associated with stress[6]. In our study, the presence of IBS compatible symptoms was not related to the presence or absence of anxiety or depression. This is similar to the findings by Cano and colleagues[2]. They, therefore, concluded that IBS in HD patients might be related to either the "uremic" state or the treatment method. This is in contrast to Kahvecioglu *et al*[4] who found that IBS in dialyzed patients was associated with depression and anxiety. In the general population, IBS seems to be more common in younger people. Our population was mostly over 50 years, and that might explain the lack of an age effect on the frequency of IBS symptoms.

Patients suffering from diabetes mellitus report a greater prevalence of gastrointestinal symptoms than controls, which is not related to glycemic control[30]. We did not observe, however, any difference in the frequency of IBS symptoms between diabetic and non-diabetic HD patients. This is in agreement with Cano *et al*[2].

It is difficult to offer an explanation for the unexpected univariate association between IBS symptoms and hemoglobin level. Patients dialyzed in central and eastern European dialysis centers have lower mean hemoglobin levels and are less likely to attain target levels than those treated in western European counterparts[31]. HD patients with higher hemoglobin levels report higher quality of life, and IBS patients in the general population have lower health-related quality of life[32]. Additionally, gastrointestinal symptoms in patients with chronic kidney disease are associated with impaired general psychological well-being[5]. However, IBS patients have a propensity to report pain and label negatively expected adverse sensations, so it is conceivable that IBS specific symptoms are "unmasked" in patients who have an overall higher quality of life.

To our knowledge, the association between serum potassium level and the frequency of symptoms compatible with IBS has not been reported before. This finding, however, has to be treated with caution. Although gastrointestinal motility is impaired in chronic pre-dialysis kidney disease[33], it is alleviated by hemodialysis[34]. Thus, another mechanism may be responsible. Hypokalemia may cause decreased motility and propulsive activity of the intestine, and even lead to ileus. We recorded potassium levels before and after a single HD session, whereas a level prevailing over a specific time period might be more appropriate. It may be, however, that episodes of hypokalemia, which are likely just after hemodialysis, are responsible for the appearance of IBS symptoms. A consistent trend of a higher prevalence of IBS compatible symptoms with lower potassium concentration, also suggests a causative role for hypokalemia. During conventional HD, large amounts of potassium are removed, approximately 40% of which originates from the extra- and the remainder from the intracellular space[35]. A change in the plasma potassium concentration during hemodialysis, however, is difficult to predict, due to the concomitant movement of the ion into cells due to correction of metabolic acidosis. After HD, plasma potassium concentration increases rapidly during the first hour and steadily thereafter. The post-dialysis rise in potassium concentration is not correlated with pre- or post-dialysis plasma K[35].

In CAPD patients, it has been reported that episodes of hypokalemia are a risk factor for developing peritonitis and bacterial overgrowth, possibly due to altered intestinal motility[36,37].

In our study, neither the dialysis session time nor the change in potassium level influenced the prevalence of IBS symptoms, what suggests that the between dialyses level rather than the intradialytic change is important. In line with this electrophysiological mechanism reasoning, is the observation that pre-dialysis potassium < 4.0 or > 5.6 mEq/L is associated with increased mortality in HD patients, most probably due to cardiac arrhythmias[38]. Despite the plausible electrophysiological mechanism, the association between potassium level and IBS symptoms might be confounded by other factors. It has been suggested for both HD and PD patients, that hypokalemia could be a surrogate marker for poor nutrition and associated comorbidities[38,39].

This study has a number of shortcomings: firstly the Bowel Disease Questionnaire was not formally validated. To that end we ensured that the Polish translation was faithful and easy to understand. Additionally, the number of subjects was rather low, the study was observational and could not prove causality in relationships. Finally, the study potentially lacked generalizability due to cross-cultural differences in the symptomatology of functional gastrointestinal disorders[15].

In summary, this study examined potential risk factors for symptoms compatible with IBS in HD patients and identified an association with acetaminophen use, serum potassium level, and KDQOL-cognitive function score. The role of hypokalemia requires further well designed and controlled clinical studies.

COMMENTS

Background

Chronic gastrointestinal symptoms are very common in patients with chronic kidney disease (CKD) treated by hemodialysis (HD) including irritable bowel syndrome (IBS). Risk factors associated with IBS in HD patients are not known.

Research frontiers

Risk factors that are associated with IBS in the general population include: somatic symptoms, female gender, psychological distress, physical and sexual abuse, food allergies, enteric infections, previous abdominal surgeries, analgesic use, and food allergies and sensitivities. Of the 196 HD patients included in this study, symptoms compatible with IBS were present in 27 (13.8%) subjects and were independently associated with low post-dialysis serum potassium, paracetamol use, and KDQOL cognitive function score.

Innovations and breakthroughs

This study showed that low post-dialysis serum potassium, paracetamol use, and KDQOL cognitive function score are independently associated with increased risk of IBS compatible symptoms.

Applications

This analysis of the risk factors for IBS may be helpful in reducing the risk of abdominal symptoms in HD patients.

Peer review

The authors report an observational study looking at various factors associated with IBS as defined by Manning criteria in haemodialysis patients.

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S- Editor Sun H **L- Editor** Webster JR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.1982

World J Gastroenterol 2011 April 21; 17(15): 1982-1988
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Viscosity of food boluses affects the axial force in the esophagus

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Received: October 13, 2010 Revised: January 4, 2011
Accepted: January 11, 2011
Published online: April 21, 2011

Abstract

AIM: To study the effect of viscosity on axial force in the esophagus during primary peristalsis using a newly validated impedance-based axial force recording technique.

METHODS: A probe able to simultaneously measure both axial force and manometry was positioned above the lower esophageal sphincter. Potable tap water and three thickened fluids were used to create boluses of different viscosities. Water has a viscosity of 1 mPa·s. The three thickened fluids were made with different concentrations of Clinutren Instant thickener. The viscous fluids were in appearance comparable to pudding (2 kPa·s), yogurt (6 kPa·s) and slush ice (10 kPa·s). Six healthy volunteers swallowed 5 and 10 mL of boluses multiple times.

RESULTS: The pressure amplitude did not increase with the bolus viscosity nor with the bolus volume whereas the axial force increased marginally with bolus volume ($0.1 > P > 0.05$). Both techniques showed that contraction duration increased with bolus viscosity ($P < 0.01$). Association was found between axial force and pressure but the association became weaker with increasing viscosity. The pressure amplitude did not increase with the viscosity or bolus volume whereas the axial force increased marginally with the bolus size.

CONCLUSION: This indicates a discrepancy between the physiological functions that can be recorded with axial force measurements and pressure measurements.

Key words: Axial force; Manometry; Esophagus; Primary peristalsis; Viscosity

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Gravesen F, Behan N, Drewes A, Gregersen H. Viscosity of food boluses affects the axial force in the esophagus. *World J Gastroenterol* 2011; 17(15): 1982-1988 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/1982.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.1982

INTRODUCTION

The primary function of the esophagus is to transport swallowed material from the pharynx to the stomach. A voluntary swallow initiates coordinated neuro-motor activity resulting in an aborally propagating contraction termed primary peristalsis. The primary effect of peristalsis is to develop force in the axial direction to pass the food into the stomach. Esophageal peristalsis depends on several factors such as body position, gravity, bolus size, and bolus viscosity[1,2].

The most common method to study esophageal peristalsis is manometry using low-compliance perfused catheters or more recently high-resolution manometry using multiple solid state transducers mounted on the catheter.

It has been shown that bolus volume affects the peristaltic contraction velocity and duration[3,4]. The interval between swallows[5,6], body position[2,4] and temperature of the bolus[7] also affect peristalsis. However, the pressure amplitude is not affected by increasing bolus viscosity whereas the duration and velocity is reduced[7,8].

Since the swallowed material is transported in the axial (longitudinal) direction of the esophagus, axial force measurements from a theoretical and practical standpoint better reflect esophageal function than pressure recordings do. Manometry measures the radial pressure which merely is an indirect measurement of the radial force (perpendicular to the axial force direction). Video-fluoroscopy is used to visually assess esophageal motor flow but it does not provide quantitative information on force in either radial or axial directions[9,10]. Several attempts have been made to develop techniques to measure the axial force in the esophagus[11-14]. Despite promising initial results based on strain gauge technology, this method has never been thoroughly tested or never made a breakthrough in clinical studies. Thus, only scarce data exist on the axial force in the esophagus. To the best of our knowledge, no studies have been published on the effect of bolus viscosity with axial force recordings.

The aim of this paper was to study the effect of bolus viscosity on axial force in the esophagus during primary peristalsis using a newly validated impedance-based force recording technique[15,16] and to compare these results with manometric recordings.

MATERIALS AND METHODS

Probe design and hardware setup

The probe was custom-made to measure axial force and pressure simultaneously at different positions on the probe (Ditens A/S, Aalborg, Denmark). The probe was 60 cm long and constructed from three different catheters. The proximal catheter of the probe was used for manometry, the middle catheter contained the transducer for axial force measurement, and the distal catheter was 2.5 cm long and contained a small bag (Figure 1).

The proximal catheter of the probe was made from an 8-lumen polyurethane catheter with an outer diameter of 4.6 mm. The 8 channels had different diameters. Three channels (diameters of 0.5 mm) were used for manometric measurements using a low compliance perfusion system. The side holes for manometric measurements were placed 6, 8 and 10 cm proximal to the tip of the catheter. Steel threads were placed in a 1.0 mm lumen to avoid elongation of the proximal catheter. One 0.5 mm lumen contained two wires connected to the force transducer electrodes in the middle catheter. Two channels with diameters of 2.0 mm were used to re-circulate saline (0.09%) in the middle catheter and to inflate the bag. The last lumen with a diameter of 1.3 mm contained a temperature sensor (TC Ltd., Uxbridge, England) for measurement 0.5 cm proximal to the force transducer. It was used to temperature compensate the impedance signal.

The middle catheter was 2 cm long and consisted of three single-lumen catheters/cylinders inside each other[15,17]. The innermost catheter was 3 mm in diameter and made of elastic Natvar catheter (Colorite Polymers, Belfast, Ireland). Two electrodes for electrical impedance measurement were placed inside this catheter. One electrode was mounted on the distal catheter and the other on the proximal catheter. Two overlapping rigid cylinders (outer diameters of 4 and 5 mm) surrounded the inner catheter. They protected the elastic catheter from radial forces and from bending, factors that would introduce errors.

The distal catheter was a non-stretchable catheter with an outer diameter of 1.5 mm through which inflation of the bag was done. The cylindrical shaped bag was made of 25 μm thick polyurethane (Ditens A/S, Aalborg, Denmark) and contained up to 13 mL. It was mounted on the outer rigid cylinder and the distal catheter. Tensile axial force applied to the bag made the rigid cylinders and electrodes move apart, resulting in increased electrical impedance. The electrical impedance, measured as the electrical potential difference (voltage), was calibrated to axial force [g] by applying precision weights in the range of 0-200 g.

Subjects

Six healthy men were included in the study (mean 38.3 years, range 25-61). Oral and written informed consent was obtained from all subjects. Ethics approval for the study was obtained from the Local Ethics Committee (Protocol No. VN 2003/120 mch).

Protocol

The catheter was first inserted through the mouth and esophagus into the stomach. The lower esophageal sphincter (LES) was located by the proximal pressure recordings. The probe was further retracted, placing the middle of the bag 5 cm proximal to the LES. Pressure was recorded 8, 10 and 12 cm proximal to the LES. Axial force was recorded 6.5 cm proximal to the LES.

Potable tap water and three thickened fluids were used to create boluses of different viscosities. Water has a viscosity of 1 mPa·s. The three thickened fluids (TF1-TF3) were made by mixing 100 mL tab water with 13.8, 16.5 and 19.3 g Clinutren Instant thickener (Nestlé, Vevey, Switzerland), respectively. An analysis of the instant thickener product was generated prior to the study by Vysera Biomedical Ltd., showing the viscosity as a function of the concentration in water (Figure 2). The selected viscosities of the thickened fluids were 2 kPa·s, 6 kPa·s and 10 kPa·s. The viscous fluids were in appearance comparable to pudding (2 kPa·s), yogurt (6 kPa·s) and slush ice (10 kPa·s). The bag was inflated with 2 mL of fluid throughout the experiments to enable peristalsis to grip the bag. The protocol included four series of five dry swallows, five 5 mL swallows and five 10 mL swallows. Each series tested a different fluid. At the end of each series the fluid used for the next series was mixed. All boluses were given at room temperature (23-26℃). The interval between swallows was at least 45 s. All subjects were studied in upright position with the upper body tilted

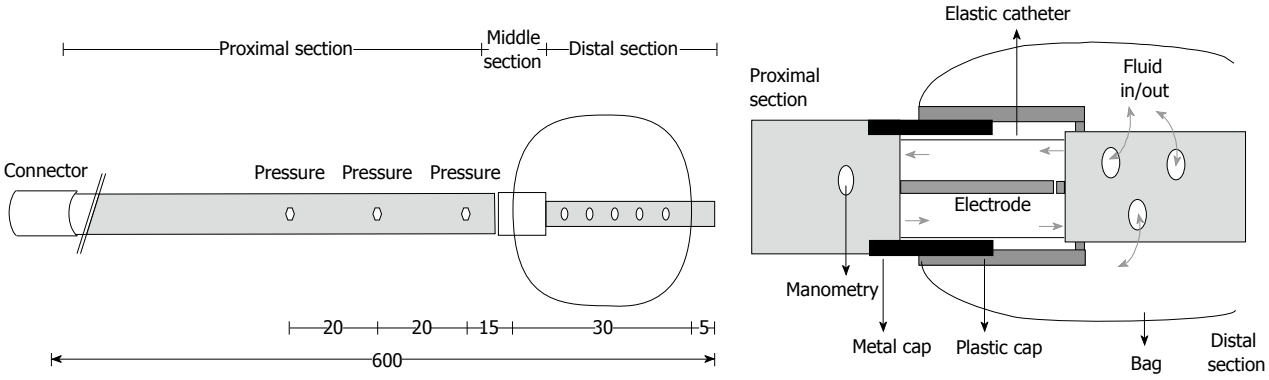

Figure 1 A sketch of the probe capable of measuring axial force and manometry simultaneously (see text for detailed explanation).

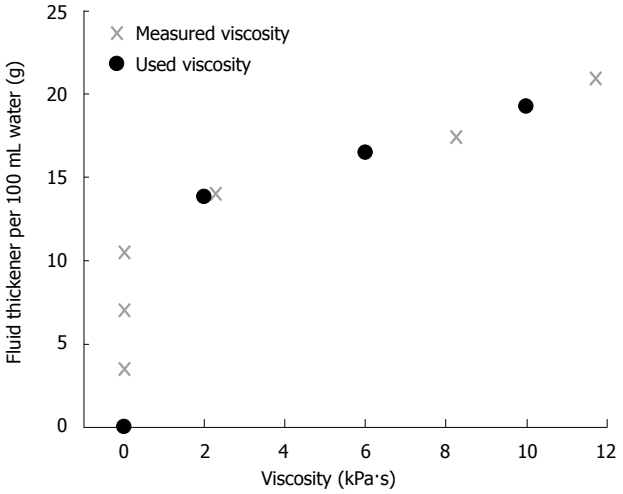

Figure 2 Viscosity recordings of the thickening powder in water. The light grey marks were measured while the black marks are used in the study.

30 degrees posterior and instructed to swallow as normally as possible. The volunteers drank 65 mL water between each series (after the five consecutive swallows) to clear the esophagus from any excess fluids of high viscosity.

Analysis

Contraction amplitude and duration were analyzed for both force and pressure measurements. The start of a contraction was defined as the interception with the x-axis for the linear fit of the steep incline of a contraction wave. The end of a contraction was defined as the interception with the x-axis for the linear fit to the steep decline of a contraction. This definition was used because the bolus itself affected the measures before the arrival of a peristaltic contraction. This definition has previously been verified by video fluoroscopy[18] and used in different manometric studies[8,19]. The linear fit was calculated using a semi-automatic program custom made for the purpose using MatLab® version 7 (Mathworks, Natick, MA, USA).

Complete absence of motor activity (manometry < 15 mmHg[20,21], force < 10 g) at a given site was termed "failure of contraction". Double-peaked, triple-peaked and repetitive waves were quantified manually during the semi-automatic analysis.

Statistics analysis

The results are given as grand mean ± SD. The correlation coefficient ϱ between the force and pressure measurements was computed with Pearson's correlation test for duration and amplitude at individual bolus size. Two-way analysis of variance (ANOVA) was used for the analysis of axial force and pressure. ANOVA was also used to analyze differences between bolus size. The axial force and pressure amplitude cannot be compared directly. Therefore, normalization was done by division with the overall mean of the axial force amplitude and pressure amplitude, respectively. The overall mean was computed from all the contraction amplitudes. $P < 0.05$ was considered significant.

RESULTS

The study was conducted without adverse events for the subjects. The age of the subjects was 27.7 ± 4.2 years. Representative recordings obtained in a subject swallowing 5 mL of water and 10 mL of thickened fluid (10 kPa·s) are shown in Figure 3. The arrival of the bolus in front of the peristaltic wave can be seen in the recording from the 10 mL swallow (marked with an arrow). The number of peristaltic contractions was higher during 5 and 10 mL swallows when compared to dry swallows; that is fewer wet swallows failed to induce contraction. The number of contractions was lower for multi-peaked contractions for both manometry and axial force during 5 mL swallows compared to dry swallows. Manometry and axial force showed an equal number of contractions. Only contractions, but not the events that did not fulfill the contraction criteria, were included in the subsequent analysis. No qualitative changes in the shape of the peristaltic contraction were found in association with the quantitative changes described. No increase or decrease in contractile amplitude or duration was found during the five subsequent swallows in a series. Thus, in the following analysis the averages were used.

Contraction amplitude

The most distal pressure recording site had the biggest amplitude for both 5 mL swallows ($F = 22.5$, $P < 0.001$) and 10 mL swallows ($F = 26.3$, $P < 0.001$). Thus, the manometric amplitude increased distally when comparing

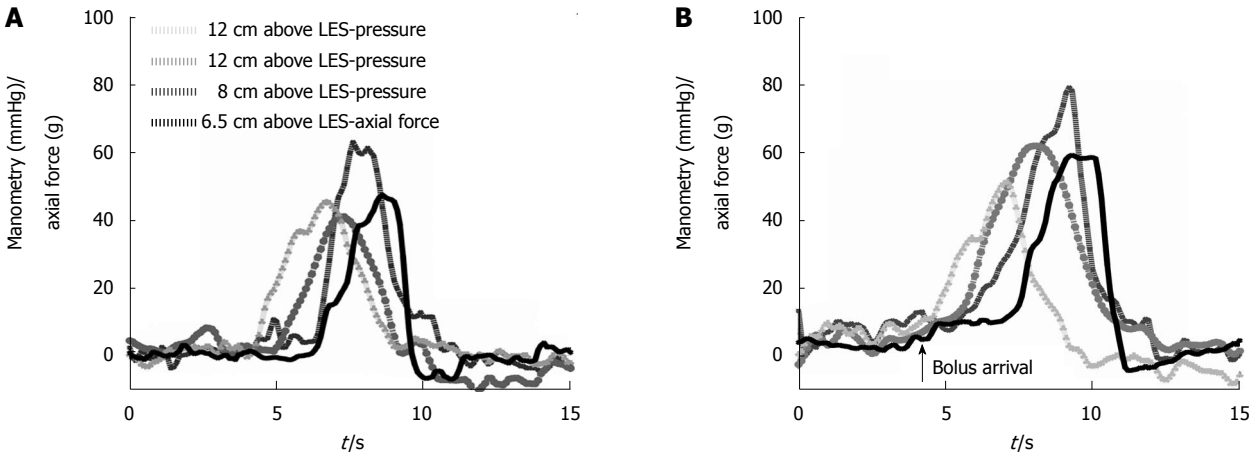

Figure 3 Two swallows (initiated at time = 0) from one subject. One swallow of 5 mL water (A) and one swallow of 10 mL thickened fluid (B). The arrival of the fluid before the actual peristaltic wave was clear during swallowing of 10 mL thickened fluid. LES: Lower esophageal sphincter.

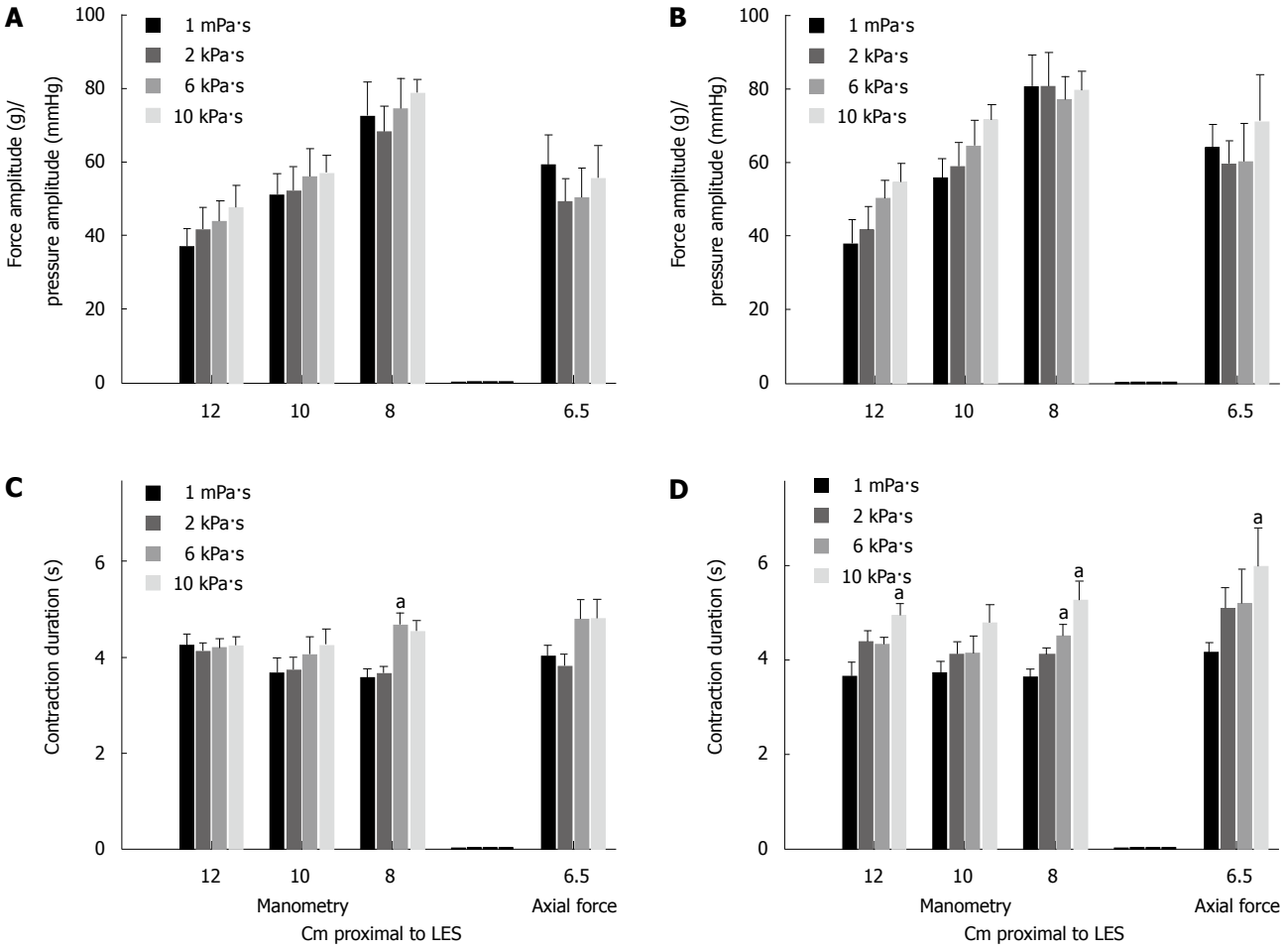

Figure 4 The mean ± SE amplitude (A, B) and duration (C, D) for manometry and axial force recordings during 5 mL swallows (A, C) and 10 mL swallows (B, D). The color of the columns represents the viscosity from 1 to 10 kPa•s. The axial force amplitude was marginally affected by bolus size (F = 3.5, P = 0.069) while pressure recordings were unaffected. The duration of the contraction increased as the viscosity increased. [a]P < 0.05 *vs* water. LES: Lower esophageal sphincter.

recordings at different levels.

The pressure amplitude did not depend on viscosity or bolus size at any recordings site (Figure 4). The axial force amplitude was 38.7 ± 17.2 g during dry swallows (not shown). The axial force amplitude was marginally influenced by bolus size (F = 3.5, P = 0.069) but did not increase with the bolus viscosity (Figure 4). Using 2-way ANOVA no difference was found when normalized amplitudes for pressure recorded 8 cm proximal to LES was compared to normalized axial force amplitudes.

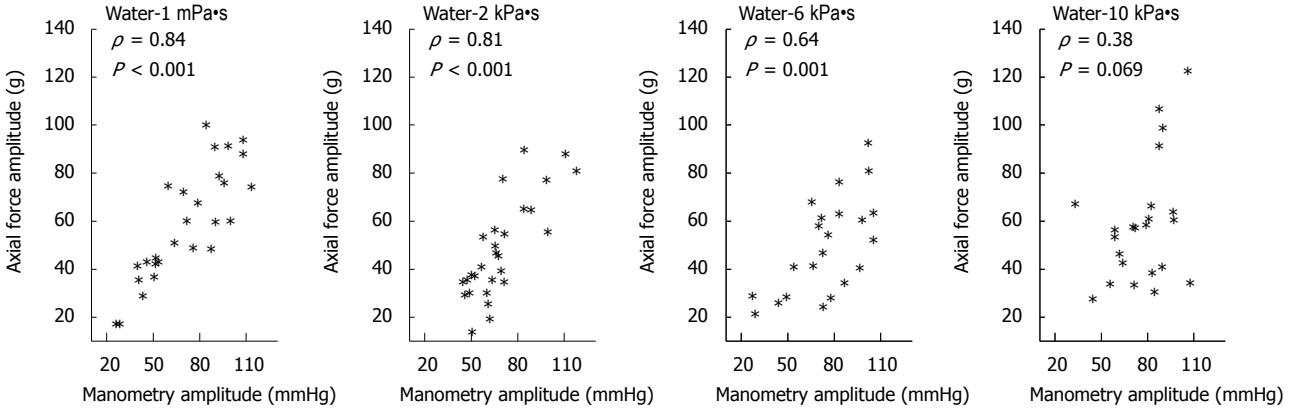

Figure 5 The correlation between manometry amplitude recorded 8 cm proximal to LES and axial force recorded 6.5 cm proximal to LES. The graphs from left to right are swallows of fluids with viscosities of 1 mPa•s, 2 kPa•s, 6 kPa•s and 10 kPa•s, respectively. LES : Lower esophageal sphincter.

Table 1 Axial force and manometry association

Volume Viscosity (Pa•s)		5 mL 1 m	5 mL 2 k	5 mL 6 k	5 mL 10 k	10 mL 1 m	10 mL 2 k	10 mL 6 k	10 mL 10 k
Amplitude									
8 cm	ρ =	0.84[a]	0.81[a]	0.64[a]	0.38	0.7[a]	0.52[a]	0.46[a]	0.7[a]
	P =	< 0.001[a]	< 0.001[a]	0.001[a]	0.069	< 0.001[a]	0.008[a]	0.02[a]	< 0.001[a]
10 cm	ρ =	0.49[a]	0.68[a]	0.57[a]	0.41	0.27	0.08	-0.7	0.26
	P =	0.016[a]	< 0.001[a]	0.021[a]	0.063	0.163	0.695	0.749	0.29
12 cm	ρ =	0.41	0.39	0.48	-0.32	-0.25	-0.06	-0.15	0.27
	P =	0.06	0.058	0.062	0.157	0.244	0.785	0.494	0.255
Duration									
8 cm	ρ =	0.36	0.34	0.47[a]	0.32	0.55[a]	0.4[a]	0.58[a]	0.53[a]
	P =	0.061	0.08	0.027[a]	0.133	0.002[a]	0.048[a]	0.002[a]	0.014[a]
10 cm	ρ =	-0.23	0.17	0.48	0.33	0.34	0.29	0.32	0.25
	P =	0.292	0.433	0.062	0.138	0.075	0.159	0.122	0.307
12 cm	ρ =	-0.32	0.31	-0.28	0	-0.35	0.03	-0.07	-0.25
	P =	0.14	0.136	0.287	0.997	0.104	0.908	0.766	0.293

The association between axial force [6.5 cm proximal to the lower esophageal sphincter (LES)] and manometry recorded 8, 10 and 12 cm proximal to the LES for both duration and amplitude. [a]Indicate a significant association ($P < 0.05$).

Contraction duration

The contraction duration recorded with manometry was not influenced by the recording site (Figure 4). The duration increased with viscosity for pressure recorded 8 cm above LES ($F = 12.3$, $P < 0.01$) (Figure 4).

The contraction duration measured with axial force increased with increasing viscosity ($F = 4.3$, $P = 0.01$) and bolus size (5 mL versus 10 mL) ($F = 4.9$, $P = 0.03$). The pressure duration recorded 8 cm proximal to the LES was lower than that for axial force for 10 mL swallows ($F = 4.9$, $P = 0.033$). Pressure recorded 8 cm proximal to the LES showed a change with viscosity ($F = 12.3$, $P < 0.001$) and the post hoc analysis showed difference between water and 10 kPa·s fluid ($P < 0.001$) and between water and 6 kPa·s fluid ($P = 0.001$). Contrary to the amplitude the duration did not change when comparing the different manometric recording sites.

Association between pressure and axial force

The association between the pressure amplitude measured 8 cm proximal to the LES and force amplitude recorded during 5 mL swallows decreased with increasing bolus viscosity (Figure 5 and Table 1). The correlation coefficients for amplitudes during 10 mL swallows were lower compared to 5 mL swallows. Association was not found at any viscosity or bolus volume when comparing axial force amplitudes to pressure amplitudes recorded 12 cm proximal to the LES (all $P > 0.05$). With regard to contraction duration no association was found for 5 mL swallows except in one case (Table 1). A weak association between axial force and manometry 8 cm proximal to LES was found for 10 mL swallows ($P < 0.58$).

DISCUSSION

The major results were that the pressure and force amplitude did not increase with the viscosity. The axial force amplitude depended on the bolus size. This was not the case for the pressure amplitude. The association between pressure and force amplitudes was weak at large bolus size (10 mL), indicating that pressure will be a less exact measure of the esophageal function.

Methodological considerations

The swallow sequence was not randomized or blinded since pilot experiments showed that the subjects could easily tell the difference between dry swallows, 5 mL and 10 mL volumes in the mouth. The same accounted for the fluids of different viscosity. However, the lack of blinding is of minor importance as a previous study did not show a learning effect regarding the swallowed bolus size[22].

The viscous fluids did not influence subsequent recordings by accumulating around the probe or bag between swallows since the parameters for dry swallows did not change and the number of contractions remained constant. Individual successive contractions showed no increase in amplitude or duration in five succeeding swallows.

In the current study the viscosity ranged from 1 mPa·s to 10 kPa·s. A previous study used somewhat lower viscosities between 1 mPa·s to 860 mPa·s[8]. Since the viscosity curve is highly non-linear this may not affect the appearance of the fluid to the same degree. However, it is important to emphasize that the exact viscosity should be given in scientific publications.

The association between manometric data recorded 8 cm proximal to the LES and axial force recorded 6.5 cm proximal to the LES was calculated. However, the distance between recording sites is of minor importance because the amplitude and duration were calculated for each curve individually. One may argue that it is important to evaluate intrabolus pressure since it will be influenced by viscosity[18]. However, we believe that the contraction pressure is more important.

Amplitudes as function of viscosity

This study confirms the results of Dooley and coworkers[7] that the contraction pressure amplitude is not affected by changes in bolus viscosity. We further show that the axial force amplitude does not depend on the viscosity.

Previous studies found a poor correlation between the axial force and manometry[23-25]. Pope and Horton found that swallowing 10 mL of salad oil reduced the axial force amplitude by 50% in the subsequent swallows[13]. This shows the frictional force is an important factor and the esophagus ability to "grip" the bolus is of great importance to a powerful forward-moving peristaltic wave. In the present study the pressure amplitude was not affected in the same way as axial force amplitude when the frictional force is changed. We believe that one of the reasons that the association between pressure amplitudes and axial force amplitudes became weaker as viscosity increased was due to a change in the frictional resistance (the frictional force resisting fluid movements). It is suspected that the range in frictional resistance between water and the viscous fluids was too low to reveal any difference when comparing axial force amplitudes and manometry amplitudes. Frictional resistance is a complex mechanism and will change depending on e.g. the bolus content, amount of mixed saliva, bolus velocity[26]. Thus the axial force amplitude (or function) generated by the esophagus may be affected.

Duration in relation to viscosity

The duration of the peristaltic wave was affected by bolus viscosity, especially for 10 mL swallows. This confirms previous manometric studies[7,8] showing a difference in the whole range of viscosities compared to water. It has been suggested that the increased duration is due to that the bolus reside longer time at the recording site[7]. However, this is not likely to happen even with very viscous fluids. As seen in the raw tracings the bolus reached the axial force transducer before the actual wave arrived. This implies that the bolus has passed the pressure recordings sites.

In conclusion, the study provided information about the pressure and axial force during peristaltic contractions when exposed to change in bolus viscosity and volume. Though the contractile patterns appeared the same for pressure and force measurements, clear differences were found between the recordings, especially at high volumes and high viscosities. It is expected that profound differences between manometry and axial force will be found between patient groups with esophageal diseases. For example it is well known that some achalasia patients show a "common cavity phenomenon" with aperistalsis. This will create a radial pressure without changes in axial force. The same may account for other esophageal diseases. Thus, it is expected that axial force measurements will have clinical relevance. This will be a subject for subsequent studies.

COMMENTS

Background

Development of diagnostic tools within esophageal motility diseases have for a long time been based on manometry. The development has mainly focused on use of multiple pressure sensors (high resolution manometry). Several authors have previously shown that relying on manometry alone can lead to erroneous conclusions.

Research frontiers

Axial force measurements, also known as traction force, provide additional information not currently available through manometry examinations alone. Axial force provides a more physiological measurement. However few studies have compared simultaneous manometry and axial force.

Innovations and breakthroughs

This is the first study of its kind to examine axial force in relation to viscosity. The data suggests that axial force can provide additional information in relation to motility.

Applications

These data are in accordance with other studies relying on axial force measurements though the areas of interest have been bolus size, temperature etc. The data, together with other papers, suggests that using axial force to assists manometry in motility examinations will provide additional information important to provide a valid diagnosis.

Peer review

This is a very interesting experimental study with important clinical implications in the diagnosis of esophageal disease.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.1989

World J Gastroenterol 2011 April 21; 17(15): 1989-1995
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Pancreatic duct guidewire placement for biliary cannulation in a single-session therapeutic ERCP

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Received: November 17, 2010 Revised: December 23, 2010
Accepted: December 30, 2010
Published online: April 21, 2011

Abstract

AIM: To investigate the technical success and clinical complication rate of a cannulated pancreatic duct with guidewire for biliary access.

METHODS: During a five-year study period, a total of 2843 patients were included in this retrospective analysis. Initial biliary cannulation method consisted of single-guidewire technique (SGT) for up to 5 attempts, followed by double-guidewire technique (DGT) when repeated unintentional pancreatic duct cannulation had taken place. Pre-cut papillotomy technique was reserved for when DGT had failed or no pancreatic duct cannulation had been previously achieved. Main outcome measurements were defined as biliary cannulation success and post-endoscopic retrograde cholangiopancreatography (ERCP) complication rate.

RESULTS: SGT (92.3% success rate) was characterized by statistically significant enhanced patient outcome compared to either the DGT (43.8%, $P < 0.001$), pre-cut failed DGT (73%, $P < 0.001$) or pre-cut as first step method (80.6%, $P = 0.002$). Pre-cut as first step method offered a statistically significantly more favorable outcome compared to the DGT ($P < 0.001$). The incidence of post-ERCP pancreatitis did not differ in a statistically significant manner between either method (SGT: 5.3%, DGT: 6.1%, Pre-cut failed DGT: 7.9%, Pre-cut as first step: 7.5%) or with patients' gender.

CONCLUSION: Although DGT success rate proved not to be superior to SGT or pre-cut papillotomy, it is considered highly satisfactory in terms of safety in order to avoid the risk of a pre-cut when biliary therapy is necessary in difficult-to-cannulate cases.

Key words: Endoscopic retrograde cholangiopancreatography; Post-endoscopic retrograde cholangiopancreatography pancreatitis; Pre-cut papillotomy; Pancreatic duct

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Xinopoulos D, Bassioukas SP, Kypreos D, Korkolis D, Scorilas A, Mavridis K, Dimitroulopoulos D, Paraskevas E. Pancreatic duct guidewire placement for biliary cannulation in a single-session therapeutic ERCP. *World J Gastroenterol* 2011; 17(15): 1989-1995 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/1989.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.1989

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is widely considered as the most demanding endoscopic interventional procedure offering the least invasive way for biliary manipulations. In terms of procedure-related safety, atraumatic biliary cannulation is the fundamental prerequisite to secure a successful therapeutic-intended ERCP[1]. Nowadays, non-invasive imaging modalities [such as magnetic resonance cholangiopancreatography (MRCP) and endoscopic ultrasound (EUS)] have superseded diagnostic ERCP, reducing the potential complication rate and giving better selection criteria for patients who would benefit from therapeutic biliary manipulations[2].

Long discussions and reports from ERCP experts have proposed a plethora of different ways to provide an uncomplicated biliary cannulation[1]. Several factors during ERCP may lead an endoscopist to use the appropriate technique according to his experience and training. Many reports have shown that selective cannulation of the common bile duct (CBD) by insertion of a hydrophilic guidewire through a papillotome may minimize procedure-related complications (particularly post-ERCP pancreatitis-PEP), as opposed to standard CBD access method with direct injection of contrast media[3-7].

In difficult-to-cannulate cases, pre-cut papillotomy has been established as the alternative method to gain CBD access when biliary therapy is strongly indicated[8,9]. However, pre-cut technique predisposes to a higher rate of post-ERCP complications including hemorrhage, pancreatitis and perforation, even in the most experienced hands[10-14].

Furthermore, several studies have documented that a pancreatic duct (PD) previously cannulated with a guidewire may facilitate selective CBD cannulation with a second wire preloaded into a papillotome (double guidewire technique - DGT)[15-19]. Placement of such a guidewire into the pancreatic duct may act as an endoscopic road map for the CBD, open a stenotic papillary orifice, stabilize the papilla or straighten the common channel when dealing with a tortuous intraduodenal segment[1].

In view of the above, we retrospectively analyzed our data concerning the use of this method (DGT) in terms of procedure-related efficacy, safety and complication rate.

MATERIALS AND METHODS

Patients

During a 5-year period (from June 2003 through July 2008), 2843 therapeutic ERCPs were performed in our hospital. A retrospective database review was conducted in order to identify all cases involving the use of DGT. Inclusion criteria were the existence of an intact papilla (no prior ERCP attempts) in patients with clinical, laboratory and radiological (transabdominal US, abdominal CT scans, MRCP) findings of pancreatobiliary pathology. Patients with previous gastrointestinal surgical operation, use of needle-knife fistulotomy for papilla impacted stones and suspected sphincter of oddi dysfunction (SOD) were excluded from our study. Endoscopic sphincterotomy (EST) was performed in all patients.

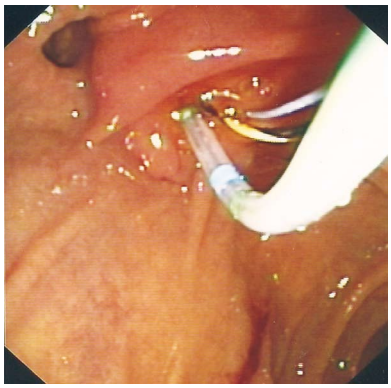

Figure 1 Endoscopic view of the papilla with a hydrophilic wire advanced into the pancreatic duct. A sphincterotome is advanced alongside the pancreatic wire with its tip oriented in the anticipated bile duct position.

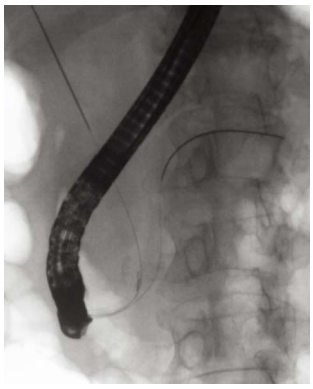

Figure 2 Biliary cannulation with the use of double-guidewire technique. One guidewire has been inserted into the distal part of the pancreatic duct and another is being moved in the direction of common bile duct through the sphincterotome inserted into the ampulla.

Methods and definitions

Our department protocol for biliary cannulation consists of a 3-step procedure undertaken in a single session. In the first step, attempts to cannulate the CBD consist of the use of a sphincterotome preloaded with a hydrophilic guidewire (single guidewire technique-SGT). Initial cannulation using SGT is both time- and cost-efficient when sphincterotomy is anticipated. The use of a guidewire seems to reduce the possibility of chemical-and pressure-related pancreatic injury by avoiding unintentional injection of contrast medium into the main PD or the papilla itself (submucosal injection). Up to 5 attempts within a 15-min period are considered adequate to provide safe cannulation without significant injury of the papillary area (i.e. trauma, edema, bleeding). If these attempts fail and repeated deep PD insertion has resulted, then the guidewire is left distally in the main PD and DGT technique is performed up to 3 times. The tip of the papillotome is positioned against the first wire placed in the PD (Figure 1) and its curve is altered in the anticipated CBD axis. The guidewire into the PD acts as a radiological marker for the PD and facilitates endoscopic location of the biliary orifice (Figure 2). Pre-cut technique with needle-knife is reserved as the 3rd step in cases when DGT has failed or no PD cannulation has

Table 1 Patient characteristics and indications *n* (%)

Dermographics	
Patient number	2332
Age (year, mean)	68.4
Gender (male/female)	1236/1096
Indications	
Choledocholithiasis	1732 (74.3)
Malignant stricture	545 (23.4)
Bile leak after cholecystectomy	42 (1.8)
Primary sclerosing cholangitis	11 (0.5)

been achieved in the first place.

Procedural success was defined as the insertion of the guidewire into the CBD. To determine safety and complication rate, all patients underwent measurement of serum amylase before and 24 h after ERCP. Asymptomatic hyperamylasemia was defined as a threefold rise in serum amylase without epigastric pain at 24 h after ERCP. Definition of PEP was the incidence of epigastric pain associated with serum amylase elevation to greater than 3 times normal values at 24 h after the procedure. Bleeding, perforation and other post-ERCP-related adverse events were recorded in detail.

Statistical analysis

Due to the fact that the distribution of the variables under study was not Gaussian, analysis of differences between these parameters, in each patient group, was performed with the non-parametric Mann-Whitney, χ^2, or Fischer's exact statistical tests, where applicable. The correlation between the employment of different cannulation methods and either final patient outcome, or presence of asymptomatic hyperamylasemia or clinical pancreatitis, was studied using univariate logistic regression analysis.

RESULTS

Of the 2843 ERCPs performed in the study period, 511 were excluded as patients had previously undergone endoscopic manipulations (EST, endoscopic papillary balloon dilation), or altered anatomy was observed due to gastrectomy, or SOD was suspected on the basis of clinical and ERCP findings. A total of 2332 patients met selection criteria. Indications for therapeutic ERCP varied, with suspected common bile duct stones and suspected malignant biliary strictures being the most common (Table 1).

Using SGT as the first step method, CBD cannulation was achieved in 2153 patients (92.3%). Unintentional PD guidewire insertion after 5 attempts was documented in 112 patients. In these cases, DGT was performed after the last effort and selective CBD access was successful in 49 out of 112 patients (43.8%). In the 63 failed DGT cases pre-cut papillotomy gained CBD access in 46 patients (73%). In 67 patients no CBD or PD cannulation was possible with the initial 5 attempts. Fifty-four of them underwent successful pre-cut papillotomy at the same session (80.6%).

SGT was characterized by statistically significant enhanced patient outcome compared to either the DGT ($P < 0.001$), pre-cut failed DGT ($P < 0.001$) or pre-cut as first step method ($P = 0.002$) (Tables 2 and 3). In addition, pre-cut as first step method offered a statistically significantly ($P < 0.001$) more favorable outcome compared to the DGT method. These observations were further confirmed using univariate logistic regression analysis (Table 4).

Thirty patients in whom therapeutic ERCP failed were referred to surgical or interventional radiological treatment. The total success rate of CBD cannulation in the study population was 98.7% (2302/2332).

The development of asymptomatic hyperamylasemia was significantly more frequent in the DGT and precut-failed DGT group of patients, compared to the SGT patients (Table 2). Employing regression analysis, patients who underwent DGT were 2.15 times [HR] more likely (95% CI 1.39-4.60, $P = 0.002$) to develop asymptomatic hyperamylasemia than the SGT patients.

The rates of PEP did not differ in a statistically significant manner between groups of patients who underwent different types of cannulation (Tables 2 and 3). The presence of PEP in the DGT group of patients was statistically significantly ($P = 0.010$) more evident in younger individuals (Median = 42.00 years) than in older ones (63.00 years), without statistical correlation with the initial pathology (Table 4).

In the group of patients who underwent SGT, a statistically significant difference ($P = 0.005$) between age and outcome was observed, as younger patients (Median = 64.00 years) were more likely to be attributed with a successful outcome than older ones (Median = 67.00 years). However, PEP was more frequently present ($P < 0.001$) in younger (Median = 62.00 years) patients than older ones (Median = 67.00 years). Patients who suffered from choledocholithiasis were more likely to present PEP than patients who suffered from malignancy, without reaching statistically significant difference (Table 5).

In the pre-cut failed DGT group of patients, PEP was more frequently present ($P = 0.021$) in younger (Median = 45.00 years) patients than older ones (Median = 63.50 years). As far as the pre-cut as first step method is concerned, the presence of PEP was found more frequently ($P = 0.017$) in younger patients (Median = 52.00 years) than older patients (Median = 68.00 years). The presence of PEP was statistically more evident in patients with choledocholithiasis compared to patients with malignancy in both groups of patients in which pre-cut technique was performed (Table 5).

Patients' gender did not seem to relate in any statistically significant way with the presence of asymptomatic hyperamylasemia and PEP within patients who underwent pre-cut first step, DGT or pre-cut failed DGT method. On the contrary, female patients who underwent SGT showed a statistically significant elevated presence of asymptomatic hyperamylasemia (14.0%) compared to male patients (Table 6).

One case of retroperitoneal perforation was recorded using SGT, and 2 patients developed massive bleeding using pre-cut as first step and sphincterotomy after successful SGT, respectively. These patients underwent immediate surgical treatment without further complications.

Table 2 Comparison between single-guidewire technique/double-guidewire technique, single-guidewire technique/pre-cut failed double-guidewire technique and single-guidewire technique/pre-cut first step methods in terms of patient outcome, the development of asymptomatic hyperamylasemia or post-endoscopic retrograde cholangiopancreatography pancreatitis *n* (%)

Variables	SGT	DGT	SGT	Pre-cut failed DGT	SGT	Pre-cut first step
Outcome (success)	2153 (92.3)	49 (43.8)	2153 (92.3)	46 (73.0)	2153(92.3)	54 (80.6)
P value[1]	< 0.001[a]		< 0.001[a]		0.002[a]	
Asymptomatic hyperamylasemia	258 (12.0)	11 (22.4)	258 (12.0)	15 (23.8)	258 (12.0)	10 (14.9)
P value[1]	0.046[a]		0.008[a]		0.591	
PEP	115 (5.3)	3 (6.1)	115 (5.3)	5 (7.9)	115 (5.3)	5 (7.5)
P value[1]	0.935		0.538		0.629	

[a]*P* < 0.05. [1]Calculated by Fisher's exact test; SGT: Single-guidewire technique; DGT: Double-guidewire technique; PEP: Post-endoscopic retrograde cholangiopancreatography pancreatitis.

Table 3 Comparison between double-guidewire technique/pre-cut failed double-guidewire technique, pre-cut failed double-guidewire technique /pre-cut first step and double-guidewire technique/pre-cut first step methods in terms of patient outcome, the development of asymptomatic hyperamylasemia or post-endoscopic retrograde cholangiopancreatography pancreatitis *n* (%)

Variables	DGT	Pre-cut failed DGT	Pre-cut failed DGT	Pre-cut first step	DGT	Pre-cut first step
Outcome (success)	49 (43.8)	46 (73.0)	46 (73.0)	54 (80.6)	49 (43.8)	54 (80.6)
P value[1]	< 0.001[a]		0.405		< 0.001[a]	
Asymptomatic hyperamylasemia	11 (22.4)	15 (23.8)	15 (23.8)	10 (14.9)	11 (22.4)	10 (14.9)
P value[1]	0.955		0.266		0.426	
PEP	3 (6.1)	5 (7.9)	5 (7.9)	5 (7.5)	3 (6.1)	5 (7.5)
P value[1]	0.999		0.588		0.928	

[a]*P* < 0.05. [1]Calculated by Fisher's exact test. SGT: Single-guidewire technique; DGT: Double-guidewire technique; PEP: Post-endoscopic retrograde cholangiopancreatography pancreatitis.

Table 4 Logistic regression analysis for every possible comparison of the cannulation method used, for the prediction of outcome (failure)

Method	HR[1]	95% CI	*P* value[2]
SGT	1		
Pre-cut first step	1.43	1.16-1.76	0.001
SGT	1		
DGT	3.93	3.21-4.81	< 0.001
Pre-cut first step	1		
DGT	1.23	1.13-1.35	< 0.001
Pre-cut first step	1		
Pre-cut failed DGT	1.063	0.95-1.20	0.307

[1]Hazard ratio; [2]Test for trend. SGT: Single-guidewire technique; DGT: Double-guidewire technique.

Table 5 Associations between the development of post-endoscopic retrograde cholangiopancreatography pancreatitis and the initial pathology within groups of patients who underwent each cannulation method *n* (%)

Method	Choledocholithiasis	Malignancy
Pre-cut first step		
PEP	5 (19.2)	0 (0.0)
P value[1]	0.007[a]	
DGT		
PEP	3 (8.3)	0 (0.0)
P value[1]	0.556	
Pre-cut failed DGT		
PEP	5 (16.1)	0 (0.0)
P value[1]	< 0.001[a]	
SGT		
PEP	97 (5.8)	17 (3.9)
P value[1]	0.146	

[a]*P* < 0.05. [1]Calculated by Fisher's exact test. SGT: Single-guidewire technique; DGT: Double-guidewire technique; PEP: Post-endoscopic retrograde cholangiopancreatography pancreatitis.

DISCUSSION

In the era of modern non-invasive imaging modalities, the role of ERCP has been focused in the therapeutic management of pancreatobiliary diseases. Several techniques and accessories have been used in order to achieve selective CBD cannulation and to decrease the rate of post-ERCP complications.

In the present study, SGT proved to be an effective and safe CBD cannulation approach. The ability to manually control the angle of orientation of the sphincterotome has been shown to be advantageous when dealing with unusually oriented or distorted papillas caused by either diverticula or surgically altered anatomy[20]. Moreover, the use of hydrophilic coated-tip guidewires seems to offer a direct way of CBD cannulation under fluoroscopy with less papillary trauma compared to the rather blind method of contrast media injection[4].

Three large prospective randomized studies comparing post-ERCP complications between the use of a papillotome with a guidewire (SGT) and papillotome with contrast media injection as cannulation methods, demonstrated significantly lower incidence of PEP in the SGT group (0%-8.6% *vs* 4.1%-16.6%)[4-6]. In addition, PEP rate was

Table 6 Associations between patient gender and the presence of PEP and asymptomatic hyperamylasemia, within groups of patients who underwent each cannulation method *n* (%)

Method	PEP	Asymptomatic hyperamylasemia
Pre-cut first step		
Male	2 (5.1)	3 (7.7)
Female	3 (10.7)	7 (25.0)
P value[1]	0.642	0.081
DGT		
Male	1 (3)	6 (18.2)
Female	2 (12.5)	5 (31.3)
P value[1]	0.086	0.467
Pre-cut failed DGT		
Male	1 (2.9)	9 (26.5)
Female	4 (13.8)	6 (20.7)
P value[1]	0.171	0.768
SGT		
Male	62 (5.5)	115 (10.2)
Female	53 (5.2)	143 (14.0)
P value[1]	0.77	0.008[a]

[a]$P < 0.05$. [1]Calculated by Fisher's exact test. SGT: Single-guidewire technique; DGT: Double-guidewire technique.

lower if unintentional guidewire cannulation of the PD occurred compared to that of unintentional PD opacification by contrast injection (0%-5.1% *vs* 4.4%-19%). In a SGT group, Lee *et al*[6] reported a total of 3 cases of PEP, two in patients with SOD diagnosis, a well-known risk factor for PEP. In these studies, the authors concluded that the reduction in PEP in SGT groups of patients was mainly the result of avoiding elevation of hydrostatic pressure into the PD, partly attributed to the contrast media injection.

Dumonceau *et al*[21] first described the successful use of PD guidewire placement as an adjunct to cannulate CBD in a case of Billroth I gastrectomy with a distorted prepapillary segment of CBD. Later on, Gyökeres *et al*[15] reported successful use of DGT in 24 difficult CBD cannulation cases with no significant difference in PEP rate when compared to conventional cannulation. Maeda *et al*[16], in a prospective study, documented a superior CBD cannulation rate (93%) with a modified technique, using a PD wire placement and a cannula instead of a papillotome, as opposed to the conventional method with cannula and contrast media (58% success rate). No episodes of pancreatitis with either method were noted, but a significantly higher incidence of hyperamylasemia occurred in the PD wire group. It should be mentioned, however, that patients of the latter group were submitted to pancreatography prior to PD wire insertion, thus increasing the risk of overinjection into the PD with possible acinarization.

In our study, using DGT, selective CBD cannulation was achieved in 43.8% of patients (49/112) with previously failed SGT. Three patients of this group developed pancreatitis (6.1%) and 11 patients asymptomatic amylasemia (22.4%), showing an acceptable safety profile comparable to SGT (5.3% and 12%, respectively). Recently published data from a prospective randomized study assessing clinical efficacy and safety of DGT *versus* conventional cannulation method reported almost equivalent CBD cannulation success in difficult cases (47.3% *vs* 54%). The DGT group showed an increased incidence of clinical pancreatitis compared to the SGT group, without, however, reaching a statistically significant difference (17% *vs* 8%)[22].

According to our results, PD reaction (i.e. PEP) seems to be affected by both iatrogenic and patient-related characteristics. Young patients suffering from choledocholithiasis proved to be statistically more prone to develop pancreatitis when pre-cut access papillotomy as first step was used, or after a failed DGT technique was performed. On the other hand, elderly patients, despite repeated PD wire insertion and use of pre-cut, demonstrated hyperamylasemia as part of a benign post-ERCP clinical course. In addition, 12.5% and 13.8% of female patients who underwent a successful DGT and pre-cut failed DGT developed PEP, respectively. Although these observations were not found to be statistically significant, patients' gender seems to carry an independent risk factor concerning pancreatic injury following PD manipulations.

Age, sex, prior PEP or recurrent history of acute pancreatitis and SOD are well known risk factors for PEP[23,24] and many experts have already proposed the use of a temporary prophylactic pancreatic stent in these situations[1,25]. Several uncertainties remain regarding patients' selection criteria for pancreatic stent use and the appropriate diameter. These may be attributed to the lack of long-term data on changes in PD anatomy and stent-induced complications in failed stent placement cases[26]. Goldberg *et al*[27] documented the aid of a 5F pancreatic stent placement for CBD cannulation in 39 patients with 97.4% success rate. To achieve biliary cannulation, 59% of patients underwent pre-cut sphincterotomy over the pancreatic stent with only 5% PEP rate. The authors, however, do not mention the existence of SOD indication, nor do they report in how many cases pancreatic sphincterotomy was performed to facilitate stent insertion.

No pancreatic stent insertion was performed in our series, as our initial aim was to investigate the contribution of a simple and non-time-consuming technique involving the use rather than abuse of PD. In addition, patients with suspected SOD were excluded due to the lack of manometry which would imply a clear therapeutic indication and the need for a pancreatic stent use. What seems technically more important in DGT technique, regarding success and complication rate, is the gentle straight deep passage of the wire into the PD. Insertion of the wire in angulated mode into a normal PD may potentially increase side-branch acinarization associated with the occurrence of PEP.

It should always be kept in mind that basic CBD cannulation techniques cannot be substituted by any available trick and only proper expertise in ERCP secures periprocedural efficacy. Based on this knowledge, the wire into the PD seems to be helpful for cases of difficult cannulation, particularly when the papilla is mobile with small orifice or disoriented due to a diverticulum or malignancy. In these cases, the anatomical axis of the common channel is stabilized by the inserted wire into the PD, offering the possibility of a new, less traumatic, wire cannulation attempt than pre-cut access papillotomy may cause.

The special incisional technique of pre-cut has been estimated to enable biliary access in about 5% to 10% of hard-to-cannulate cases, according to several reports[28]. However, pre-cut has been described to be an independent risk factor for post-ERCP complications (variation between 8% and 35.3%) and only recommended as an expert's alternative method[14]. In full agreement with these reports, 5.6% of patients in the period of our study underwent pre-cut papillotomy (77% overall success rate) and 10 patients experienced PEP (7.7%). Data analysis showed no statistically increased possibility of PEP in patients with previously failed DGT followed by pre-cut (7.9% of cases), compared to patients who underwent pre-cut as initial cannulation technique (7.5% of cases).

The decision as to the appropriate CBD cannulation method in specialized situations should to be dependent on a patient's individual clinical and anatomical basis as well as on an endoscopist's experience in the available techniques. In the present study, while proposing the SGT as the standard technique for achieving CBD cannulation, DGT offered a remarkable alternative in difficult cases before proceeding to pre-cut papillotomy. DGT success and low complication rate is considered highly satisfactory in order to avoid the risk of a pre-cut when biliary therapy is necessary. Further randomized prospective trials comparing various pre-cut techniques and pancreatic duct cannulation approaches, as rescue methods to facilitate biliary access, will eventually offer an evidence-based approach.

ACKNOWLEDGMENTS

We particularly wish to thank El Papakonstantinou for her valuable aid with fluoroscopic procedures. There are no potential conflicts of interest for any of the authors.

COMMENTS

Background

Biliary cannulation is the fundamental prerequisite to secure a successful therapeutic-intended magnetic resonance cholangiopancreatography (MRCP). Selective biliary cannulation by insertion of a hydrophilic guidewire tends to be the standard method of choice in terms of efficacy and safety [single guidewire technique (SGT)]. In difficult-to-cannulate cases, pre-cut papillotomy has been established as the alternative method to gain biliary access. However, pre-cut technique predisposes to a higher rate of post-ERCP complications, even in the most experienced hands.

Research frontiers

Several studies have documented that a pancreatic duct previously cannulated with a guidewire may facilitate selective biliary cannulation with a second wire (double guidewire technique-DGT).

Innovations and breakthroughs

In a single-center, retrospective study using DGT, selective biliary cannulation was achieved in 43.8% of patients with previously failed SGT. Pre-cut papillotomy gained biliary access in 73% of failed DGT cases and 80.6% of cases as first step method, respectively. Univariate analyses revealed no statistically significant difference in terms of complication rate between patients with different types of cannulation.

Applications

Although the DGT success rate proved not to be superior to that of SGT or pre-cut papillotomy, it is considered a highly satisfactory technique in terms of safety in order to avoid the risk of a pre-cut when biliary therapy is necessary.

Peer review

In this study, the authors conclude that the DGT may be safely performed before proceeding to pre-cut if repeated pancreatic duct cannulation occurs.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.1996

World J Gastroenterol 2011 April 21; 17(15): 1996-2002
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Microscopic colitis as a missed cause of chronic diarrhea

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Received: July 8, 2010 Revised: September 7, 2010
Accepted: September 14, 2010
Published online: April 21, 2011

Abstract

AIM: To determine the prevalence of increased intraepithelial lymphocytes, using immunohistochemistry in patients with normal colonoscopy and near normal biopsy.

METHODS: We retrospectively reviewed all non-malignant colon mucosal biopsies between 2005 and 2007, reported as normal, chronic inflammation or melanosis coli in patients who were undergoing routine colonoscopy. Immunohistochemistry using CD3 was performed on all mucosal biopsies and an intraepithelial lymphocyte count (IEL) was determined. Cases with an IEL count of $\geqslant$ 20 IELs per 100 surface epithelial cells were correlated with demographic, clinical and follow-up data. A further subgroup was evaluated for lymphocytic colitis.

RESULTS: Twenty (8.3%) of 241 cases revealed an IEL count $\geqslant$ 20. Six (2.5%) patients were identified as having lymphocytic colitis ($P < 0.001$), of whom, five were missed on initial evaluation ($P = 0.01$). Four of these five patients were labeled with diarrhea-predominant irritable bowel syndrome (IBS). On follow-up, three of the remaining 20 cases were diagnosed with malignancy (renal cell carcinoma and myelodysplastic syndrome) and one had an unknown primary tumor with multiple liver metastases. Two cases of collagenous colitis with an IEL count < 10 were included in this study. Increased IELs were not confined to patients with diarrhea as a primary presenting symptom, but were also present in patients with abdominal pain ($n = 7$), constipation ($n = 3$) and loss of weight ($n = 1$).

CONCLUSION: Immunohistochemistry using CD3 is of value in identifying and quantifying IELs for the presence of microscopic colitis in patients with diarrhea-predominant IBS.

Key words: Microscopic colitis; Lymphocytic colitis; Collagenous colitis; CD3 immunohistochemistry; Intraepithelial lymphocytes

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Mohamed N, Marais M, Bezuidenhout J. Microscopic colitis as a missed cause of chronic diarrhea. *World J Gastroenterol* 2011; 17(15): 1996-2002 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/1996.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.1996

INTRODUCTION

Microscopic colitis is regarded as a common cause of chronic watery diarrhea, accounting for approximately

4%-13% of patients presenting with this symptom[1]. By definition the colon appears normal or nearly normal on colonoscopy, with set histopathological criteria required for the diagnosis on mucosal biopsy. Lymphocytic colitis and collagenous colitis constitute the two major subtypes of microscopic colitis that share many similarities, including almost identical clinical symptoms, together with a macroscopically normal colonic mucosa. Both entities demonstrate colonic intraepithelial lymphocytosis, increased inflammatory cells within the lamina propria, and preserved crypt architecture, but are distinguished by the presence of a thickened basement membrane in collagenous colitis.

In the past, microscopic colitis was thought to be a rare disorder and very little was known about its etiology or epidemiology. It has become apparent that microscopic colitis is now regarded as common cause of diarrhea in middle-aged and elderly patients. Many recent publications have shown that the incidence of microscopic colitis is on the increase. Epidemiological data have now been reported from seven major regions[2], with most of the reported data coming from North American and European studies. The incidence rates for collagenous colitis is 0.8-6.2/100 000 and lymphocytic colitis is 0.5-12.9/100 000[2]. According to various studies, the prevalence of collagenous colitis and lymphocytic colitis is 10-15.7/100 000 and 14.4/100 000, respectively[1,3-5]. There are very few data available from developing countries, with a few case series reported from India[6], Turkey[7] and Sri Lanka[8].

Currently there are no data regarding this disease in South Africa, where infectious diseases are more prevalent. Isolated cases have been reported from Nigeria[9,10]. In this region, microscopic colitis is underdiagnosed because of a lack of colonoscopic facilities and the assumption that most cases of chronic diarrhea are likely to be infective, therefore, most patients self medicate and do not present to a hospital[10,11].

At Tygerberg Hospital, a tertiary referral center, approximately 1700 colonoscopies are performed each year, which include colonoscopies for non-infective diarrhea-related causes. Colonic biopsies at our institution are often reported as chronic inflammation, indeterminate colitis, chronic colitis or normal in the investigation of diarrheal disease. It is possible that the diagnosis of LC may have been missed in a proportion of these cases, because under-reporting of cases is common and has been documented in other studies[12]. According to a Swedish study, in a third of cases the diagnosis was missed in the primary histological examination[13,14]. The important role of the pathologist was clearly illustrated in this study that showed the difficulties in diagnosing microscopic colitis, especially the lymphocytic subtype[14]. According to Nielson, terms such as "unspecific chronic inflammation" or "signs of chronic inflammatory bowel disease but not diagnostic" should be avoided[14].

Immunostaining does not seem to play a major role in the diagnosis of LC. According to Tysk *et al*[2] and Chang *et al*[15], in some uncertain cases, immunostaining may facilitate the assessment of intraepithelial counts. There have been no studies to validate the benefit of performing immunohistochemistry in uncertain cases. Currently, the histological diagnosis is based on hematoxylin and eosin (H and E) assessment of criteria: (1) increase in intraepithelial lymphocytes (IELs > 20/100 surface epithelial cells); (2) surface epithelial damage; and (3) infiltration of lymphocytes and plasma cells into the lamina propria, with no subepithelial collagen deposition, as identified in collagenous colitis[16,17]. The diagnosis of lymphocytic colitis remains a challenge because it is often difficult to identify and quantify lymphocytes due to orientation of the biopsy, or nuclei having similar cytological features to those of columnar cell nuclei. Immunohistochemistry staining with CD3 has been shown to play a role in the counting and identification of IELs in celiac disease; incidentally, there is also an association between microscopic colitis and celiac disease[18].

Only one recent study has examined the reproducibility of histological diagnosis in microscopic colitis. That study found excellent correlation in distinguishing microscopic colitis and non-microscopic colitis amongst pathologists using H and E- stained slides. That study claimed κ values of 0.90 and 0.83 for inter-observer agreement and 0.89 for intra-observer agreement[19]. However, the authors have stated that their high rate of concordance was due to their particular expertise within the field of gastroenterology. In their study, immunohistochemistry was not performed to assess whether it allows easier identification of IELs.

In the present study, we aimed to determine the prevalence of increased IELs with the use of immunohistochemistry and described the spectrum of disease in all non-malignant biopsies reported as normal or chronic inflammation over a 3-year period. By facilitating counting of IELs, we hoped to identify cases that may have the lymphocytic colitis subtype.

MATERIALS AND METHODS

Study design and population

The study design was a retrospective analysis of all non-malignant colonoscopic biopsies diagnosed as normal or chronic inflammation in patients who underwent colonoscopy at the Tygerberg Hospital Gastroenterology Unit, for the period 2005-2007. Cases were retrieved from the Department of Pathology, Division of Anatomical Pathology, DisaLAB database for the 3-year period. Of the 1212 cases identified, only 247 met the criteria necessary for our analysis: (1) normal or chronic colitis on histology; (2) melanosis coli; or (3) microscopic colitis including collagenous and lymphocytic colitis. The melanosis coli category was incorporated as it is possible that reported cases of diarrhea might not be due to laxative abuse. Cases that were excluded were: (1) known cases of inflammatory bowel disease, malignancy, radiotherapy, infective diarrhea, rectal bleeding, and an abnormal colonoscopy.

Table 1 Microscopic colitis cases identified by IHC using CD3

	No. of cases	[1]*P* value
Cases marked as normal, chronic inflammation or melanosis coli	241	
IELs > 20	20	< 0.001
Known case of LC	1	0.158
Missed LC	5	< 0.001
Collagenous colitis	2	0.078
Microscopic colitis (Total No. of cases)	8	< 0.001

[1]*P* value for comparing whether the proportion was different from 0. LC: Lymphocytic colitis.

Immunohistochemistry

Immunohistochemistry using antibodies against CD3 was performed on all cases as the primary evaluation method for IELs. The staining was performed on 4-μm thick, formalin-fixed, paraffin-embedded tissue sections, using the Bond max autoimmune stainer with the Bond Polymer Refine Detection system (DS9800). Antibodies against CD3 (Leica Biosystems, Newcastle, UK; NCL-L-CD2-565, dilution 1:300) were applied to each case. For epitope retrieval ER2 (Leica Biosystems) was used for 20 min.

All immunohistochemical slides were randomly assigned a study number and an IEL count was performed. For a lymphocyte to be counted, the nucleus had to be visible with cytoplasmic and membrane staining. Intercryptal areas were counted and areas overlying lymphoid follicles were avoided. Only cases with ⩾ 20 per 100 IELs were further investigated. In addition, all H and E-stained sections were re-evaluated for basement membrane thickening. In suspected cases, a Masson Trichrome stain was performed and the basement membrane measured with an Olympus ocular micrometer. Poorly orientated biopsies were excluded from evaluation.

Data regarding presenting history, microscopic diagnosis, patient age, sex and follow-up of patients with ⩾ 20 IELs were recorded. A subcategory of patients presenting with chronic diarrhea was identified and further evaluated for microscopic colitis. The results were correlated with clinical findings.

Histological criteria

Histological diagnosis of lymphocytic colitis was confirmed with ⩾ 20 IELs per 100 surface epithelial cells, with normal being < 5[16,19,20]. In addition, a mixed inflammatory infiltrate in the lamina propria that consisted of lymphocytes and plasma cells with surface epithelial damage was noted[3,16]. Diagnosis of collagenous colitis was established with a subepithelial collagen layer reaching or exceeding 10 μm[16,21,22].

Ethics

The study protocol was approved by the University of Stellenbosch Ethics committee.

Statistical analysis

Microsoft Excel was used to capture the data and STATISTICA version 9 was used to analyze the data. Summary statistics were used to describe the demographic variables and certain laboratory parameters. Medians and means were used as the measures of central location and SDs and quartiles as indicators of spread. For demographic variables such as laboratory parameters, 95% CIs were calculated. Incidence rates in the population studied were determined as proportions and these were compared to determine whether they were significantly different from zero. $P < 0.05$ represented statistical significance in hypothesis testing.

RESULTS

Clinical and immunohistochemical findings

Immunohistochemical evaluation of 241 cases revealed a mean lymphocyte count of 7.7 (95% CI = 6.4-8.9). Twenty cases (8.3%) were identified as having an IEL count of ⩾ 20 per 100 surface epithelial cells ($P < 0.001$) (Table 1).

These 20 patients were further categorized and the clinicopathological features summarized in Table 2. Six (2.5%) of the 241 patients were identified as having lymphocytic colitis ($P < 0.001$). Five (2%) of these patients were only diagnosed in this review and were therefore missed on initial evaluation ($P = 0.01$). Four of the five patients were labeled with irritable bowel syndrome (IBS). On follow-up, 3/5 patients had persistent diarrhea, despite ongoing investigations. On review, their diagnosis was changed to microscopic colitis. The remaining 2/5 patients were lost to follow-up. Clinical symptoms that were not in keeping with irritable bowel syndrome in this group included increase stool frequency of up to three times per day and abdominal pain that woke the patient at night. These patients were not evaluated for response to treatment.

We included two patients who were later diagnosed with malignant disease (myelodysplastic syndrome and renal cell carcinoma) after a 2-year follow-up. A third patient developed multiple liver lesions with an unknown primary tumor. In addition, three patients initially reported as having normal colonoscopy were diagnosed with diverticular disease ($n = 2$) and ulcerative colitis ($n = 1$). Subsequent review of the surgical notes indicated an error in documentation.

Among the remaining eight patients, the primary presenting symptom resolved in four. Two patients with abdominal pain were later diagnosed with pancreatitis and Behcet's disease. It is not clear if the latter patient's abdominal pain was related to her condition. Two patients were lost to follow-up.

The two (0.8%) cases of collagenous colitis that were included in the total study population of 241 ($P = 0.07$) had an IEL count of 7 and 8, respectively. No additional cases of collagenous colitis were identified by selective staining with the Masson Trichrome technique.

The most common presenting complaint was chronic diarrhea in 9/20 cases, abdominal pain in 7/20, and constipation in 3/20, followed by loss of weight in 1/20. Seventeen cases were originally reported as normal on histology; one of lymphocytic colitis and two of melanosis coli (Table 2).

Table 2 Clinicopathological features and follow-up data in patients with > 20 intra-epithelial lymphocytes

Age	Sex	IEL count	Presenting symptom	Original biopsy diagnosis	Follow-up period for 2 yr
32	F	22	Chronic diarrhea	Normal	Lymphocytic colitis[1,2]
32	F	27	Chronic diarrhea	Normal	Lymphocytic colitis[2]
55	F	27	Constipation	Normal	Resolve
65	M	26	Abdominal pain	Normal	Myelodysplastic syndrome
22	F	31	Constipation	Normal	Lost to follow up
35	M	38	Chronic diarrhea	Normal	Lymphocytic colitis[1]
42	F	30	Constipation	Normal	Lost to follow up
59	F	28	Abdominal pain	Normal	Resolve
59	F	40	Chronic diarrhea	Normal	Multiple liver lesions
56	M	31	Chronic diarrhea	Normal	Ulcerative colitis
79	F	20	Abdominal pain	Melanosis coli	Diverticulitis
19	F	28	Abdominal pain	Normal	Behcet's disease
24	F	24	Chronic diarrhea	Normal	Lymphocytic colitis[2]
51	M	48	Chronic diarrhea	Normal	Metastatic renal cell carcinoma
56	F	30	Chronic diarrhea	Normal	Lymphocytic colitis[2]
34	F	30	Abdominal pain	Normal	Resolved
33	F	30	Chronic diarrhea	Lymphocytic colitis	Lymphocytic colitis[3]
71	M	20	Loss of weight	Normal	Diverticulitis
59	F	25	Abdominal pain	Normal	Resolve
35	F	35	Abdominal pain	Melanosis coli	Pancreatitis

[1]Lost to follow-up; [2]On review diagnosis changed from irritable bowel syndrome to lymphocytic colitis; [3]Known patient included in the study. IEL: Intra-epithelial lymphocyte.

Table 3 Clinicopathological features and follow-up data on patients with chronic diarrhea and 10-19 intra-epithelial lymphocytes

Age	Sex	IEL count	Original diagnosis	Comorbid disease	Follow-up period for 2 yr	
					Diagnosis	Diarrhea
54	F	10	Chronic inflammation	Diabetic hypertension	Lactose intolerant	Persistent
70	F	12	Melanosis coli	Diabetic	Autonomic neuropathy	Persistent
22	M	13	Chronic inflammation		Lost to FU	Lost to FU
64	M	15	Melanosis coli	Diabetic, asthmatic	Autonomic neuropathy	Persistent
59	F	16	Melanosis coli	Schistosomiasis contact	Irritable bowel syndrome	Persistent
64	F	18	Normal	Diabetic, asthmatic, previous sigmoidectomy for benign stricture	Hypothyroid	Persistent

IEL: Intra-epithelial lymphocyte; FU: Follow-up.

In addition, patients with an IEL > 10 and < 19 who presented with chronic diarrhea were documented to identify possible cases of paucicellular lymphocytic colitis (Table 3). Although no patients could be confidently diagnosed in this subgroup, comorbid disease such as diabetes accounted for several cases of diarrhea within this subgroup.

Histological findings

The histological findings among the different groups were very similar with IELs apparent in all 20 cases by H and E staining. However, the lymphocytes were more easily identified with the aid of CD3 (Figure 1). Poor staining quality and tangential biopsies accounted for misidentification of lymphocytes by H and E staining (Figure 2). Chronic inflammation was mild to moderate within the lamina propria.

In the lymphocytic colitis group, chronic inflammation was regarded as moderate in the lamina propria and 3/6 cases showed surface epithelial damage. Crypt branching was absent. Again, lymphocytes were more easily counted and identified with the immunohistochemical stain compared to H and E.

The two cases of collagenous colitis had a thick collagen band that measured 10 and 11 μm with visible entrapment of capillaries and mild to moderate chronic inflammation in the lamina propria.

Pigment was confirmed in two cases of melanosis coli but was subtle. The site of most biopsies could not be verified because it was not documented. None of the patients with microscopic colitis was evaluated for response to medical treatment.

DISCUSSION

An increase in the awareness of the entity of microscopic colitis has resulted in it being recognized as a known cause of chronic watery diarrhea[2,13,23]. Although the incidence of this disease seems to be rising, there has been very little documentation of this entity from our hospital. The importance of recognizing this condition is crucial, firstly, because chronic diarrhea is a debilitating illness, and secondly, treatment of this condition is no longer empirically based, as several recent randomized, double-blind, placebo-controlled trials have shown budesonide to be ef-

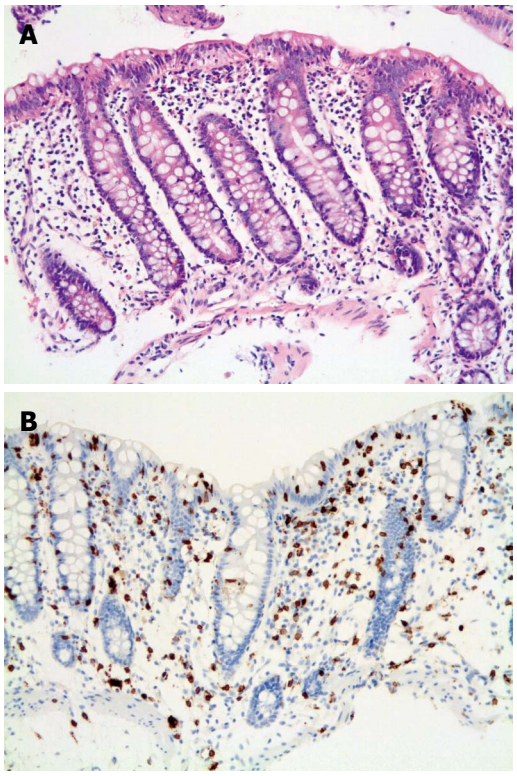

Figure 1 Lymphocytic colitis. A: Classic form. Colonic biopsy showing typical findings of diffuse increase in intraepithelial lymphocytes, mild inflammation with surface epithelial damage (H and E stain × 200); B: CD3 immunohistochemistry highlighting lymphocytes (× 200).

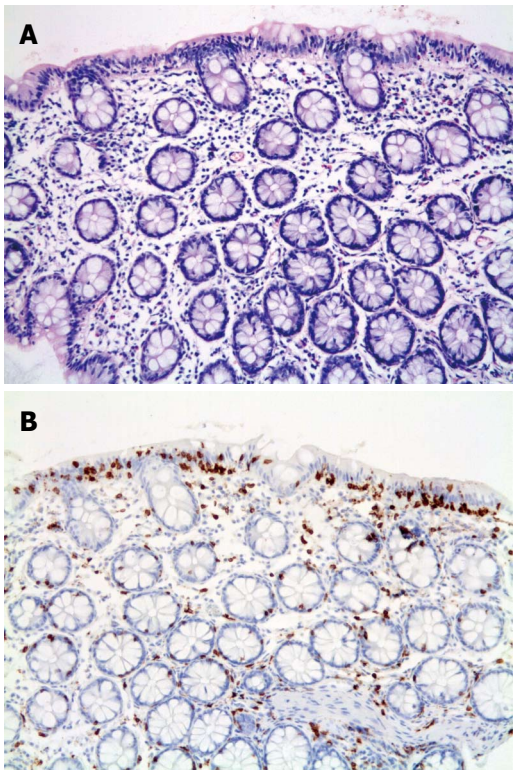

Figure 2 Lymphocytic colitis. A: Tangential colonic biopsy showing possible intraepithelial lymphocytes (H and E, original magnification × 200); B: The intraepithelial lymphocytes are more prominent with CD3 immunostaining (× 200).

fective in the treatment of this disorder[24-27].

Our study is novel in the sense that we reviewed all our non-malignant colon biopsies reported as normal or chronic inflammation, to identify patients with chronic diarrhea that might have had microscopic colitis. Using this bottom up approach, we focused on lymphocytic colitis. Neither the incidence nor the prevalence of this disease could be estimated using this approach, because our sample population did not consist of patients presenting exclusively with chronic diarrhea. Instead, we identified secondary causes of intraepithelial lymphocytosis that included diverticular disease, ulcerative colitis, and malignancy. These secondary causes need to be excluded before making a diagnosis of microscopic colitis[14]. Other secondary causes of intraepithelial lymphocytosis, not identified in this study but described by Nielson, include Crohn's disease, colonic infections and amyloidosis[14]. According to Fenoglio-Preiser[28], there have also been reports of lymphocytic-colitis-like histology in patients with constipation, which is similar to our findings. We have also identified patients with abdominal pain as another group presenting with lymphocytosis. Although the clinical symptoms of constipation and abdominal pain resolved in a few cases, others were later identified as having significant pathology. Our results suggest that any normal colonoscopy with a finding of intraepithelial lymphocytosis should be carefully monitored for future disease.

In the present study, the diagnosis of lymphocytic colitis was missed in five patients at the initial histological evaluation. It is particularly interesting to note that four of these patients were labeled as having IBS, in view of the biopsy being reported as normal. In a population-based cohort from Olmsted County, approximately one half of patients with microscopic colitis met the symptom-based criteria for IBS[29]. It is therefore not surprising that there is symptomatic overlap between these two entities. The recommendations from the Olmsted County study are that patients with diarrhea-predominant IBS should undergo colonoscopy to exclude microscopic colitis[29]. Similarly, Madisch *et al*[30]have shown that 30% of patients with microscopic colitis had clinical symptoms that overlap with IBS. We can therefore conclude that patients with microscopic colitis can be misdiagnosed with IBS.

Even though there is very little inter- and intra-observer variability in the histological diagnosis[19], the diagnosis of microscopic colitis can be challenging at times, especially due to the morphological heterogeneity described in microscopic colitis. Since the initial description of lymphocytic colitis in 1989, there have been several atypical forms of microscopic colitis described[15], including a paucicellular variant[31]. In this variant, patients still have the same clinical symptoms, but the IEL count is less, with only 10-12 IELs/100 enterocytes cited[32]. We feel that, in these cases, immunostaining might be of more diagnostic value in determining a low IEL that is not so apparent by H and E staining. A recent study has challenged the notion of regarding paucicellular lymphocytic colitis as a variant of classical lymphocytic colitis, based on the dem-

onstration of a distinct immunological difference[33]. This group also has indirectly claimed that immunostaining displays a clear contrast between immunoreactive lymphocytes and negative epithelial cells. However, the comparison between H and E staining and immunohistochemistry was not directly evaluated in their study[33]. Our study was not designed to identify cases of paucicellular lymphocytic colitis, but it is an area that requires further study.

As stated earlier, the prevalence of microscopic colitis is difficult to estimate from our study due to our selection criteria and referral bias. However, this study does indicate that microscopic colitis, especially the lymphocytic colitis subtype, is underdiagnosed at our institution ($P < 0.05$). For a true estimate of the prevalence of this disorder, further studies are needed, combining data from all referral centers in the region. Other factors not taken into account in this study are the site of the biopsy and drug history. It is well known that lymphocytic colitis and collagenous colitis can be patchy in distribution, and the topographic gradient of IELs decreases from the right colon to the rectum[34]. Therefore, representative biopsies should be taken from each part of the colon and submitted in a separate container. Concomitant drug use can cause or worsen drug-induced microscopic colitis. It is important to recognize these drugs because drug withdrawal may improve symptoms. Among the more common drugs implicated are non-steroidal anti-inflammatory drugs, lansoprazole, clozapine, ranitidine, ticlopidine, carbose and flutamide[32]. Future studies at our institution need to take these factors into account.

We identified that intraepithelial lymphocytosis may be an early manifestation of a disease other than microscopic colitis within our defined population. IEL count alone is not specific for microscopic colitis and the biopsy findings need to be correlated with clinical information for a more specific diagnosis. In cases in which there is a history of chronic watery diarrhea, the use of CD3 immunohistochemistry may be of additional value in making the diagnosis of lymphocytic colitis. We suggest that patients with diarrhea-predominant IBS should have a routine colonoscopy and be evaluated for microscopic colitis.

COMMENTS

Background

Microscopic colitis was previously considered a rare disorder, but it now accounts for approximately 10% of cases of chronic watery diarrhea. Cases are often under-recognized despite there being well-established histopathological criteria. It is suspected that colon mucosal biopsies are often under-reported as chronic inflammation, normal or colitis, not otherwise specified.

Research frontiers

Intra-epithelial lymphocytes (IELs) are crucial to the histological diagnosis of the lymphocytic colitis subtype. Immunohistochemistry has been shown to be of value in the quantification of IELs in celiac disease, but not in the identification and quantification of IELs in lymphocytic colitis.

Innovations and breakthroughs

A recent randomized, double-blind, placebo-controlled study has confirmed that budesonide is effective in the treatment of lymphocytic colitis. It has therefore become increasingly important to recognize this condition. This is believed to be the first time that normal colon biopsies were retrospectively reviewed and evaluated for IELs. The authors demonstrated that a subset of patients with chronic diarrhea was identified as having lymphocytic colitis using this approach.

Application

The value of this study demonstrates that immunohistochemistry is a useful adjunct to hematoxylin and eosin staining in the evaluation of IELs required for the diagnosis of lymphocytic colitis.

Terminology

Microscopic colitis is an umbrella term that comprises lymphocytic and collagenous subtypes. Although the latter is distinguished histologically by a thickened membrane, the clinical symptoms and colonoscopy findings are identical.

Peer review

This study illustrates well that patients with diarrhea-predominant irritable bowel syndrome should have a colon biopsy with close scrutiny of mucosal lymphocytes to exclude microscopic colitis.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.2003

World J Gastroenterol 2011 April 21; 17(15): 2003-2006
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Extracapsular invasion as a risk factor for disease recurrence in colorectal cancer

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Supported by Grants-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science, and Technology
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Received: November 4, 2010 Revised: November 25, 2010
Accepted: December 2, 2010
Published online: April 21, 2011

Abstract

AIM: To evaluate the presence of extracapsular invasion (ECI) in positive nodes as a predictor of disease recurrence disease in colorectal cancer.

METHODS: Two hundred and twenty-eight consecutive patients who underwent colorectal resection were identified for inclusion in this study, of which 46 had positive lymph nodes. Among 46 cases with stage Ⅲcolorectal cancer, 16 had ECI at positive nodes and 8 had disease recurrence. The clinical and pathological features of these cases were reviewed.

RESULTS: In the univariate analysis, the number of positive lymph nodes and depth of tumor invasion were significantly associated with the presence of ECI at positive nodes. Multivariate analysis demonstrated that only ECI was a predictor of recurrence. The recurrence-free interval differed significantly among patients with ECI at positive nodes.

CONCLUSION: Our results suggest that ECI at metastatic nodes can identify which cases are at high risk of short-term disease recurrence in colorectal cancer.

Key words: Extracapsular invasion; Lymph node; Metastasis; Colorectal cancer; Risk factor; Adjuvant therapy

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Fujii T, Tabe Y, Yajima R, Yamaguchi S, Tsutsumi S, Asao T, Kuwano H. Extracapsular invasion as a risk factor for disease recurrence in colorectal cancer. *World J Gastroenterol* 2011; 17(15): 2003-2006 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/2003.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.2003

INTRODUCTION

The role of systemic adjuvant chemotherapy in colorectal cancer patients with lymph node involvement has been established in a large number of clinical trials[1-3]. Lymph node status is one of the most important prognostic factors for colorectal carcinoma. However, patients with TNM stage Ⅲ colorectal cancer are a heterogeneous group. Some patients with stage Ⅲ colorectal cancer have good prognoses, similar to that of patients with stage Ⅱ disease, whereas others develop disease recurrence. It is of utmost importance to develop markers that can predict which patients are at high risk for disease recurrence.

Previous studies have demonstrated and confirmed that the presence of extracapsular invasion (ECI) at metastatic lymph nodes is significantly related to prognosis in various types of carcinoma including colorectal cancer[4-11]. We have also recently demonstrated that ECI at metastatic nodes in breast and colorectal cancer was strongly associated with

further regional nodes metastasis[12]. The purpose of this study was to investigate the correlation between the presence of ECI in positive lymph nodes and disease recurrence in cases of colorectal cancer undergoing curative operation. It will be advantageous to be able to tailor therapy individually, using ECI as an indicator of the risk of recurrence.

MATERIALS AND METHODS

Two hundred and twenty-eight consecutive patients who underwent colorectal resection in the Department of General Surgical Science, Graduate School of Medicine, Gunma University, from January 2007 to December 2009 were identified for inclusion in this study. Patients with recurrence or metastasis at operation, neo-adjuvant chemotherapy, radiation, or incomplete clinical information were excluded. Of the eligible cases, 46 (20.2%) with positive lymph nodes, identified as TNM stage Ⅲ colorectal, were analyzed in this study. The clinical features of these cases were reviewed according to the presence or not of ECI at positive lymph nodes, and statistical analysis was performed. ECI was defined as extracapsular growth of tumor cells, invasion into perinodal fat or extranodal location of tumor cells[12]. Informed consent was obtained from all patients.

Age, sex, primary tumor size, location, depth of tumor invasion, histological type, lymphovascular invasion at the primary tumor site, number of metastatic lymph nodes, ECI at positive lymph nodes, administration of adjuvant therapy and serum tumor markers (carcinoembryonic antigen) were tested as possible predictors of disease recurrence. Recurrence-free interval was defined as the interval from surgery to the time disease recurrence was diagnosed. The overall median follow-up period was 1.7 years and none of the patients died of surgical complications. Fisher's exact test, the Chi-squared test, and the Student t-test were used to compare the 2 groups. Multivariate analysis was performed with logistic regression analysis to select covariates (primary tumor size and ECI at positive lymph nodes). The recurrence-free interval was calculated by the Kaplan-Meier method. The log-rank test was used to evaluate differences between recurrence-free intervals. Differences were considered to be significant at $P < 0.05$.

RESULTS

Table 1 summarizes the characteristics of the patients who underwent colorectal resection with TNM stage Ⅲ colorectal cancer. The series consisted of 16 cases with ECI at positive nodes and 30 with no ECI at positive nodes. Table 1 also summarizes the results of the univariate analysis conducted to determine the relationship between the clinicopathologic variables and the presence of ECI at positive nodes. The number of positive lymph nodes and the depth of tumor invasion were significantly associated with the presence of ECI at positive nodes.

The 46 cases with metastatic lymph nodes were divided into 2 groups based on the presence of disease recurrence. Among 46 cases with stage Ⅲ colorectal cancer, 8 (17.4%) had disease recurrence. Table 2 summarizes the results of the univariate analysis conducted to determine the relationship between the clinicopathologic variables and disease recurrence. In the univariate analysis ECI at positive nodes and the depth of tumor invasion were the factors significantly associated with disease recurrence. Among those, multivariate analysis demonstrated that only ECI was a predictor of the recurrence ($P = 0.016$). Time to tumor recurrence by Kaplan-Meier curves was significantly shorter among patients with ECI at positive nodes (Figure 1).

Table 1 Patients characteristics and clinicopathological features associated with the presence of extracapsular invasion at lymph node metastases

	ECI	Positive *n* = 16	Negative *n* = 30	*P* value
Age (yr)		65.3 ± 16.1	66.5 ± 13.8	0.798
Sex				
Male		7	21	0.082
Female		9	9	
Location				
Colon		13	20	0.295
Rectum		3	10	
Histological type				
Tub		15	28	0.957
Muc		1	2	
pT category				
T 1,2		1	10	0.040
T 3,4		15	20	
Tumor size (mm)		47.3 ± 15.1	40.4 ± 21.2	0.270
Number of positive LNs		3.63 ± 2.29	1.70 ± 1.27	0.001
Lymphovascular invasion		16	29	0.460
CEA ⩾ 3.0		3	3	0.041
Adjuvant treatment	13	0.720	23	0.720

LN: Lymph node; ECI: Extracapsular invasion; CEA: Carcinoembryonic antigen.

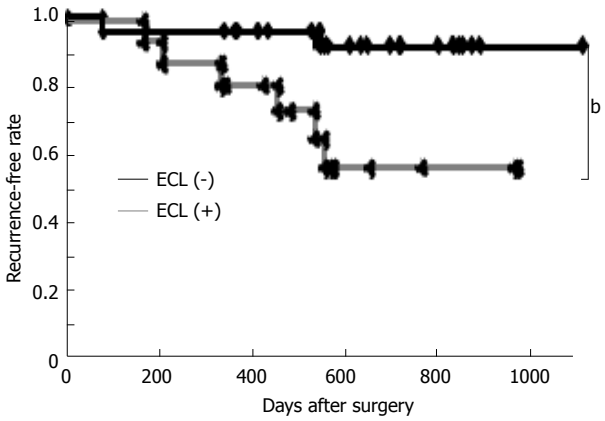

Figure 1 Impact of the presence of extracapsular invasion at positive nodes on postoperative recurrence-free interval. Recurrence-free interval by Kaplan-Meier curves differed significantly among patients with and without extracapsular invasion at positive nodes. $^{b}P < 0.01$.

DISCUSSION

The key observations made in this study can be summarized as follows: (1) the presence of ECI at positive nodes

Table 2 Patient characteristics and clinicopathological features associated with recurrent disease

	Recurrent disease	Positive 8	Negative 38	*P* value
Age (yr)		68.0 ± 17.2	65.7 ± 14.0	0.780
Sex				
Male		3	25	0.278
Female		5	13	
Location				
Colon		6	27	0.795
Rectum		2	11	
Histological type				
Tub		7	36	0.451
Muc		1	2	
PT category				
T 1, 2		0	11	< 0.001
T 3, 4		8	27	
Tumor size (mm)		44.9 ± 11.6	42.5 ± 20.8	0.679
Number of positive LNs		2.38 ± 1.41	2.37 ± 2.02	0.906
ECI		6	10	0.009
Lymphovascular invasion		8	37	0.643
CEA	⩾ 3.0	3	3	0.093
Adjuvant treatment		5	31	0.473

ECI: Extracapsular invasion; CEA: Carcinoembryonic antigen.

was significantly associated with the number of positive lymph nodes and depth of tumor invasion; (2) multivariate analysis demonstrated that only ECI was a predictor of recurrence; and (3) the recurrence-free interval by Kaplan-Meier curves was significantly shorter among patients with ECI at positive nodes. These findings suggest that the presence of ECI at positive lymph nodes is a strong predictor for short-term recurrence in cases with colorectal cancer undergoing curative surgery.

The surgical stage remains the most accurate predictor of survival for colorectal cancer[13]. Pathologic prognostic factors of primary tumor invasion and regional node involvement predict the risk of relapse of cases with colorectal cancer undergoing curative operation. In the current study, both the number of positive lymph nodes and the depth of tumor invasion were significantly associated with the presence of ECI at positive nodes. Lymph node metastasis is one of the most important prognostic factors in patients with colorectal cancer, and many studies have indicated that the location and number of metastatic nodes affect prognosis[5,14-16]. Regarding ECI, previous studies have demonstrated and confirmed that the presence of ECI at metastatic lymph nodes is significantly associated with prognoses in various types of carcinoma including colorectal carcinoma[4-11]. The ability of metastatic nodes to recruit degradation factors that permit cancer cells to break through the lymph node capsule is indicative of a very aggressive cancer. We previously demonstrated that the presence of ECI in positive lymph nodes is significantly related to the nodal spread of tumor cells in colorectal and breast cancer patients[12]. These studies imply that ECI is a biologic marker of aggressive cancer and essentially support our findings. Tumor cells are thought to invade the lymphovascular vessels, which enables tumor cells to spread metastatic or recurrent disease.

Adjuvant therapy is systemic treatment administered with the intent of reducing the risk of recurrence. The benefit of adjuvant therapy in patients with lymph node involvement (stage Ⅲ) has been well established in large prospective randomized trials[1-3]. In Japan, the oxaliplatin plus 5-fluorouracil/leucovorin (LV) (FOLFOX) regimen has not been approved for adjuvant therapy for patients with stage Ⅲ colorectal cancer at this time. In this Japanese population study, oral chemotherapeutic agents, including capecitabine or UFT (tegafur plus uracil) with oral LV, were used for adjuvant therapy for stage Ⅲ colorectal cancer. Oral chemotherapeutic agents are advantageous because of their ease of administration. However, in the current series, 13 (81.3%) of the 16 cases with ECI in positive nodes had oral adjuvant therapy but 6 of 16 cases (37.5%) had disease recurrence. These results imply that high-risk patients with ECI at positive nodes should receive stronger adjuvant chemotherapy, including FOLFOX with or without monoclonal antibody.

This study has several potential limitations. The major limitation of our study is that it used retrospective methods of data collection. In addition, the number of cases in our study was relatively small and the follow-up periods were relatively short. However, the clinical implications of this data are very important, and these findings serve to emphasize that ECI at metastatic lymph nodes is an important prognostic factor for stage Ⅲ colorectal carcinoma and will be advantageous in tailoring therapy to the individual case. In patients treated in phase Ⅲ adjuvant clinical trials, disease-free survival and overall survival have been highly correlated, both within studies and across trials[17]; however, in a number of patients, improving the quality of life and the length of recurrence-free intervals may be more important statistical parameters than median overall survival. Additional research is needed to explore this putative association between the presence of ECI and the risk of recurrence.

In conclusion, we have demonstrated that ECI at met-

astatic lymph nodes may predict which cases are at high risk of short-term disease recurrence in colorectal cancer. Thus, it will be possible to tailor therapy individually, using ECI as an indicator of the risk of recurrence. Analyses from large randomized trials or experimental data are warranted to evaluate this relationship between the presence of ECI and disease recurrence.

ACKNOWLEDGMENTS

The authors would like to thank Saitoh Y, Yano T, Ohno M, Matsui Y, Muraoka S and Sekiguchi M for their secretarial assistance

COMMENTS

Background

The authors have demonstrated that extracapsular invasion (ECI) at metastatic nodes in breast and colorectal cancer was strongly associated with further regional nodes metastasis. The purpose of this study was to evaluate the presence of ECI in positive nodes as a predictor of disease recurrence disease in colorectal cancer.

Research frontiers

Lymph node status is one of the most important prognostic factors for colorectal carcinoma. However, patients with TNM stage Ⅲ colorectal cancer are a heterogeneous group. It is of utmost importance to develop markers that can predict which patients are at high risk for disease recurrence.

Innovations and breakthroughs

This study suggests that the presence of ECI at positive lymph nodes is a strong predictor for short-term recurrent-free interval in cases with colorectal cancer undergoing curative operation.

Applications

It will be possible to tailor therapy individually, using ECI as an indicator of the risk of recurrence.

Terminology

ECI was defined as extracapsular growth of tumor cells, invasion into perinodal fat or extranodal location of tumor cells.

Peer review

The key observations made in this study suggest that the presence of ECI at positive lymph nodes is a strong predictor of short-term recurrent-free interval in cases with colorectal cancer undergoing curative operation.

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S- Editor Sun H **L- Editor** Cant MR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.2007

World J Gastroenterol 2011 April 21; 17(15): 2007-2012
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Impact of disease severity on gastric residual volume in critical patients

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Supported by Kaohsiung Veterans General Hospital, Grant No. VGHKS 94-082
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Received: September 10, 2010 Revised: December 14, 2010
Accepted: December 21, 2010
Published online: April 21, 2011

Abstract

AIM: To investigate whether illness severity has an impact on gastric residual volume (GRV) in medical critically ill patients.

METHODS: Medical intensive care unit (ICU) patients requiring nasogastric feeding were enrolled. Sequential Organ Failure Assessment (SOFA) score was assessed immediately preceding the start of the study. Acute Physiology and Chronic Health Evaluation (APACHE) Ⅱ scores were recorded on the first, fourth, seventh, and fourteenth day of the study period. GRV was measured every 4 h during enteral feeding. The relationship between mean daily GRV and SOFA scores and the correlation between mean daily GRV and mean APACHE Ⅱ score of all patients were evaluated and compared.

RESULTS: Of the 61 patients, 43 patients were survivors and 18 patients were non-survivors. The mean daily GRV increased as SOFA scores increased (P < 0.001, analysis of variance). Mean APACHE Ⅱ scores of all patients correlated with mean daily GRV (P = 0.011, Pearson correlation) during the study period. Patients with decreasing GRV in the first 2 d had better survival than patients without decreasing GRV (P = 0.017, log rank test).

CONCLUSION: GRV is higher in more severely ill medical ICU patients. Patients with decreasing GRV had lower ICU mortality than patients without decreasing GRV.

Key words: Critical care; Outcome; Residual volume; Severity of illness index; Tube feeding

Peer reviewer: Maxim Petrov, MD, MPH, Department of Surgery, University of Auckland, Private Bag 921019, Auckland 1142, New Zealand

Hsu CW, Sun SF, Lee DL, Lin SL, Wong KF, Huang HH, Li HJ. Impact of disease severity on gastric residual volume in critical patients. *World J Gastroenterol* 2011; 17(15): 2007-2012 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/2007.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.2007

INTRODUCTION

Malnutrition is prevalent in intensive care unit (ICU) patients and is associated with increased morbidity and mortality[1]. Early administration of enteral nutrition to

critically ill patients has been associated with a significantly lower incidence of infections and a reduced length of hospital stay[2,3]. However, intragastric enteral nutrition often is complicated by intolerance, as indicated by elevated volumes of aspirated gastric residuals[4]. Disordered upper gastrointestinal (GI) tract motility occurs frequently in ICU patients. Intolerance to nasogastric delivery of feeding is the most important consequence of the abnormal upper GI motility that occurs in critically ill patients[5]. Several factors related to critical illness have been reported to be associated with gastric dysmotility and feeding intolerance including age, admission diagnosis, hyperglycemia, the nature of the acute illness, mechanical ventilation, sedatives, cytokine release and splanchnic hypoperfusion due to shock and sepsis[6]. Two studies have shown that "upper digestive intolerance" and "enteral feeding intolerance" are linked to adverse outcomes, suggesting that decreased gastric emptying (GE) is related to clinical deterioration and worsening of patient outcomes[7,8]. Direct measurement of GE is usually inconvenient and impractical in routine clinical practice. Clinically, gastric residual volumes (GRVs) are easier to measure than GE, and GRV measurements are by far the most frequently recommended assessment for GE[9]. They are used as a surrogate marker to determine the success or failure of nutrition delivered *via* a nasogastric route. However, the relationship between GRV and disease severity is not clear. The aim of this study is to investigate whether disease severity has an impact on GRV and whether GRV is a predictor of ICU mortality.

MATERIALS AND METHODS

This prospective, observational study was conducted during a 2-year period from January 2005 to December 2006 in a medical ICU of a tertiary medical center. Patients who required enteral feeding were enrolled. Criteria for exclusion included abdominal surgery, acute pancreatitis, GI bleeding, intestinal obstruction, and patients with subtotal or total gastrectomy. The protocol was approved by the Human Investigation and Research Committee of the hospital.

After informed consent was obtained, the following demographic data were collected: primary ICU admission diagnosis, age, gender, body mass index (BMI), use of mechanical ventilation, Sequential Organ Failure Assessment (SOFA) score[10], Acute Physiology and Chronic Health Evaluation (APACHE) Ⅱ score[11], blood glucose level, number of ICU days, ventilator days, hospital days, and Glasgow Coma Scale score. A standard 12 French enteral feeding tube (Abbott, Chicago, IL, USA) for general patients was placed into the stomach. The correct position of the nasogastric tube was confirmed by injecting 50 mL of air with a syringe into the tube and auscultating the epigastric area, or by radiograph if necessary. We checked the tube position by measuring the exposed portion of the tube and compared the length with previous measurements. The patients were fed in a semi-recumbent position, and the patient's position and tube length were kept the same in each measurement. As soon as the feeding tube was inserted, continuous tube feeding using enteral feeding pumps (Abbott) was started. Enteral feeding was initiated at 20 mL/h. The rate was increased by 20 mL/h every 4 h until the volume required to meet the patient's optimum caloric support was achieved. The rate of continuous enteral feeding was controlled by the pumps. GRV was measured by aspirating with a 50-mL syringe every 4 h until the end of enteral feeding. Feeding was stopped for 30 min before GRV was measured. After measurement, the nurses stopped enteral tube feedings if residual volume was higher than 500 mL or residual volume was between 200 to 500 mL and patients had abdominal distension, absence of bowel sounds, or presence of nausea or vomiting[12]. Feeding re-started immediately at original rate if GRV < 200 mL and there was low risk of aspiration. Daily GRV was calculated by summation of each GRV measurement. Serum glucose was controlled by an intensive insulin control protocol in order to reach the target glucose level of 140 mg/dL.

APACHE Ⅱ scores[11] were recorded on the first, fourth, seventh, and fourteenth day of the study period. Study observations continued from start of enteral feeding until one of the following events occurred: the enteral tube was removed, the patient was discharged from the ICU, or he/she expired. SOFA scores[10] were assessed within a 24-h period preceding the start of study as the presence or absence of prospectively defined cardiovascular, respiratory, renal, hepatic and hematologic dysfunction, as well as level of consciousness. Dysfunction of cardiovascular, respiratory, renal, hepatic, hematologic and central nervous systems was determined based on laboratory data, vasopressor dosage, Glasgow Coma Scale score, and PaO_2/FiO_2.

Patients were classified as diabetic on the basis of their medical history. Survivors were defined as patients who were alive when discharged from the ICU or transferred to a general medical ward; this was determined at time of ICU discharge.

Statistical analysis

All the statistical analyses were done with the SPSS (Inc., Chicago, IL, USA) version 12.0. mean ± SE were recorded for all continuous variables. For discrete variables, the frequencies were reported. One way analysis of variance (ANOVA) was used to compare differences among more than 2 groups. Pearson correlation was used to compare correlation between daily GRV and APACHE Ⅱ score. Kaplan-Meier curves were used to estimate the probability of survival. Log-rank test was used to compare the difference between the patients with decreasing daily GRV and patients without decreasing daily GRV in the first 2 d. Cox model was used to construct the relative risk among the percentage change of daily GRV in the first 2 d. All P values were two-tailed. A P value < 0.05 was considered as significant.

RESULTS

Demographics

Sixty-one patients were enrolled in this study. Patient char-

Table 1 Demographic data of the 61 study subjects (mean ± SE) *n* (%)

Average age (yr)	67.9 ± 2.0
Gender (male)	43/61 (70.1)
Body mass index (kg/m^2)	23.1 ± 0.5
Diabetic patients	22/61 (36.1)
Mechanically ventilated patients	61/61 (100)
Study days	11.7 ± 0.8
ICU days	19.1 ± 1.6
Ventilator days	23.8 ± 2.3
Hospital days	31.7 ± 2.7
SOFA score	7.5 ± 0.6
APACHE Ⅱ score	20.2 ± 0.9
Blood glucose level (mg/dL)	184.7 ± 8.6
ICU mortality rate	18/61 (29.5)
Admission diagnosis	
Pneumonia	19/61 (31.1)
Sepsis	18/61 (29.5)
CHF	6/61 (9.8)
ARDS	6/61 (9.8)
Stroke	5/61 (8.2)
COPD or asthma	5/61 (8.2)
Myocardial infarction	1/61 (1.6)
Toxic overdose	1/61 (1.6)

ICU: Intensive care unit; SOFA: Sequential organ failure assessment; APACHE: Acute physiology and chronic health evaluation; CHF: Congestive heart failure; ARDS: Acute respiratory distress syndrome; COPD: Chronic obstructive pulmonary disease.

acteristics are summarized in Table 1. The mean patient age was 67.9 ± 2.0 years, and 70.1% of the patients were male. The mean BMI was 23.1 ± 0.5 kg/m^2. Thirty-six percent of patients had diabetes and all patients were mechanically ventilated. The mean number of study days was 11.7 ± 0.8 d, mean number of ICU days was 19.1 ± 1.6 d, mean number of ventilator days was 23.8 ± 2.3 d, mean number of hospital days was 31.7 ± 2.7 d, mean SOFA score was 7.5 ± 0.6, mean APACHE Ⅱ score was 20.2 ± 0.9, and mean blood glucose was 184.7 ± 8.6 mg/dL. The ICU mortality rate was 29.5%. The 4 most common admission diagnoses were pneumonia (n = 19), sepsis (n = 18), congestive heart failure (n = 6) and acute respiratory distress syndrome (n = 6).

Relationship between mean daily GRV and SOFA score

We stratified study patients into 4 groups by SOFA score. There were 27 patients with SOFA scores below 6; 18 patients with scores in the range of 6-10; 10 patients with scores in the range of 11-15; and 6 patients with scores above 15. Figure 1 demonstrates that the mean daily GRV was 7.8 ± 1.3 mL in the patients with a SOFA score below 6, 26.6 ± 4.8 mL in the patients with SOFA scores in the range of 6-10, 59.8 ± 4.5 mL in the patients with SOFA scores in the range of 11-15, and 133.2 ± 40.0 mL in the patients with a SOFA score above 15. Patient with higher SOFA scores had significantly higher daily GRV (P < 0.001, ANOVA).

Relationship between daily GRV and APACHE Ⅱ score during study period

During the study period, the mean APACHE Ⅱ scores

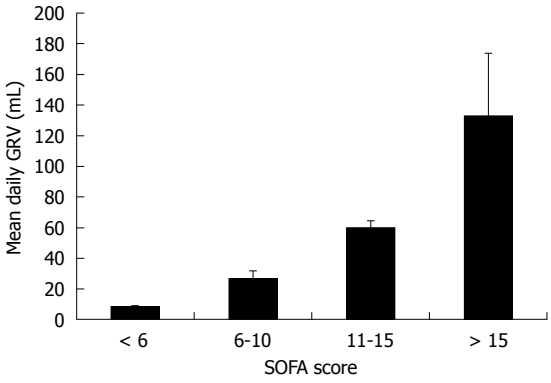

Figure 1 Mean daily gastric residual volume of patients with different Sequential Organ Failure Assessment scores (P < 0.001). SOFA: Sequential Organ Failure Assessment.

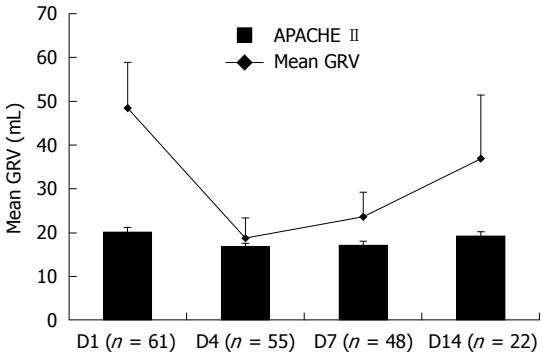

Figure 2 The relationship of mean daily Acute Physiology and Chronic Health Evaluation Ⅱ score to mean daily gastric residual volume on different study dates (P = 0.011).

and mean daily GRV of all patients on the first, fourth, seventh, and fourteenth day are shown in Figure 2. The mean APACHE Ⅱ scores of all patients on the first, fourth, seventh, and fourteenth day were 20.2 ± 0.9, 16.8 ± 0.7, 17.0 ± 0.9 and 19.1 ± 1.1, respectively. The mean daily GRVs of all patients on the first, fourth, seventh, and fourteenth day were 48.4 ± 10.4, 18.6 ± 4.6, 23.6 ± 5.5 and 36.9 ± 14.4 mL, respectively. The mean daily GRV fluctuated simultaneously with the mean APACHE Ⅱ scores during the study period. There was a significant correlation between daily GRV and APACHE Ⅱ score (P = 0.011, Pearson correlation = 0.338).

Difference of mean daily GRV between survivors and non-survivors

We divided study patients into survivors and non-survivors. There were 43 patients in the survivor group and 18 patients in the non-survivor group. Figure 3 shows the mean daily GRV of survivors and non-survivors during the study period. The mean daily GRV of non-survivors was higher than that of survivors in the early and late stages of the study period. Non-survivors had a trend of increasing mean daily GRV in the first 2 d, while survivors had a decreasing trend of GRV.

Table 2 demonstrates that the mean GRV1 (daily GRV on the first day) was 74.4 ± 31.0 mL in non-survivors

Table 2 Difference of GRV1 and PDay12 between survivors and non-survivors (mean ± SE)

	Non-survivors (n = 18)	Survivors (n = 43)
GRV1 (mL)	74.4 ± 31.0	38.0 ± 6.9
PDay12	-1.1 ± 0.6	0.3 ± 0.1

GRV1: Gastric residual volume on the first day; GRV2: Gastric residual volume on the second day. PDay12 = (GRV1 - GRV2)/GRV1.

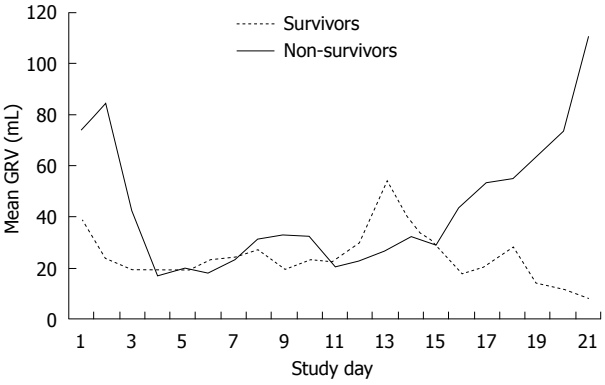

Figure 3 Mean daily gastric residual volume change of survivors and non-survivors during the study period.

and 38.0 ± 6.9 mL in survivors. Additionally, the mean [GRV1-GRV2 (daily GRV on the second day)]/GRV1 was -1.1 ± 0.6 mL in non-survivors and 0.3 ± 0.1 mL in survivors. If we define the percentage of daily GRV change from day 1 to day 2 (PDay12) as (GRV1-GRV2)/GRV1, non-survivors had a negative PDay12 while survivors had a positive PDay12.

Change of daily GRV in first 2 d and ICU mortality

We categorized patients into 2 groups; one group was patients without a decreasing daily GRV (PDay12 ≤ 0) in the first 2 d, and the other group was patients with a decreasing daily GRV in the first 2 d (PDay12 > 0). Thirty patients did not have a decreasing daily GRV (PDay12 ≤ 0) and 31 patients had a decreasing daily GRV (PDay12 > 0). Patients with PDay12 > 0 had a significantly higher ICU survival rate than patients with PDay12 ≤ 0 (P = 0.017) (Figure 4). Relative risk among the percentage change of daily GRV in the first 2 d was constructed by Cox model. The relative risk is equal to exp {-1.211x} where x represents the percentage change of daily GRV in the first 2 d. If the daily GRV decreased by 10% in the second day, the relative risk is equal to 0.886 (= exp {-0.1211}). If the daily GRV increased by 10% in the second day, the relative risk is equal to 1.129 (= exp {0.1211}). There was no evidence indicating that the proportional hazards assumption was not fixed in the data set.

DISCUSSION

In this prospective study, we found disease severity was associated with GRV in these medical ICU patients. This

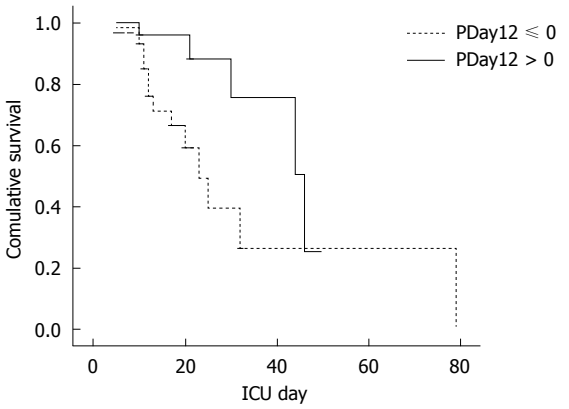

Figure 4 Kaplan-Meier estimates of the probability of survival between patients with decreasing change of daily gastric residual volume (PDay12 > 0) and patients without decreasing change of daily gastric residual volume (PDay12 ≤ 0) (P = 0.017).

study showed patients with higher SOFA scores had higher GRV, i.e. GRV tended to be higher in more severely ill patients. These results are similar to those of the study by Mentec *et al*[8], in which patients with high gastric aspirate volume had a higher ICU mortality rate. Slow GE may partly explain why patients with more severe illness had higher GRV. GRV is determined by the balance between the amount of infused formula plus endogenous secretions (saliva and gastric secretions), and the amount of fluid emptied from the stomach[9]. GE is influenced by many factors, including admission diagnosis, nature of illness, age, medications and mechanical ventilation[5,6,8,9]. Nguyen *et al*[6] reported that slow GE was more common in patients who were older, had higher admission APACHE Ⅱ scores, admission blood glucose and bilirubin concentrations, and were ventilated with synchronized intermittent mandatory ventilation. Of these, APACHE Ⅱ scores correlated best with GE, suggesting that illness severity is an important determinant of GE in critically ill patients. Patients with severe illnesses have high levels of circulating catecholamines which are likely to have an impact on GI motor function. Adrenaline reduces GE *via* a β-adrenergic effect[13].

During the study period, we also found a significant correlation between mean APACHE Ⅱ scores and mean GRV. There was a trend that patients with higher APACHE Ⅱ scores had higher daily GRV. Our results also demonstrated that illness severity was related to GRV, and illness severity varied during the ICU course. Concurrent day-to-day variation in illness severity and daily GRV were seen in this study. GRV was the greatest in the first few days of tube feedings. Some of the severely ill patients with high APACHE Ⅱ scores expired in the first few days, thus the overall illness severity of patients declined from the first day to the fourth day, so mean APACHE Ⅱ score also decreased from the first day to the fourth day. Mean daily GRV decreased as mean APACHE Ⅱ score decreased. As the study went on, some patients improved, they were extubated, and their enteral feeding stopped; therefore the number of

study patients decreased gradually. The rest of the study patients were more complicated or severely ill and they had higher APACHE Ⅱ scores, thus the mean APACHE Ⅱ score increased gradually after the seventh day of the study. From that time on, mean daily GRV also increased gradually. Mean daily GRV exhibited a good correlation with APACHE Ⅱ scores ($P < 0.05$).

In the interim analysis, we also found that patients with the same SOFA score had widely different daily GRV. This suggests that there was wide variability of daily GRV among patients with the same disease severity, since GRV is not only influenced by disease severity but also by other factors such as GE, medications, size of the feeding tube and timing of measurement.

GE itself is influenced by many factors. Admission diagnosis had modest impact on GE in critically ill patients[6]. Most patients with increased intracranial pressure after a head injury have been found to have slow GE, and elevated intracranial pressure is thought to be the main mediator of impaired gastric motility and emptying[14]. Other diseases associated with delayed GE include burns, multiple trauma, sepsis, chronic liver disease, and renal disease[6,15]. Patients with myocardial injury and non-intestinal post-operative respiratory failure have the lowest incidence of delayed GE[6]. Hyperglycemia has also been shown to result in delayed GE[16].

Medications used routinely in ICU patients are also likely to have clinically important effects on GI motor function. Opioids and benzodiazepines can impair gastric motility and reduce GE[17,18]. High gastric aspirate volume was more frequently seen in patients who received at least 1 d of sedation[8]. Both endogenous and administered opiates, acting *via* μ receptors, may contribute to abnormal upper GI motor function[19,20]. Dopamine slows GE by reducing antral contractions[21]. Its negative effect on GI motility can be seen at doses as low as 5 μg/kg per minute, and the effect increases with increasing rates of infusion[22]. Proton pump inhibitors[23] and cimetidine[24] can delay GE. Other medications such as phenothiazines, diltiazem, verapamil and anticholinergic drugs also cause GI hypomotility[5]. Use of promotility agents is associated with reduced GRV[25], e.g. erythromycin is a powerful stimulator of gastric contractions[26].

The size of enteral feeding tube has also been shown to influence the measurement of GRV. Higher GRVs are found in patients with larger enteral feeding tubes[27]. Timing of measurement of GRV also affects the value of GRV. GRV typically increases and then plateaus in the first 3 to 6 h after feeding. The highest GRV tends to occur in the 2 to 4 h after initiation of feedings. Additionally, GRV may vary with different infusion rate. To avoid this confounding factor, we stopped feeding for 30 min prior to measurement of GRV. This is the reason why the measured GRV values in this study are lower than in previous studies[28,29].

As discussed above, there are many factors that influence GRV. This results in wide variability of daily GRV in patients with the same illness severity. For this reason, we used daily GRV change rather than a single daily GRV to predict a patient's ICU outcome. This may avoid some of the confounding factors such as admission diagnosis, nature of illness, medications and age, as it reflects the change of illness severity more accurately. In addition, we also found that there was a different trend of GRV change between survivors and non-survivors in the first 2 d. Daily GRV tended to increase in non-survivors and decrease in survivors. We found that patients with a decreasing change of daily GRV had better ICU survival than those without a decreasing change of daily GRV in the first 2 study days. Because daily GRV correlated with illness severity, decreasing changes of daily GRV in the earlier ICU days represented decreasing illness severity; thus could indicate a positive sign for the medical ICU patients.

There are limitations in this study. Firstly, this was not a double-blind study. However, nurses who measured GRV were not aware of the study purpose. Secondly, all patients in this study were mechanically ventilated and enrolled from a medical ICU; thus, the results may not be applicable to all critically ill patients. Further larger studies including all critically ill patients are needed. Thirdly, we did not know how many patients had diabetes with gastroparesis which is characterized by severely slow GE; such patients may have increased GRVs secondary to gastroparesis[30].

In conclusion, illness severity has an impact on daily GRV. There was a trend that more severely ill medical ICU patients had higher daily GRV. Patients with decreasing change of daily GRV in the earlier ICU days had better ICU survival than patients without decreasing change of daily GRV.

ACKNOWLEDGMENTS

The authors would like to thank Shiu-Ping Kang and the medical staff (physicians, nurses and dietitians) in the intensive care units of Kaohsiung Veterans General Hospital for their assistance in this study.

COMMENTS

Background

Disordered upper gastrointestinal tract motility occurs frequently in critical patients, resulting in intolerance to nasogastric delivery of feeding. Upper digestive intolerance and enteral feeding intolerance are linked to adverse outcomes, suggesting that decreased gastric emptying (GE) is related to clinical deterioration. It is easier to measure gastric residual volume (GRV) than GE, but it is not clear whether illness severity has an impact on GRV.

Research frontiers

GE was influenced by age, Acute Physiology and Chronic Health Evaluation (APACHE) Ⅱ score, nature of illness, medication and mechanical ventilation. Of these, APACHE Ⅱ score correlated best with GE. This study found disease severity had an impact on GRV. Concurrent day-to-day variation in illness severity and daily GRV were seen during hospitalization course. There is a trend that GRV increases when disease severity increases, and *vice versa*.

Innovations and breakthroughs

Patients with decreasing change of GRV in the earlier intensive care unit (ICU) had better ICU outcome than patients without decreasing change. We emphasized that change of daily GRV, not a single daily GRV, can predict ICU

outcome. Since GRV was influenced by many factors, change of daily GRV can avoid some confounding factors other than illness severity.

Applications

Increasing change of daily GRV is a negative sign for critical patients. In the clinical situation, we should be careful if a patient has increasing change of daily GRV day by day as it may indicate the patient getting gradually worse.

Terminology

GRV is determined by the balance between the amount of infused formula plus endogenous secretion and the amount of fluid emptied from the stomach. APACHE Ⅱ score, a system for classifying disease severity, is widely used to predict hospital mortality based on a number of laboratory values and patient characteristics. Sequential Organ Failure Assessment scores were assessed as presence or absence of cardiovascular, respiratory, renal, hepatic, hematologic and central nervous systems dysfunction. A higher score means more severe illness.

Peer review

The manuscript is of good quality.

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S- Editor Sun H **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.2013

World J Gastroenterol 2011 April 21; 17(15): 2013-2018
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Effect of multidisciplinary team treatment on outcomes of patients with gastrointestinal malignancy

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Received: November 22, 2010 Revised: January 4, 2011
Accepted: January 11, 2011
Published online: April 21, 2011

Abstract

AIM: To evaluate the effect of multidisciplinary team (MDT) treatment modality on outcomes of patients with gastrointestinal malignancy in China.

METHODS: Data about patients with gastric and colorectal cancer treated in our center during the past 10 years were collected and divided into two parts. Part 1 consisted of the data collected from 516 consecutive complicated cases discussed at MDT meetings in Peking University School of Oncology (PKUSO) from December 2005 to July 2009. Part 2 consisted of the data collected from 263 consecutive cases of resectable locally advanced rectal cancer from January 2001 to January 2005. These 263 patients were divided into neoadjuvant therapy (NT) group and control group. Patients in NT group received MDT treatment, namely neoadjuvant therapy + surgery + postoperative adjuvant therapy. Patients in control group underwent direct surgery + postoperative adjuvant therapy. The outcomes in two groups were compared.

RESULTS: The treatment strategy was altered after discussed at MDT meeting in 76.81% of gastric cancer patients and in 58.33% of colorectal cancer patients before operation. The sphincter-preservation and local control of tumor were better in NT group than in control group. The 5-year overall survival rate was also higher in NT group than in control group (77.23% *vs* 69.75%, $P = 0.049$).

CONCLUSION: MDT treatment modality can significantly improve the outcomes of patients with gastrointestinal malignancy in China.

Key words: Multidisciplinary team; Rectal cancer; Neoadjuvant radiotherapy; Prognosis

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Du CZ, Li J, Cai Y, Sun YS, Xue WC, Gu J. Effect of multidisciplinary team treatment on outcomes of patients with gastrointestinal malignancy. *World J Gastroenterol* 2011; 17(15): 2013-2018 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/2013.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.2013

INTRODUCTION

Treatment of cancer has evolved toward a multidisciplinary

team (MDT) approach[1-3]. The effect of MDT treatment modality on cancer is significantly better than that of conventional treatment modalities[4-6]. Although the MDT treatment modality has been successfully implemented in Western countries for decades, no report is available on its application in China. We conducted a study on the MDT treatment modality in a representative cancer center of China to evaluate its effect on outcomes of patients with gastrointestinal malignancy in China.

MATERIALS AND METHODS

Clinical data

Data about patients with gastric and colorectal cancer were collected and divided into two parts. Part 1 consisted of the data collected from 516 consecutive complicated cases discussed at MDT meetings in Peking University School of Oncology (PKUSO) from December 2005 to July 2009. Complicated cases were defined as those with synchronic distant metastasis, marginally resectable or unresectable lesions, postoperative progression, and other conditions leading to difficulty in making treatment strategy. Records and treatment plans or recommendations for MDT treatment were used to investigate the effect of MDT treatment modality on clinical decision making and outcomes of patients with gastrointestinal malignancy (Table 1).

Part 2 consisted of the data collected from 263 consecutive cases of resectable locally advanced rectal cancer from January 2001 to January 2005. Patients included in this study were those with resectable rectal cancer located 12 cm or less from the anal verge, histologically identified primary carcinoma of the rectum, no clinical evidence of preoperative distant metastasis, transabdominal radical resection based on the principle of total mesorectal excision (TME)[7], and R0 resection. Finally, 263 eligible patients included in this study (Table 2) were divided into neoadjuvant therapy (NT) group and control group according to whether they underwent neoadjuvant radiotherapy.

Treatment strategy

Patients in NT group received neoadjuvant therapy + surgery + postoperative adjuvant therapy. The total preoperative radiation dose was 30 Gy (30 Gy/10 fractions, bioequivalent dose 36 Gy) recommended by the Chinese Anti-Cancer Association (CACA)[8], and 5-FU or capecitabine was used in postoperative chemotherapy.

Contrast to MDT treatment, the conventional treatment strategy for locally advanced rectal cancer in China is surgery followed by postoperative chemoradiotherapy which was commonly used in China 5 years ago. Patients in NT group were evaluated before operation by special MDT members while those in control group were not evaluated.

Follow-up

Patients were followed-up every three months for the first 2 years after surgery followed by every six months for 5 years. Serum carcinoembryonic antigen (CEA) level was measured and abdominal ultrasound, pelvic MRI, chest radiograph were performed every six months, and colonoscopy was performed annually during the follow-up. The follow-up time ranged from six to ninety-six months, with a median time of seventy-two months. The outcomes of

Table 1 Complicated cases of different types of cancer discussed at multidisciplinary team meetings *n* (%)

Variables	Rectal cancer	Colon cancer	Gastric cancer
Pre-operation	68 (33.10)	40 (30.77)	69 (38.12)
Postoperative progression[1]	121 (59.02)	62 (47.69)	101 (55.80)
Other condition	16 (7.80)	28 (21.54)	11 (6.08)
Total	205 (100)	130 (100)	181 (100)

[1]Including patients with both local recurrence and distant metastasis.

Table 2 Baseline characteristics of 263 patients with locally advanced rectal cancer *n* (%)

Baseline characteristics	NT group (*n* = 101)	Control group (*n* = 162)	*P* value
Sex			
Male	57	88	0.737
Female	44	74	
Age (yr)[1]	55 (51-59)	55 (50-60)	0.664
Distance of tumor from anal verge			
< 5 cm	35 (34.7)	37 (22.8)	0.051
5-12 cm	66 (65.3)	125 (77.2)	
Surgery			
APR	25	32	0.422
LAR	76	130	
Preoperative serum CEA level			
Normal	52 (51.5)	82 (50.6)	0.745
Abnormal	35 (34.7)	52 (32.1)	
Unknown	14 (13.9)	28 (17.3)	
Pretreatment staging tools			
MRI	61 (60.4)	66 (40.7)	< 0.001
ERUS	28 (27.7)	34 (21.0)	
CT	12 (11.9)	62 (38.3)	
Pretreatment TNM stage			
ⅡA(T3 N0)	24 (23.8)	54 (34.0)	0.278
ⅡB (T4 N0)	4 (4.0)	4 (2.5)	
ⅢA (T1-2 N1)	3 (3.0)	8 (4.9)	
ⅢB (T3-4 N1)	32 (31.7)	37 (22.8)	
ⅢC (AnyT N2)	38 (37.6)	58 (35.8)	
Pathologic TNM stage			
Ⅰ (T1-2 N0)	35 (34.7)	12 (7.4)	< 0.01
ⅡA (T3 N0)	26 (25.7)	54 (33.3)	
ⅡB (T4 N0)	1 (1.0)	0 (0)	
ⅢA (T1-2 N1)	6 (5.9)	7 (4.3)	
ⅢB (T3-4 N1)	17 (16.8)	40 (24.7)	
ⅢC (AnyT N2)	16 (15.8)	49 (30.2)	
Histologic differentiation			
High	2 (2.0)	20 (12.3)	0.013
Moderate	70 (69.3)	110 (67.9)	
Poor	24 (23.8)	24 (14.8)	
Mucinous and signet	5 (5.0)	8 (4.9)	
Lymphovascular invasion			
Present	21 (20.8)	50 (30.9)	0.074
Absent	80 (79.2)	112 (69.1)	

[1]Values are medians (interquartile ranges). NT: Neoadjuvant therapy; APR: Abdominal-perineal resection; LAR: Low anterior resection; MRI: Magnetic resonance imaging; ERUS: Endorectal ultrasonography; CT: computed tomography; CEA: Carcinoembryonic antigen.

patients with gastrointestinal malignancy were evaluated at the end of 5-year follow-up with a follow-up rate of 87.8% (231/263).

Statistical analysis

Demographic and clinicopathologic data were analyzed by χ^2 test. Kaplan-Meier life table and log-rank test were used to compare the disease-free survival (DFS) and overall survival (OS) rates. Cox proportional hazards regression was used in multivariate analysis. Statistical analysis was performed using the SPSS version 16.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

MDT treatment modality

The working model of MDT in our center includes two major components: weekly MDT meetings to discuss complicated clinical cases and interdisciplinary consultations for preoperative and postoperative evaluation and therapy. Most patients receive MDT therapy according to interdisciplinary consultations while only complicated cases are discussed at MDT meetings. Although the MDT team modalities are different, the treatment strategies for patients are made by the same team in our center. The key members of MDT team include a surgeon, a medical oncologist, a radiation oncologist, a radiologist, a pathologist, and specialized nurses. Attendance of the key members at MDT meetings is not compulsory but enhanced by a special coordinator who is responsible for organizing and recording the MDT meetings. The discussion processes and conclusions for each patient are recorded in special tables.

Effect of MDT meetings on clinical decision making and outcomes of cancer patients

Complicated cases of gastric cancer (n = 181), colon cancer (n = 130), and rectal cancer (n = 205) were discussed at MDT meetings during the last 5 years (Table 1). Among the discussed cases, outpatients accounted for 84.69% (n = 437) and inpatients accounted for 15.31% (n = 79), respectively. For each disease classification, patients with postoperative recurrence or metastasis accounted for 48%-59%, suggesting that such patients are needed to be discussed at MDT meetings.

The MDT team modality directly influenced the clinical decision making. Of the 69 preoperative patients with gastric cancer discussed at MDT meetings, 53 (76.81%) underwent neoadjuvant chemotherapy instead of direct surgery. Of the 63 preoperative patients with extensive lesions or synchronous distant metastasis of colorectal cancer who underwent MDT treatment, including chemotherapy, chemoradiotherapy, or target therapy, 7 with initially inoperable liver metastasis underwent radical resection after MDT treatment.

Effect of MDT treatment on clinical outcomes of rectal cancer patients

To verify the comparability of outcomes in NT and control groups, the major demographic and tumor variables were analyzed (Table 2). No difference was found in gender and age of the patients, tumor location, preoperative serum carcinoembryonic antigen (CEA) level, pretreatment clinical stage and lymphovascular invasion (LVI) of tumor between the two groups. The histological differentiation of tumor appeared poorer in control group than in NT group, implying that the prognosis of patients with gastrointestinal malignancy is potentially better in control group than in NT group. However, multivariate analysis demonstrated that it was not a major factor for the clinical outcome of such patients, indicating that the outcomes of patients in the two groups are comparable.

Table 3 Clinical outcome of patients in two groups n (%)

Clinical outcome	NT group (n = 101)	Control group (n = 162)	Odds ratio (95% CI)	P value
Sphincter preservation[1]	13 (37.14)	5 (13.51)	3.78 (1.18-12.13)	0.041
Local recurrence	4 (3.96)	18 (11.11)	0.33 (0.11-1.00)	0.042
Distant metastasis	22 (21.78)	36 (22.22)	0.87 (0.48-1.57)	0.933
5-yr disease-free survival rate	77 (76.24)	109 (67.28)	-	0.039
5-yr overall survival rate	78 (77.23)	113 (69.75)	-	0.049

[1]Within the patients whose distance of tumor from anal verge were less than 5 cm, n = 72 (Table 1). NT: Neoadjuvant therapy.

Different pretreatment evaluation strategies for the outcomes of patients in two groups

The staging tools used for pretreatment evaluation of the two groups differed significantly. Magnetic resonance imaging (MRI) was more frequently used in NT group than in control group (60.4% *vs* 40.7%, $P < 0.05$), while computed tomography (CT) was more commonly used in control group than in NT group.

Effect of MDT treatment on the clinical outcomes of patients in two groups

Although no significant difference was found in pretreatment stage between the two groups, the proportion of pathologic stage I was higher in NT group than in control group (34.7% *vs* 7.4%), while that of stage Ⅲ was higher in control group than in NT group (Table 2).

Among the patients with low rectal cancer less than 5 cm from the anal verge (n = 72), the sphincter preservation rate was 37.14% (13/35) and 13.51% (5/37), respectively, for the NT group and control group ($P < 0.05$, Table 3).

The local recurrence rate was 3.96% (4/101) and 11.11% (18/162), the 5-year DFS rate was 76.24% and 67.28% ($P < 0.05$, Figure 1), and the 5-year OS rate was 77.23% and 69.75% (Figure 1, Table 3), for the NT group and control group, respectively.

Multivariate analysis demonstrated that the pretreat-

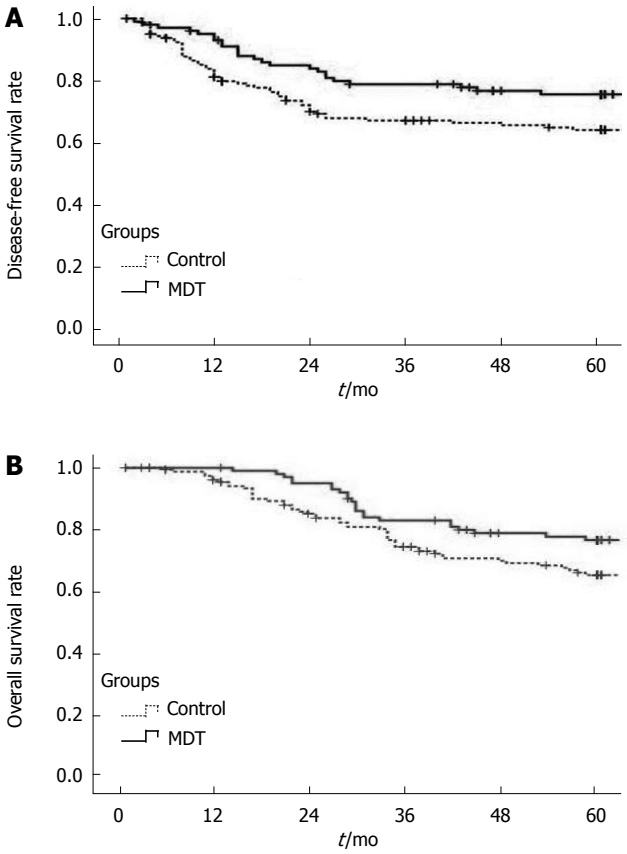

Figure 1 Disease-free survival rate (A) and overall survival rate (B) for patients in two groups. MDT: Multidisciplinary team.

ment serum CEA level, pathologic TNM stage, and LVI were the major factors for the long-term survival rate of patients with gastrointestinal malignancy (Table 4). Other variables, including neoadjuvant radiotherapy, were not the independent factors for the OS rate.

DISCUSSION

Treatment of cancer increasingly requires the cooperation of specialists from various disciplines[9], although surgery still plays a critical role in cancer treatment. Currently, most doctors around the world have recognized the effect of MDT approach[10,11] and endorse it as a principal treatment modality for cancer[1,2]. Although the composition of MDT in China is similar to that in Western countries, there are many distinct differences in working models of China. First, no special rules or guidelines are available on MDT in China, thus it is not compulsory for all cancer patients to receive MDT treatment. Second, not all but some big cancer centers adopt MDT treatment modality without consistent indications for discussion at MDT meetings in different hospitals. In general, MDT is still under development in China[12,13].

Our cancer center is one of the earliest hospitals adopting MDT approach in China. It is difficult to quantify improvement in outcomes of cancer patients, especially those with complicated clinical conditions, after MDT treatment. In this study, data on cases of locally advanced

Table 4 Multivariate analysis of overall survival rate by COX model (enter method)

Variables	Hazard ratio	95% CI	*P* value
Pretreatment CEA level	1.429	1.044-1.956	0.026
Pathologic TNM stage	1.440	1.137-1.825	0.002
Lymphovascular invasion	0.468	0.286-0.765	0.002
Sex	1.164	0.726-1.867	0.529
Age	0.700	0.424-1.156	0.163
Distance of tumor from anal verge	0.994	0.854-1.157	0.934
Pretreatment TNM stage	0.949	0.727-1.239	0.703
Surgery form (LAR or APR)	0.853	0.575-1.264	0.427
Histologic differentiation	0.969	0.822-1.142	0.706
NT	0.878	0.519-1.483	0.626

CEA: Carcinoembryonic antigen; LAR: Low anterior resection; APR: Abdominal-perineal resection; NT: Neoadjuvant therapy.

rectal cancer, which is considered the most successful and mature model of MDT approach[14-16], were collected to evaluate the effect of MDT treatment on the clinical outcomes of patients with gastrointestinal malignancy. Cases discussed at MDT meeting were reviewed to assess the influence of MDT treatment modality on the treatment strategy for patients with gastrointestinal malignancy. The data included in the two parts were completely independent without any overlap.

Several studies demonstrated that MDT approach can optimize the decision making, enhance the quality of cancer care, and improve the clinical outcomes of cancer patients[1,11,17,18]. Our data indicate that MDT meetings change a considerable proportion of treatment strategies, including neoadjuvant therapy for preoperative patients and MDT treatment modality for patients with tumor recurrence and metastasis. In this study, 7 patients with inoperable liver metastasis of colorectal cancer underwent R0 resection after MDT treatment. However, the limited time of MDT meetings and the large number of patients who need to be discussed at MDT meetings made it impossible to discuss and evaluate all patients, thus the vast majority of patients were evaluated before operation and neoadjuvant therapy was evaluated according to the interdisciplinary consultations.

It is widely believed that accurate and integrative evaluation before operation, as well as active strategies for adjuvant therapy used by MDT members, are the primary factors for improving the clinical outcomes of cancer patients[2,3,16,19,20]. The meticulous and reliable assessment of patients with locally advanced rectal cancer before operation by MDT members is closely associated with the treatment strategy. It was reported that MRI is more accurate in clinical staging of tumor and in predicting of circumferential resection margin (CRM) when it is used in evaluation of rectal cancer[21-23]. In this study, the strategy for preoperative evaluation of the two groups differed significantly. MRI was used more frequently in NT group than in control group (60.4% *vs* 40.7%, $P < 0.01$), suggesting that MRI can improve the accuracy of clinical tumor staging in NT group.

It has been shown that neoadjuvant radiotherapy for

rectal cancer can improve the local control of cancer before operation[24-27], which constituted the major difference between MDT and traditional treatment modalities in this study. Neoadjuvant radiotherapy can decrease the size or stage of low rectal cancer, thus preserving the anus[28-30]. In this study, the sphincter preservation, the local control of cancer, and the 5-year OS rate were better in NT group than in control group, suggesting that patients may benefit from MDT treatment.

Finally, although our study showed the advantages of MDT treatment modality for gastrointestinal cancer, its widespread use in China is still problematic. First, administrative support is insufficient in some places, leading to organizational problems and even its discontinuation. Second, MDT meetings are time-consuming and incomplete attendance is a barrier to success. However, these problems will not hinder the popularity and application of MDT treatment modality in China.

In conclusion, MDT treatment modality can significantly improve the clinical strategies for the treatment of gastrointestinal malignancy, and Chinese patients can benefit from it.

ACKNOWLEDGMENTS

The authors thank all the doctors and nurses who attended our MDT meetings and those who gave us constructive advices for the MDT treatment modality.

COMMENTS

Background

Treatment of cancer has evolved toward a multidisciplinary team (MDT) approach. Although the MDT treatment modality has been successfully implemented in Western countries for decades, it is not widely applied in China. The authors conducted a study on the MDT treatment modality in a representative cancer center in China to evaluate its effect on clinical decision making and outcomes in patients with gastrointestinal malignancy.

Research frontiers

MDT treatment modality is the major concern in cancer treatment, and this study addressed it for cancer in China.

Innovations and breakthroughs

MDT treatment modality, systematically introduced in this study, can significantly improve the clinical strategies for the treatment of gastrointestinal malignancy, and Chinese patients can benefit from it.

Applications

MDT treatment modality is of high values for patients with gastrointestinal malignancy and can be commonly used in hospitals of China in treatment of cancer patients.

Peer review

This paper is very good and shows that neoadjuvant therapy can improve the outcomes of patients with gastrointestinal malignancy, thus providing a novel therapy for gastrointestinal cancer.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.2019

World J Gastroenterol 2011 April 21; 17(15): 2019-2027
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Gastric cancer cells induce human CD4$^+$Foxp3$^+$ regulatory T cells through the production of TGF-β1

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Supported by Shanghai Municipal Natural Science Foundation, No. 10ZR1420000 and National Natural Science Foundation of China, No. 81072009
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Received: December 19, 2010 Revised: December 28, 2010
Accepted: January 4, 2011
Published online: April 21, 2011

Abstract

AIM: To elucidate the molecular and cellular features responsible for the increase of regulatory T cells (Tregs) in gastric cancer.

METHODS: The frequencies of CD4$^+$Foxp3$^+$ Tregs and the level of transforming growth factor-β1 (TGF-β1) were analyzed from 56 patients with gastric cancer by flow cytometry and enzyme-linked immunosorbent assay respectively. *Foxp3* gene expression was analyzed by real-time polymerase chain reaction. The gastric cancer microenvironment was modeled by establishing the co-culture of gastric cancer cell line, MGC-803, with sorting CD4$^+$ T cells. The normal gastric mucosa cell line, GES-1, was used as the control. The production of TGF-β1 was detected in supernatant of MGC and GES-1. The carboxyfluorescein diacetatesuccinimidyl ester (CFSE) dilution assay was performed to evaluate the proliferation characteristics of induced Tregs. Neutralizing anti-TGF-β1 antibody was added to the co-culture system for neutralization experiments.

RESULTS: The level of serum TGF-β1 in gastric cancer patients (15.1 ± 5.5 ng/mL) was significantly higher than that of the gender- and age-matched healthy controls (10.3 ± 3.4 ng/mL) ($P < 0.05$). Furthermore, the higher TGF-β1 level correlated with the increased population of CD4$^+$Foxp3$^+$ Tregs in advanced gastric cancer ($r = 0.576$, $P < 0.05$). A significant higher frequency of CD4$^+$Foxp3$^+$ Tregs was observed in PBMCs cultured with the supernatant of MGC than GES-1 (10.6% ± 0.6% *vs* 8.7% ± 0.7%, $P < 0.05$). Moreover, using the purified CD4$^+$CD25$^-$ T cells, we confirmed that the increased Tregs were mainly induced from the conversation of CD4$^+$CD25$^-$ naive T cells, and induced Tregs were functional and able to suppress the proliferation of effector T cells. Finally, we demonstrated that gastric cancer cells induced the increased CD4$^+$Foxp3$^+$ Tregs *via* producing TGF-β1. Gastric cancer cells upregulated the production of TGF-β1 and blockade of TGF-β1 partly abrogated Tregs phenotype.

CONCLUSION: Gastric cancer cell can induce Tregs development *via* producing TGF-β1, by which the existence of cross-talk between the tumor and immune cells might regulate anti-tumor immune responses.

Key words: Transforming growth factor-β1; Regulatory

T cells; Gastric cancer; Immune suppression

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Yuan XL, Chen L, Zhang TT, Ma YH, Zhou YL, Zhao Y, Wang WW, Dong P, Yu L, Zhang YY, Shen LS. Gastric cancer cells induce human $CD4^+Foxp3^+$ regulatory T cells through the production of TGF-β1. *World J Gastroenterol* 2011; 17(15): 2019-2027 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/2019.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.2019

INTRODUCTION

Gastric cancer (GC) is a common fatal malignancy from cancer worldwide[1,2]. Although the incidence of GC is declining in most developed countries, it remains one of the most common causes of cancer-related death in many Asian countries, such as China, Japan, and Korea[3,4]. Certain tumors, including GC, have developed the capacity to escape immune surveillance or to inhibit immune functions. Recently, emerging evidence suggests that $CD4^+$ regulatory T cells (Tregs) play an important role in tumor escape from immunological control by suppressing the activation and proliferation of T cells, B cells, and natural killer (NK) cells[5,6].

Our recent results have showed that the existence of Tregs maintained immune tolerance in gastric tumor microenvironments[7]. In addition, we found increased expression of Foxp3 protein per cell in tumor-infiltrating Tregs and Tregs can mediate immune suppression *via* COX-2 production[8]. Interestingly, our and others data showed that after patients received curative resection for GC, the increased proportion of Tregs was significantly restored to normal levels[7,9,10]. These results strongly suggest that gastric cancer-related factors induce and/or expand the accumulation of Tregs. However, the detailed mechanism underlying the induction of Tregs during GC progress remains undefined.

Transforming growth factor-β1 (TGF-β1), as well as other mediators such as prostaglandin E2 and H-ferritin, has been reported to induce Treg cells[11]. In vitro, studies have shown that TGF-β1 can impose a regulatory phenotype on $CD4^+CD25^-$ T cells through the induction of Foxp3 expression[12,13]. In contrast, other studies have shown that the development and functional capacity of $CD4^+CD25^+$ Tregs is normal in TGF-β1 deficient mice[14], questioning a role for TGF-β1 in mediating Treg development and function. Over the past few years, significant progress has been made in defining the cellular and molecular basis for these protumorigenic effects of TGF-β1 within tumor microenvironment[15]. The mechanism of TGF-β1 function in gastric cancer is believed to be mediated primarily by increasing the deposition of extracellular matrix and immunosuppression. However, the underlying mechanism of TGF-β1 responsible for regulating gastric cancer immunosuppression has not been fully elucidated yet.

In this study, we examined the serum level of TGF-β1 in gastric cancer patients and analyzed the correlation of TGF-β1 with the prevalence of Tregs. We confirmed that serum level of TGF-β1 was elevated in GC and correlated with increased $CD4^+Foxp3^+$ Treg cells. By the co-culture system in vitro, we evaluated the contribution of GC cell supernatant to $CD4^+$ T cell dysfunction. Our results indicated that MGC supernatant can induce the increase of Tregs, which especially from the conversation of $CD4^+CD25^-$ naive T cell and blockade of TGF-β1 production partly impaired the development of Tregs. These results suggested that the gastric cancer cells played a pivotal role in impairing the antitumor T cell response by induction of Tregs.

MATERIALS AND METHODS

Patients

Fifty six patients with gastric cancer, who underwent surgery at Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine, China, were included in this study. Prior to the sample collection, appropriate permission was granted from the research ethical committee of Xinhua hospital, Shanghai Jiao Tong University School of Medicine. Peripheral bloods were collected from each patient and from 20 healthy volunteers as previously described[7]. Sera were frozen at -80℃ immediately after centrifugation for later determination of concentrations of TGF-β1. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient. All patients were diagnosed by pathological analyses based on the UICC (International Union Against Cancer) criteria. At the time of sample collection, none of the patients had suffered other cancer, acute and chronic infections, autoimmune diseases, inflammatory diseases and none were receiving concomitant mediations. The laboratory characteristics of patients were as follow: WBC 4.2-10.6 × 10^9/L; RBC 3.9-5.7 × 10^{12}/L; platelets 181-350 × 10^9/L; neutrophils 47.8%-73.9%; lymphocytes 15.2%-44.9%; monocytes 2.9%-10.2%. The clinicopathologic characteristics of the tumors are summarized in Table 1.

Cell culture and supernatant collection

Human GC cell lines (MGC-803, SGC-7901) were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and normal gastric mucosa cell line (GES-1), derived from a human fetal gastric mucosa epithelium, was obtained from Beijing Institute for Cancer Research. The cells were routinely cultured in DMEM media (GIBCO, Invitrogen, USA) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco) in 5% CO_2 at 37℃. MGC, SGC and GES cells were washed twice with PBS when they grew to 60%-80% confluence and then

Table 1 Serum levels of transforming growth factor-β1 and the population of $CD4^+Foxp3^+$ Tregs in patients with gastric cancer according to clinicopathological findings

Variables	*n*	TGF-β1 (ng/mL)	*P*	Tregs (%)	*P*
Gender			> 0.05		> 0.05
Male	38	16.1 ± 6.8		8.0 ± 3.2	
Female	18	14.0 ± 5.1		7.6 ± 2.5	
Age			> 0.05		> 0.05
< 55	25	14.1 ± 4.1		8.2 ± 4.1	
> 55	31	15.9 ± 4.6		7.7 ± 3.5	
TNM stage			< 0.05		< 0.05
Early stage (Ⅰ / Ⅱ)	22	12.4 ± 5.0		6.3 ± 1.2	
Advanced stage (Ⅲ / Ⅳ)	34	18.1 ± 7.8		8.8 ± 2.4	
Histological type			> 0.05		> 0.05
Well and Moderately	20	14.1 ± 4.9		7.0 ± 3.7	
Poor	36	16.8 ± 7.9		8.6 ± 4.5	
Lymph node metastasis			< 0.05		< 0.05
Negative	18	11.2 ± 5.2		6.5 ± 2.4	
Positive	38	17.4 ± 7.2		8.6 ± 2.9	

TGF: Transforming growth factor; Tregs: Regulatory T cells.

kept in serum-free culture medium for an additional 48 h. Supernatant was collected and debris was removed by centrifugation at 1500 *g* for 10 min, and then passed through a 0.45 mm filter (BD, USA). 100 μL supernatants were stored at -80℃ for later determination of concentrations of TGF-β1. For co-culture assay, all the remaining supernatants were further concentrated 20-fold with a Microcon Ultracel YM-10 filter (Millipore, USA) according to the manufacturer's instructions. In the induction experiments, different volumes of supernatant protein concentrate from MGC or GES were added to sorted naive T cell culture system.

TGF-β1 measurement

The cell supernatants of MGC, GES-1 and GC patients' sera previously stored at -80℃ were thawed, and measured for TGF-β1 concentration by enzyme-linked immunosorbent assay using human TGF-β1 immunoassay kit (R&D, USA) in triplicate following the manufacturer's protocol. The minimum detectable dose of this assay is 30.0 pg/mL. The intra-assay coefficient of variation (CV) was 5.7% and the interassay CV was 10.6%.

Treg cells analysis and sorting by FCM

Phenotype analysis of regulatory T cells (Tregs) and cell sorting were performed by BD FACS Aria flow cytometer (BD, USA) as previously described[8]. Briefly, the cells were labeled with CD3-PC7, CD127-PE, CD4-APC, and CD25 PerCP. Intracellular staining for Foxp3 was performed using Alexa Fluor® 488 anti-human Foxp3 Antibody and Foxp3 Fix/Perm Buffer Set (BioLegend, USA) following the manufacturer's protocol. In the preliminary experiments, we found that CD25 expression was nonspecific and was higher in $CD4^+$ T cells after coculturing with GC cells. Therefore, for consistency, the gating strategy for Tregs was based on the expression of CD4 and Foxp3. To analyze the prevalence of Treg cells, $CD4^+Foxp3^+$ Treg cells were evaluated after gating on $CD3^+CD4^+$ T cells and expressed as a percentage of the total $CD4^+$ T cells. The FACS Aria was adjusted with Accudrop Fluorescent Beads (BD bioscience, USA) for optimum sorting conditions which allowed $CD4^+CD25^+CD127^{low/-}$ T cells and $CD4^+CD25^-CD127^+$ T cells to be sorted. The purity of the isolated T cells was greater than 95%.

Induction and neutralization experiments

The purified $CD4^+CD25^-CD127^+$ T cells (1×10^5) were cultured with conditioned medium described above in 96-well plates at 37℃ and 5% CO_2 in the presence or absence of soluble anti-human CD3 (10 μg/mL; eBioscience) plus anti-CD28 (10 μg/mL; eBioscience) and IL-2 (100 U/mL; Sigma, USA). After 72 h of cultivation, the proportion of $CD4^+Foxp3^+$ T cells was detected by FCM, and Foxp3 gene expression was analyzed by real-time PCR. For anti-TGF-β1 antibody neutralization experiments, neutralizing mouse anti-TGF-β1 antibody (500 μg/mL; Clone: 27235; R&D Systems, USA) and normal mouse IgG1 (500 μg/mL; Clone: 11711; R&D Systems, USA) were added to the culture medium with a final concentration of 0.1 μg/mL at the beginning of the culture.

Real-time quantitative RT-PCR

Foxp3 mRNA expression was performed using the SYBR Premix Ex Taq™ (Takara) according to the manufacturer's instructions. Amplification reactions were performed by primers specific for Foxp3 (forward, 5'-CAGCACATTCCCAGAGTTCCTC-3'; reverse, 5'-GCGTGTGAACCAGTGGTAGATC-3'). The relative quantity of the Foxp3 mRNA was normalized to the level of the internal control GAPDH mRNA level.

CFSE-based suppression assay in vitro

For the proliferation inhibition assay, the carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay was performed per standard technique. Briefly, the sorted $CD4^+CD25^-CD127^+$ T cells were co-cultured with MGC-803, GES-1 or medium only for 2 d. As suppressor cells, equal numbers of cells were removed and placed in co-culture with CFSE-labeled $CD4^+CD25^-$ T cells at a ratio of 1:1 in the presence of soluble 10 μg/mL mouse anti-human CD3 and 10 μg/mL mouse anti-human CD28 antibodies (eBioscience, USA). After 4 d, the cells were harvested and proliferation was measured by loss of CFSE dye with flow cytometry. Cell proliferation indices were calculated with Modfit software (Topsham, USA) based on the reduction of CFSE positive cells.

Statistical analysis

Data were expressed as mean ± SD. The statistical significance of the difference between the two means was assessed using Student's *t*-test, and the one-way ANOVA with Tukey's post test was performed for multiple comparisons. Correlation between variables was evaluated by Pearman's rank correlation coefficients. All the statistical analyses were performed using GraphPad Prism version 5.0

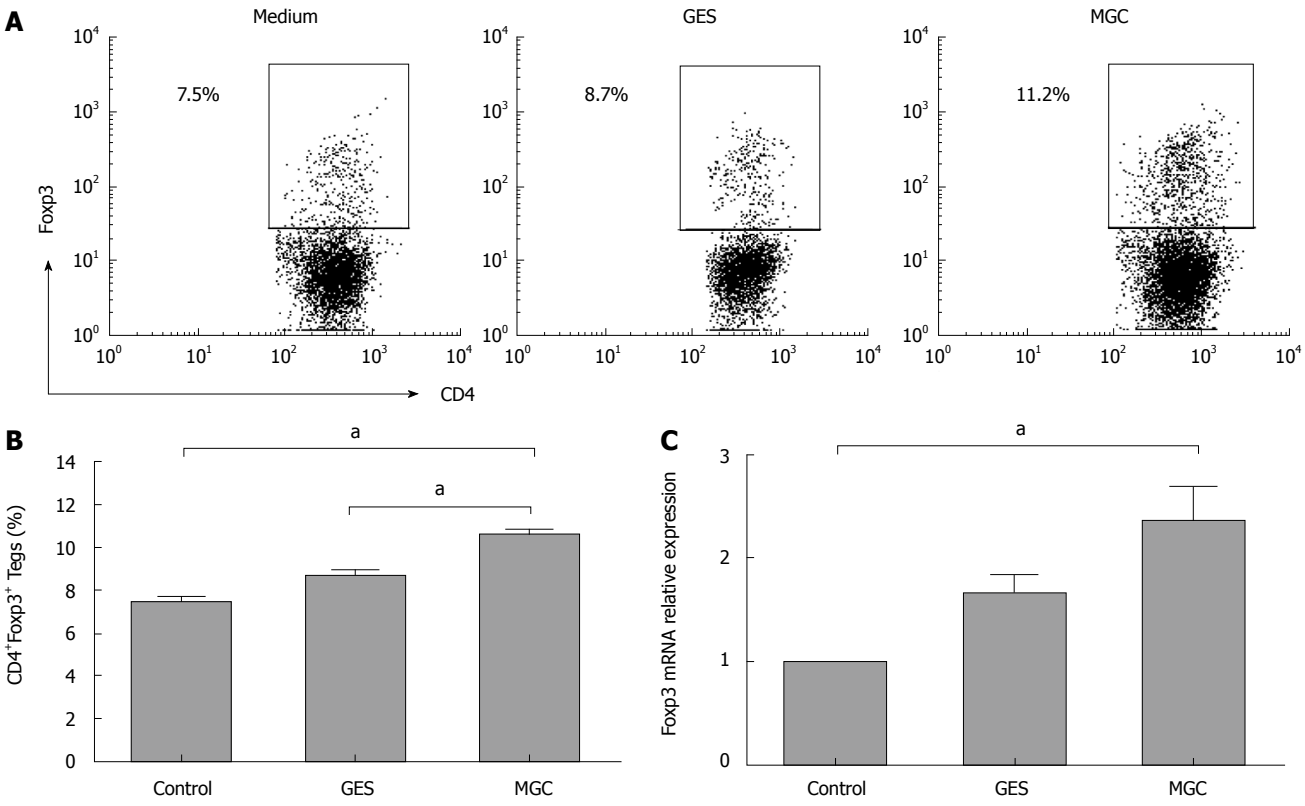

Figure 1 Gastric cancer cell supernatant induces the increased Tregs in coculture with peripheral blood mononuclear cells. A: Representative flow cytometry analysis of $CD4^+Foxp3^+$ Tregs frequency in $CD4^+$ T cells population following peripheral blood mononuclear cells co-culture with medium, GES, or MGC supernatants. Rectangles show double positive gating and numbers reflect percentage of cells in that gate; B: Summarized data from all subjects showed that $CD4^+Foxp3^+$ Tregs increased in coculture with MGC supernatants ($^aP < 0.05$); C: Relative quantity of Foxp3 mRNA was measured by real-time polymerase chain reaction before and after culture with medium, GES, or MGC supernatants.

for Windows (GraphPad Software, USA) and a significant difference was considered as $P < 0.05$.

RESULTS

Elevated serum level of TGF-β1 in gastric cancer correlated with increased $CD4^+Foxp3^+$ Treg cells

To determine whether serum TGF-β1 correlated with the clinicopathological findings, we summarized the mean values of TGF-β1 in patients with GC according to clinical variables as shown in Table 1. The mean level of serum TGF-β1 in GC patients (15.1 ± 5.5 ng/mL) was significantly higher than that of the gender- and age-matched healthy controls (10.3 ± 3.4 ng/mL) ($P < 0.05$), which was consistent with previous reports[16,17]. Furthermore, the serum TGF-β1 levels increased as GC stage progressed. Compared to those with early stage disease, patients with advanced stage disease had significantly elevated serum TGF-β1 ($P < 0.05$). As shown in Table 1, no significant differences in serum TGF-β1 levels were found in GC patients with different age, genders, and histological types ($P > 0.05$). However, the serum concentration of TGF-β1 was positively correlated with lymph node metastasis ($P < 0.05$). The results also showed that the population of $CD4^+Foxp3^+$ Tregs in the peripheral blood of advanced stage GC patients was significantly higher than that in healthy controls or early stage GC patients ($P < 0.05$) (Table 1).

The consistency of Tregs and serum TGF-β1 level in patients with GC encouraged us to perform a correlation study, and the results showed that the increased TGF-β1 was correlated with the Treg cells ($r = 0.576$, $P < 0.05$) in advanced stage patients, but not in early stage patients ($r = 0.248$, $P < 0.05$). The present results indirectly suggested the relationship of TGF-β1 and Tregs in gastric cancer.

GC cell supernatant induces the increase in $CD4^+$ $Foxp3^+$ Treg cells

Based on the above results, we hypothesized that gastric cancer-derived stimulators may contribute to increased Tregs. To address this hypothesis, we established a co-culture system with human GC cells and PBMCs from healthy donors to model the gastric cancer microenvironment *in vitro*. After 3 d of culture, our data showed that a higher frequency of Tregs was observed in PBMCs cultured with the supernatant of MGC. However, the frequency of Tregs had almost no significant difference in PBMCs cultured with GES-1 cell supernatants and medium control (Figure 1A and B). When cocultured with MGC cell culture supernatant, Foxp3 mRNA expression level was higher than that with GES-1 and medium (Figure 1C).

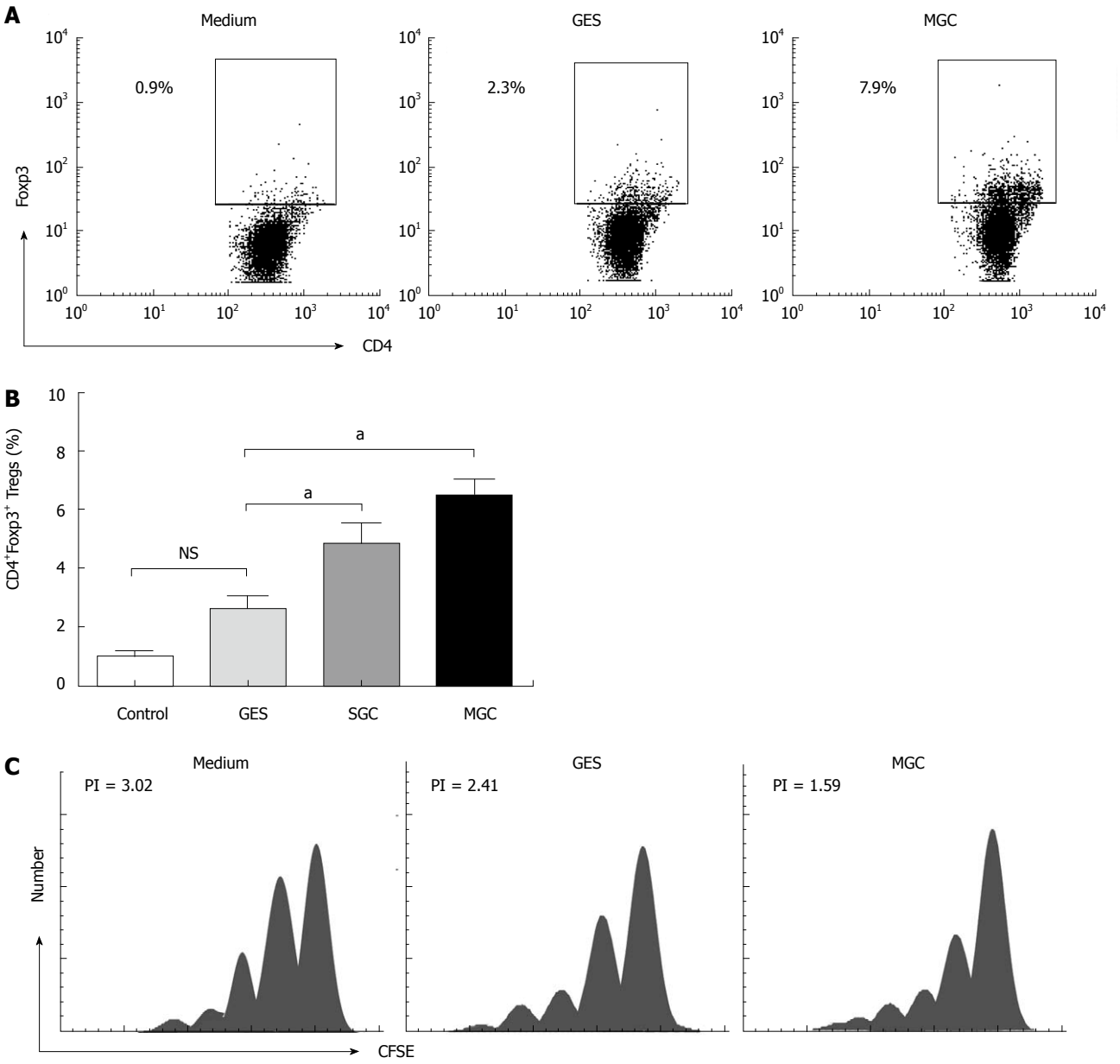

Figure 2 Gastric cancer cell supernatant mediates the conversion of $CD4^+CD25^-$ T cell to $CD4^+Foxp3^+$ Tregs. A: Representative flow cytometry analysis of CD4+Foxp3+ Tregs frequency in $CD4^+$ T cells population following sorted $CD4^+CD25^-CD127^+$ T cells co-culture with medium, GES, or MGC supernatants; B: Summarized data showed that both MGC and SGC supernatants induced higher $CD4^+Foxp3^+$ Tregs ($^aP < 0.05$); C: After co-culture with MGC, GES or medium, $CD4^+CD25^-$ T cells were placed in coculture with CFSE-labeled $CD4^+CD25^-CD127^+$ T cells at a ratio of 1:1 in the presence of soluble anti-CD3/CD28 as well as IL-2. The representative data from three independent experiments are shown.

GC cell supernatants induce the conversion of $CD4^+CD25^-$ naive T cells to $CD4^+$ $Foxp3^+$ Tregs

To investigate whether the supernatant of GC cell culture induced the increased Tregs from the conversion of natural $CD4^+CD25^-$ T cells, we performed co-culture experiments with the sorted natural $CD4^+CD25^-$ T cells using our previous method[7]. Because naive $CD4^+$ T cells were more susceptible to the induction of Foxp3 by TGF-β1[18] and to minimize any potential contaminating $CD25^-Foxp3^+$ nTregs, we used the $CD4^+CD25^+CD127^{low/-}$ population for all of our coculture experiments. To ensure consistency, the same cell culture supernatants were used for conversion experiments. Our results showed that MGC cell supernatant can induce a higher population of $CD4^+Foxp3^+$ Tregs than GES-1 and medium ($P < 0.05$) (Figure 2A and B) and, the $CD4^+CD25^-$ cells decreased respectively in coculture system. More interesting is that the induced Tregs correlated with the TGF-β1 level in different repeated experiments ($r = 0.635$, $P < 0.05$).

To further confirm that the supernatant of GC cell culture could increase Tregs population and Foxp3 expression, the cell culture supernatant from another GC cell strain, SGC-7901, was collected. As observed in the culture with supernatant of MGC cell culture, the supernatant of SGC cell cultures also increased Foxp3 expression in naive T cells (Figure 2B). Collectively, our results suggested

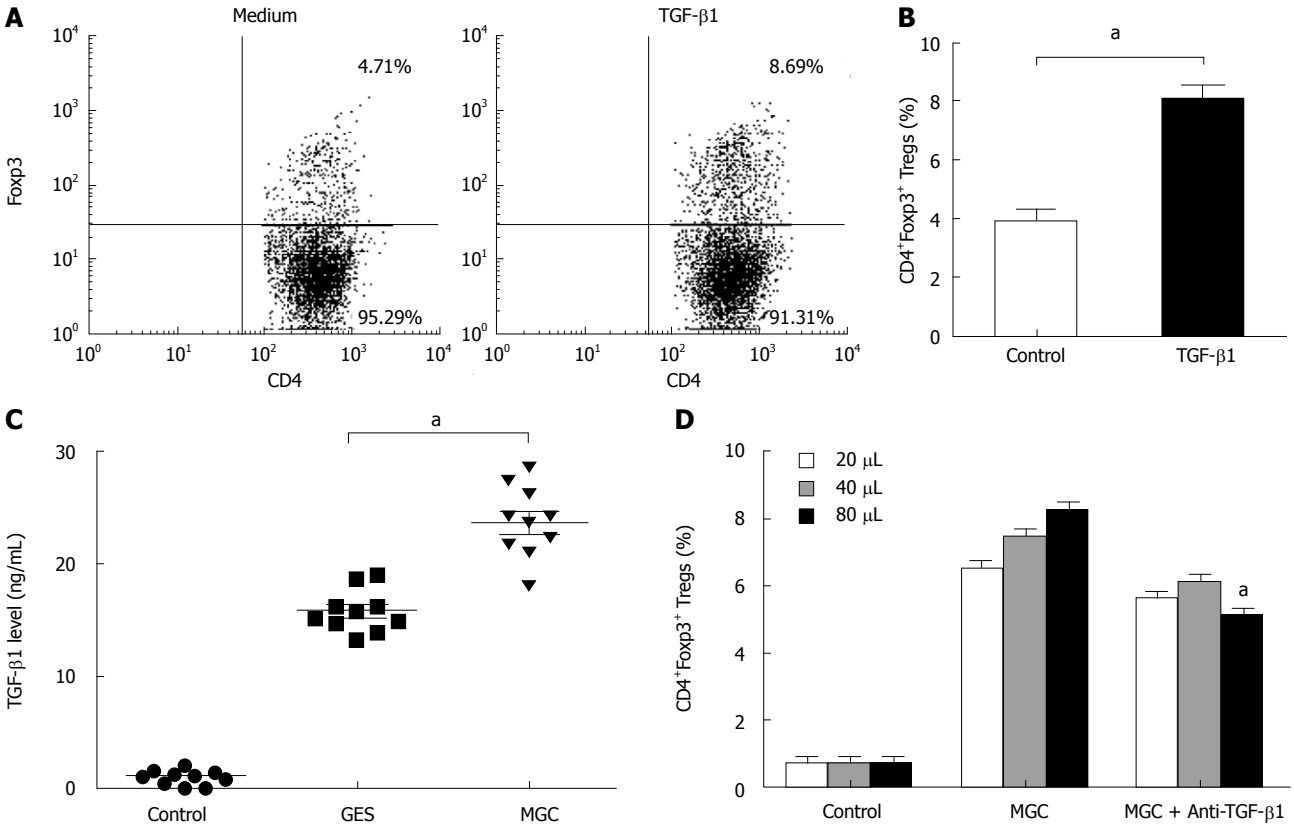

Figure 3 Gastric cancer cells producing transforming growth factor-β1 can partially mediate the conversion of $CD4^+CD25^-$ T cells to $CD4^+Foxp3^+$ Treg cells. A: Representative flow cytometry analysis of $CD4^+Foxp3^+$ Tregs frequency in the presence or absence of transforming growth factor-β1 (TGF-β1); B: Summarized data showed that $CD4^+Foxp3^+$ Tregs frequency increased in presence of TGF-β1 in comparison with that in absence of TGF-β1 ($^aP < 0.05$); C: Gastric cancer cell TGF-β1 production was assessed by enzyme-linked immunosorbent assay in supernatants. ($^aP < 0.05$); D: TGF-β1 blocking antibody or control IgG1 antibody was added to the co-culture in order to monitor impairment of Tregs development within the coculture system. Different volumes of supernatant protein concentrate (20, 40, and 80 μL) from MGC were added to the co-culture system. ($^aP < 0.05$).

that the GC supernatant can induce the increased Tregs, which was mainly because of the conversion of natural $CD4^+CD25^-$ T cells.

Gastric cancer cell induced $CD4^+Foxp3^+$ Treg cells that can suppress T cell activation

In order to understand whether GC cell supernatant induced $CD4^+Foxp3^+$ Tregs can inhibit effector T cells, we analyzed the suppressive function of GC cell induced Tregs. To eliminate the influence of cell culture related factors, the induced co-cultured supernatants were removed after coculture with MGC, GES-1, or medium. Then the CFSE dilution assay was used to evaluate the proliferation characteristics of T cells. Compared with medium, a significant decrease in the proliferative response of responder $CD4^+CD25^-$ T cells could be found with MGC after being cocultured (Figure 2C). These results demonstrated that GC cell induced Treg cells displayed the suppressive activity *in vitro*.

Gastric cancer cells induce conversion of naive T cells into Treg through TGF-β1

To further elucidate the possible mechanism of the conversion, we presumed that TGF-β1 from gastric cancer cells can serve as a key factor in the induction of Foxp3 expression of natural $CD4^+CD25^-$ T cells. To address this possibility, we firstly performed the inducing experiments for TGF-β1. Consistent with other studies, compared with control Ab, TGF-β1 can induce an increase in Tregs (Figure 3A and B). MGC cells secreted significant higher TGF-β1 into supernatant than that GES-1 and medium (Figure 3C).

To test the role of TGF-β1 in GC cell mediated increasing Tregs, we compared the effects of neutralizing monoclonal antibody with activity against TGF-β1 and isotypic control antibody. To some extent, blocking TGF-β1 activity in MGC supernatant with anti-TGF-β1 mAb, instead of the isotypic control antibody, reduced the frequency of induced $CD4^+FOXP3^+$ T cells. But our results also showed that this blocking effect was not complete because the converted numbers of $CD4^+Foxp3^+$ T cells in MGC were still higher than that of the control (Figure 3D). Our data indicated that gastric cancer-derived TGF-β1 played a certain role in the conversion of natural $CD4^+CD25^-$ T cells to $CD4^+Foxp3^+$ Treg cells.

DISCUSSION

In the current study, we showed that higher levels of TGF-β1 in gastric cancer patients have been correlated with the frequency of $CD4^+Foxp3^+$ regulatory T cell. Nu-

merous studies have respectively reported an increased frequency of circulating Tregs and higher level of TGF-β1 during GC progression[7,9,16,19]. However, to date, there has been no report to directly demonstrate the relationship of higher TGF-β1 levels and increased frequency of Tregs in GC. Given that TGF-β1 is a key factor for Foxp3 expression maintenance, regulatory function, and homeostasis in peripheral $CD4^+CD25^+$ Treg cells[20], tumor-derived TGF-β1 may contribute to the development of Tregs during GC progression. Indeed, our work supports this possibility by demonstrating a mechanism of $CD4^+Foxp3^+$ Tregs development mediated through TGF-β1 production by GC cells.

TGF-β1 is a tumor suppressor growth factor, anti-inflammatory cytokine, and immunosuppressant. Therefore, the levels of TGF-β1 were different according to the carcinogenic process, the stage of carcinogenesis, and organ. It has been reported that high levels of TGF-β1 are produced by many types of tumors, including melanomas and cancers of the colon, stomach, liver, and prostate, as well as other malignancies[16,21,22]. Generally defective TGF-β1 signaling seems to be essential in the carcinogenic process, but the level of TGF-β1 is increased in advanced cases or some types of cancer. TGF-β1 levels were significantly increased in gastric cancer tissue compared with adjacent normal tissues[17]. In this study, our data confirmed the higher level of serum TGF-β1 in patients with gastric cancer. Furthermore, compared to early stage patients, elevated serum TGF-β1 was observed in patients with advanced stages. However, the role of TGF-β1 varied in different tumor stages, in which TGF-β1 seems to act as a tumor suppressor in early stages of tumorigenesis and during later stages of tumorigenesis, TGF-β can foster tumor progression, and metastasis[23,24]. Our results showed that the serum concentration of TGF-β1 was positively correlated with lymph node metastasis in GC. This study reinforced the role of TGF-β1 in promoting GC progression. An increase in the Treg population has been observed in both the periphery and tumor microenvironment in patients with cancer[25]. We find a positive correlation between TGF-β1 and Tregs in advanced stage GC patients. To our knowledge, this is the first report to show the correlation of TGF-β1 level with increased Treg cells in GC.

In this report, an *in vitro* co-culture system was used to understand the underlying mechanisms responsible for the upregulation of Tregs observed in our clinical cohorts. After co-culture with GC cell supernatants, an increased population of $CD4^+Foxp3^+$ T cells was found in PBMCs. Of note, upregulation of Foxp3 mRNA expression supported that Tregs increased in the culture system. This increase was observed using a different GC cell line, suggesting that the induction of Tregs is a feature common to GC cells. Multiple mechanisms have been involved in production of increased Treg cells in the tumor microenvironment including expansion, conversion and recruitment. Mizukami *et al*[26] found that CCL17 and CCL22 are related to the increased population of $Foxp3^+$ Tregs in early GC[26]. Using the optimized conditions for sorting effector and Treg cells, we provided evidence that the conditioned medium obtained from GC supernatant was capable of inducing the conversion of $CD4^+CD25^-$ T cells to $CD4^+Foxp3^+$ Tregs, which is different from the effects of chemokines on Treg infiltration in GC microenvironment. Moreover, the induced Tregs were functional and inhibited the proliferative response of $CD4^+CD25^-$ effector T cells. Although a prior study showed that increased Treg frequency was derived from natural Treg self expansion by factors secreted by hepatocellular carcinoma cell[27], our data clearly demonstrated that the conversion of natural $CD4^+CD25^-$ T cells may be an important pathway of Treg cell maintenance in GC. Of course, we cannot discount the important role of chemokines in inducing Tregs migration to the microenvironment of GC.

Tumor cell supernatants include a complex protein component. Based on the fact that GC cells could produce a higher level of TGF-β1 and TGF-β1 correlated with Tregs from our data, we questioned if gastric cancer derived TGF-β1 induced Treg increases in the coculture system. Although TGF-β1 can promote the generation of Tregs in vitro, it has been controversial whether TGF-β1 is involved in the generation or maintenance of Tregs under pathologic conditions, especially in tumor environments[28]. Some studies showed that tumor-derived factors such as TGF-β1 may contribute to $CD4^+CD25^+$ Treg cell expansion[29], but also enhance their suppressor ability[27]. In contrast to them, Zhao *et al*[30] recently found that neutralization of TGF-β1 did not affect Foxp3 expression in ovarian carcinoma cells. The role of TGF-β1 needs to be elucidated in GC. Our results demonstrated that a higher TGF-β1 level was found in GC cell supernatant and TGF-β1 can induce increased Treg cells. Surprisingly, although blocking TGF-β1 could decrease the conversion activity in our study, it was not completely abrogated. Accordingly, we could not rule out that a fraction of converting Treg cells may be generated in the presence of other unknown tumor-derived soluble factors besides TGF-β1. It is likely that multiple cytokines are involved in the induction of Foxp3 expression[30]. TGF-β1, together with other factors, seemed to account for the induction of Treg cells in the GC microenvironment. Further research is needed to elucidate the potential mechanism of GC derived other factors for induction of Tregs. Additionally, what should be noted is that TGF-β1 derived from GC can also account for immunosuppression of other cell types, and besides GC cells, macrophages and stromal cell also can secrete TGF-β1 in tumor environments[31]. It is documented that TGF-β1 is important for the inhibition of $CD8^+$ CTL and NK cells, which play a critical role in the prevention and clearance of tumors[32]. A better understanding of the mechanisms of the Treg increase in GC may allow for future immunotherapeutic and diagnostic opportunities in this population. Recent reports have shown that functional polarization of Th subsets of lymphocytes has been implicated in tumor promotion. Tumor derived TGF-β1 in the tumor microenvironment could promote tumor eradication by influencing the polarization of Th1/Th2 and controlling Treg/Th17 cell polarization[33]. This study

only focuses on the specific role of TGF-β1 in the induction of Tregs. However, the polarization of other Th cells in the tumor environment by TGF-β1 needs to be further elucidated. A complete understanding the role of TGF-β1 in controlling T cell polarization in tumors is crucial for dissecting the beneficial use of TGF-β1 in future immunotherapies against gastric cancer.

In conclusion, we provide evidence that a higher TGF-β1 level is related to the increased population of $CD4^+Foxp3^+$ Tregs. GC cell supernatants can stimulate induction of human $CD4^+Foxp3^+$ Treg cells. This study suggests that gastric cancer cell supernatants can induce the conversation of Tregs from $CD4^+CD25^-$ naive T cells partly *via* mechanisms involving TGF-β1. Our data support the existence of intercellular cross-talk between the tumor cell and Tregs that might regulate anti-tumor immune responses.

ACKNOWLEDGMENTS

We thank Guo-Hua Xie, Mei-Xing Li, Lin Deng, Dan-Yan Yu, and Xiu-Jun Pan for providing excellent technical assistance.

COMMENTS

Background

Regulatory T cells (Tregs) accumulate in the tumor environment and suppress tumor-specific T-cell responses. Previous studies have suggested that Tregs were elevated in gastric cancer and increased populations of Tregs impaired anti-tumor immunity. However, the molecular and cellular features responsible for the increase and maintenance of Treg cell levels in gastric cancer remain elusive.

Research frontiers

Recently, emerging evidence suggests that Tregs play an important role in tumor escape from immunological control. Although the precise mechanism causing the increased numbers of Treg cells is unknown, transforming growth factor-β1 (TGF-β1), as well as other mediators, has been reported in inducing Treg cells. However, there are contradictory reports regarding the role of TGF-β1 in the induction of Tregs in cancer. The research aimed to explore whether or not gastric cancer cells producing this cytokine would account for the increased Treg and promote tumor progression.

Innovations and breakthroughs

In this study, the data confirmed the higher level of serum TGF-β1 in patients with gastric cancer. Moreover, the higher TGF-β1 level correlated with the increased population of $CD4^+Foxp3^+$ Tregs in advanced gastric cancer. Gastric cancer cell induced the increase of functional $CD4^+Foxp3^+$ Tregs, mainly from the conversion of $CD4^+CD25^-$ naive T cells. Furthermore, gastric cancer cells can induce Tregs development *via* production of TGF-β1.

Applications

The results indicated that the gastric cancer cell played a pivotal role in impairing the antitumor T cell response by induction of Tregs. This study supports the existence of intercellular cross-talk between the tumor cell and Tregs that might regulate anti-tumor immune responses. A complete understanding of the role of TGF-β1 in tumors is crucial for dissecting the beneficial use of TGF-β1 in future immunotherapies against gastric cancer.

Terminology

Regulatory T cell (Treg cell) is functionally defined as a T cell that inhibits an immune response by influencing the activity of another cell type. Tregs are characterized by specific expression of the forkhead transcription factor Foxp3, and make up 5%-10% of the normal peripheral $CD4^+$ T cell population. Treg cells within the tumor microenvironment are a crucial component of the tumor immunosuppressive network.

Peer review

The authors examined the level of serum TGF-β1, and found that it was higher in patients with gastric cancer than in healthy controls. In addition, they also found that gastric cancer cells induced the increased $CD4^+Foxp3^+$ Tregs *via* production of TGF-β1. Gastric cancer cells upregulated the production of TGF-β1 and blockage of TGF-β1 partly abrogated Tregs phenotype. These experiments are well-designed and results are clear.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.2028

World J Gastroenterol 2011 April 21; 17(15): 2028-2036
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK are prognosis-related in colorectal cancer

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Supported by Grants from the Major State Basic Research Development Program of China, 973 Program No. 2004CB518707; the Zhejiang Provincial Natural Science Foundation of China, No. R2090353; the Fundamental Research Funds for the Central Universities, No. KYJD09007
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Received: November 10, 2010 Revised: December 17, 2010
Accepted: December 24, 2010
Published online: April 21, 2011

Abstract

AIM: To investigate the expression of markers that are correlated with the prognosis of colorectal cancer (CRC) patients.

METHODS: One hundred and fifty-six CRC patients were followed up for more than 3 years after radical surgery. Immunohistochemical (IHC) analysis was performed to detect the expression of 14 pathway-related markers (p53, APC, p21ras, E-cadherin, endothelin-B receptor, Shp2, ADCY-2, SPARCL1, neuroligin1, hsp27, mmp-9, MAPK, MSH2 and rho) in specimens from these patients. Bioinformatics analysis involving a Support Vector Machine (SVM) was used to determine the best prognostic model from combinations of these markers.

RESULTS: Seven markers (SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK) were significantly related to the prognosis and clinical pathological features of the CRC patients ($P < 0.05$). Prognostic models were established through SVM from combinations of these 7 markers and proved able to differentiate patients with dissimilar survival, especially in stage Ⅱ/Ⅲ patients. According to the best prognostic model, the p53/SPARCL1 model, patients having high p53 and low SPARCL1 expression had about 50% lower 3-year survival than others ($P < 0.001$).

CONCLUSION: SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK are potential prognostic markers in CRC. A p53/SPARCL1 bioinformatics model may be used as a supplement to tumor-nodes-metastasis staging.

Key words: Colorectal cancer; Prognosis; SPARCL1; p53; Bioinformatics

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Yu SJ, Yu JK, Ge WT, Hu HG, Yuan Y, Zheng S. SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK are

prognosis-related in colorectal cancer. *World J Gastroenterol* 2011; 17(15): 2028-2036 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/2028.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.2028

INTRODUCTION

The incidence and mortality of colorectal cancer (CRC) are in the forefront of all cancers in western developed countries[1]. In China, the incidence of CRC has also increased in recent years, with 177 000 new cases and 99 000 deaths every year and a 5-year survival rate of 63.4%[2]. Tumor-nodes-metastasis (TNM) staging is helpful in predicting the survival of most patients. However, the heterogeneity of patients in their clinical outcome and their response to adjuvant chemotherapy calls for more useful prognostic pooled/panel molecular markers that will provide evidence for the choice of adjuvant therapy, especially for stage Ⅱ and Ⅲ patients.

Recently, Parsons *et al*[3] analyzed DNA mutations in CRC patients, and found that genetic changes of tumors are based on signaling pathways. Additionally, Wood *et al*[4] listed the number of mutations of all 140 genes included in 38 groups or pathways, which provided an impetus for the ongoing research on markers in CRC. After ranking these genes and pathways by the number of mutations listed by Wood *et al*[4], we selected three genes (p53, APC, ras) and 11 pathways which included genes with several mutations, and 14 genes were ultimately chosen from the pathways as candidate markers for our study. The genes are p53, APC, p21ras, E-cadherin, endothelin-B receptor, Shp2, ADCY-2, SPARCL1, neuroligin1, hsp27, mmp-9, MAPK, MSH2 and rho.

To identify prognosis-related markers of CRC, 156 patients who were followed up for more than 3 years after radical surgery were included in our survey. Immunohistochemical (IHC) analysis was performed to individually detect the expression of the 14 candidate markers in the specimens. The survival status of these patients was also analyzed. We found that seven tumor markers were found to be significantly related to the prognosis and clinical pathological features of these patients.

With the rapid development of the life sciences, bioinformatics has been developed and applied to collect, deposit and analyze large datasets and screen for useful information. In order to select molecular biomarkers more intelligently, we used a bioinformatics tool, the Support Vector Machine (SVM) classifier, to discriminate patients with different prognoses. SVM is based on the principles of Structure Risk Minimization and Vapnik-Chervonenkis Dimension as statistical learning theory, and thus provides a good generalization control[5]. SVM applications are actively used in various areas, from face recognition to genomics[6], and SVM is also a powerful tool for analyzing multiple markers. In this study, the seven prognostic markers were randomly combined, and SVM was used to evaluate which combination model was the best for predicting the prognosis of CRC patients.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University, College of Medicine, along with the patients' informed consent.

Patients and specimens

Tumor specimens included in this study were from 156 CRC patients who underwent a radical resection operation in the Second Affiliated Hospital of Zhejiang University, College of Medicine, between 1999 and 2004, with a median age of 60 years (range 20-92 years) at diagnosis. The clinical data of all patients are presented in Table 1. Tumor specimens for IHC were from filed blocks in the histopathological department.

Living patients were all followed up for > 36 mo after the radical operation, with a median follow-up of 62 mo (range 36-108 mo). The follow-ups were performed by history and physical surveillance every 3-6 mo for 2 years, then every 6 mo up to 5 years and every year after 5 years (conforming to NCCN V.2.2010). No patient was lost during the follow-up.

IHC

All 156 specimens in paraffin blocks were made into tissue arrays using a ZM-1 tissue array machine[7]. Sections (4-μm thick) were cut, and immunostaining for each antigen was conducted using the avidin-biotin peroxidase complex technique (MaxVision™ HRP-Polymer IHC Kit, MAIXIN-Bio), following the manufacturer's instructions. The antibodies used were p53 (monoclonal mouse, ZhongShan), APC (polyclonal rabbit, ZhongShan), p21ras (monoclonal mouse, MAIXIN), E-cadherin (monoclonal mouse, ZhongShan), endothelin-B receptor (polyclonal rabbit, CHEMICON), Shp2 (monoclonal rabbit, Abcam), ADCY-2 (monoclonal rabbit, Abcam), SPARCL1 (polyclonal goat, R & D), neuroligin (polyclonal rabbit, CHEMICON), HSP27 (monoclonal mouse, ZhongShan), mmp9 (polyclonal rabbit, ZhongShan), ERK1 + ERK2 (monoclonal mouse, ZhongShan), MSH2 (monoclonal mouse, ZhongShan) and Rho(-A,-B,-C) (monoclonal rabbit, MILLIPORE).

The IHC results were assessed using a semi-quantitative system, as previously described[8]. According to the percentages of positive cells (0: none, 1: < 25%, 2: 25%-50%, 3: 50%-75% and 4: > 75%) and staining intensity (0: negative, 1: weak, 2: moderate and 3: strong), the expression levels of the proteins were divided into four groups by the sum of the two scores above: 0 (0, negative expression), 1 (2-3, low expression), 2 (4-5, medium expression) and 3 (6-7, high expression).

Bioinformatics analysis

Experimental data were then analyzed by the Zhejiang University ProteinChip Data Analysis System (ZUCIPDAS, www.zlzx.net). We constructed a non-linear SVM classifier (with a radial based function kernel, a parameter Gamma of

Table 1 Clinicopathologic data of patients

Terms	*n* (%)
Sex	
Male	85 (54.5)
Female	71 (45.5)
Location	
Right hemicolon	45 (30.1)
Transverse colon	3 (1.9)
Left hemicolon	8 (5.8)
Sigmoid colon	32 (20.5)
Rectum	67 (41.7)
Differentiation	
Well	95 (60.9)
Moderately	40 (25.6)
Poorly	17 (10.9)
Unknown	4 (2.6)
Bowel wall invasion (pT)	
T1	7 (4.5)
T2	30 (19.2)
T3	116 (74.4)
T4	3 (1.9)
Lymph node metastasis (pN)	
N0	82 (52.6)
N1	43 (27.5)
N2	31 (19.9)
Distant metastasis (pM)	
M0	144 (92.3)
M1	12 (7.7)
TNM staging	
Ⅰ	29 (18.6)
Ⅱ	52 (33.3)
Ⅲ	63 (40.4)
Ⅳ	12 (7.7)
Post-surgery event	
Recurrence or metastasis	51 (32.7)
Survival status	
Dead	51 (32.7)[1]
Alive	105 (67.3)

[1]Among patients who have died, 40 patients died from recurrence or metastasis, while 11 patients died from causes such as heart or lung failure, or reasons unknown. TNM: Tumor-nodes-metastasis.

0.6, and a cost of the constraint violation of 19) to distinguish groups with different prognoses, and validated results by a 10-fold cross validation method.

One hundred and thirty-one patients with complete data were then filtered for the ongoing bioinformatics analysis. The seven prognostic biomarkers (SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK) were combined randomly to build 127 SVM models. For each model, the expression of these markers was the input, and the 3-year survival status of each patient was the evaluation criteria. The model with the highest accuracy for predicting the 3-year survival of the patients was selected as the best prognostic model, and the accuracy of the models was then validated by 10-fold cross validation between training sets and test sets.

Of the 131 patients, 44 died within 3 years after surgery, but the other 87 were still alive after 3 years. Because the number of patients dead at 3 years was about half of the number of living ones, the model showed low sensitivity due to the unbalanced data. Obviously, sensitivity is important for a prognostic model, so we next defined an adjusted accuracy [accuracy = (sensitivity+specificity)/2 + sensitivity]/2; sensitivity = true positive/(true positive + false negative), specificity = true negative/(true negative + false positive). This increased the weight of sensitivity and allowed SVM to select models with higher sensitivity.

Table 2 Expression of candidate markers in 156 colorectal cancer patients

Markers	Numbers of patients with different expression[1]			
	Negative	Low	Medium	High
P53	42	36	32	40
APC	80	37	21	10
MAPK	114	27	8	0
E-cadherin	44	47	36	22
Mmp9	109	40	6	1
Hsp27	96	33	18	6
MSH2	17	52	47	39
P21ras	102	43	8	2
ADCY-2	45	67	36	3
Shp2	108	34	13	0
ETB	59	60	30	5
Neuroligin	53	52	43	4
Rho	28	68	45	9
SPARCL1	23	52	61	20

The immunohistochemical (IHC) results were assessed using a semi-quantitative system, as previously described[8]. According to the percentages of positive cells and staining intensity, the expression levels of the proteins were divided into four groups as negative, low, medium and high expression. [1]Data were missing because some specimens were lost during sectioning and staining of tissue arrays.

Statistical analyses

Kaplan-Meier survival analysis (log-rank test) was used to evaluate the relationship between marker expression and the survival of patients. Kruskal-Wallis test was used to evaluate the relationship between the expression of candidate markers and some pathologic features in IHC analyses. SPSS Version 13.0 software (SPSS Inc., Chicago, IL) was used for all statistical analyses. $P < 0.05$ was considered to be statistically significant, and all P values were two-sided.

RESULTS

Association between the expression of candidate markers and the survival of CRC patients

The expression of candidate markers in CRC was investigated by IHC (listed in Table 2). It should be noted that some specimens were lost during sectioning and staining of tissue arrays, resulting in an average of 3.6 specimens per marker (2.3%). Representative examples of immunoassayed slides for each marker are shown in Figure 1.

Kaplan-Meier survival analysis revealed that markers significantly related with survival were SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK. The higher protein expression of SPARCL1, Shp2, MSH2, E-cadherin, and MAPK in CRC patients was related to better survival, while the higher expression of p53 and ADCY-2 was related to worse survival. The Kaplan-Meier survival curves of these markers are shown in Figure 2.

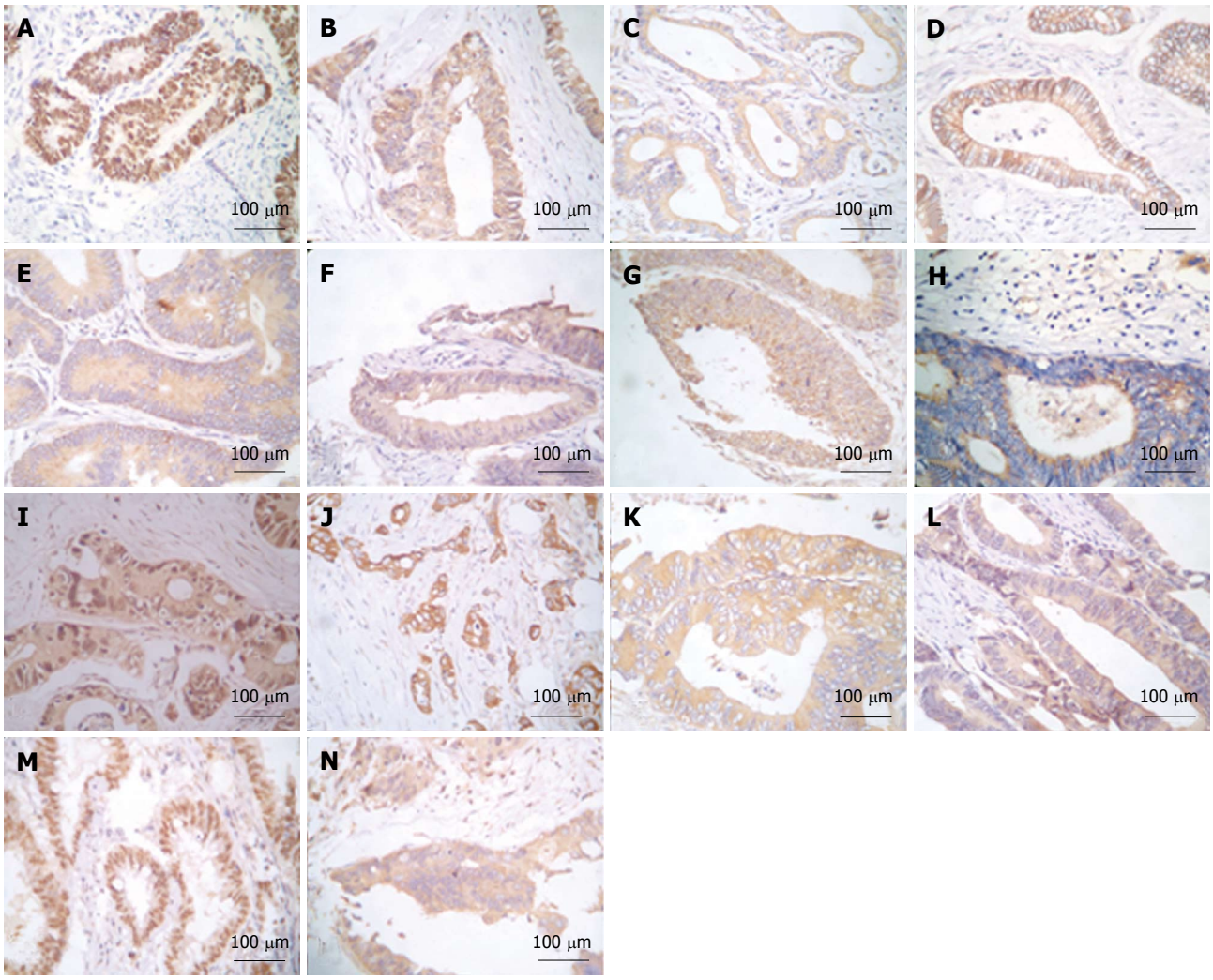

Figure 1 Immunohistochemical expression of 14 markers. The markers and the cellular location of positive staining are listed below: A: P53: nuclear; B: APC: cytoplasm; C: P21ras: cytoplasm; D: E-cadherin: membrane or cytoplasm; E: Endothelin B receptor: cytoplasm; F: Shp2: cytoplasm; G: ADCY-2: cytoplasm; H: SPARCL1: cytoplasm; I: neuroligin1: nuclear or cytoplasm; J: hsp27: nuclear or cytoplasm; K: MMP9: cytoplasm; L: MAPK: cytoplasm; M: MSH2: nuclear; N: Rho: cytoplasm. (Scale bar = 100 μm).

Kruskal-Wallis tests revealed that among these markers, SPARCL1, Shp2 and MSH2 were noticeably associated with the most clinical pathological features of CRC patients, including differentiation, bowel wall invasion (pT), lymph node metastasis (pN), distant metastasis (pM), TNM stage, post-surgery recurrence or metastasis. P53 was mainly related to TNM staging; E-cadherin and MAPK were mainly related to post-surgery recurrence and metastasis (Table 3). However, other markers, such as endothelin B receptor, APC and rho, were just related to differentiation or stages (data not shown).

Prognostic bioinformatics model established by combining the seven markers and evaluated by survival analysis

By SVM, the seven markers can randomly form 127 combinations. After being validated by 10-fold cross validation, the model with the highest accuracy (65.3) was the p53/SPARCL1 combination among all these combinations.

According to the prediction result (PR) given by the p53/SPARCL1 model, patients can be divided into two groups: "high risk" (PR > 0) and "low risk" (PR < 0). Three-year survival of the low risk group (88.30%) was more than twice as high as that of the high risk group (37.84%). Kaplan-Meier analysis revealed that the difference of survival was significant between these two groups ($P < 0.001$) (Figure 3A).

Prognostic value of the p53/SPARCL1 model for stage II and III CRC patients

Among these 131 patients, 99 patients were classified as stage Ⅱ or Ⅲ. We found that the difference in 3-year survival was not great between stage Ⅱ ($n = 43$) and Ⅲ ($n = 56$) patients, i.e. 88.40% *vs* 62.50% ($P = 0.039$) (Figure 3B). However, when these 99 patients were grouped by the PR of the p53/SPARCL1 model, the 3-year survival rate was very different between the low risk ($n = 70$) and high risk ($n = 29$) groups, i.e. 87.14% *vs* 37.93% ($P < 0.001$) (Figure 3C). Thus, the survival difference was much greater between low and high risk groups than between stage Ⅱ and

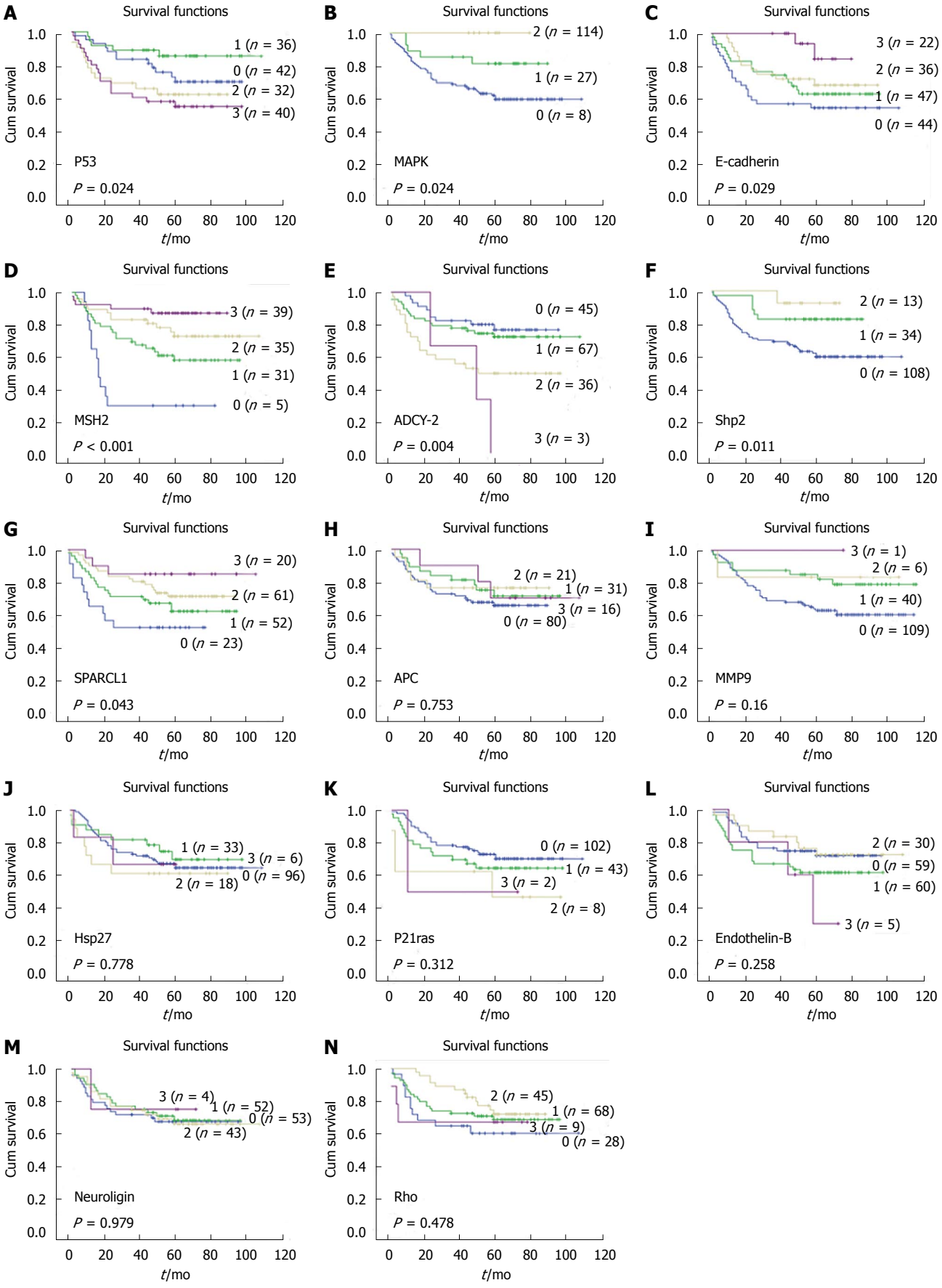

Figure 2 Kaplan-Meier curves of 14 markers. A: P53; B: MAPK; C: E-cadherin; D: MSH2; E: ADCY-2; F: Shp2; G: SPARCL1; H: APC; I: MMP9; J: Hsp27; K: P21ras; L: endothelin-B receptor; M: Neuroligin1; N: Rho.

Table 3 Relationship between marker expression and clinical features (*P* values)

Markers	*P* values					
	Differentiation	pT	pN	pM	TNM	Post-surgery
P53	0.671	0.654	0.003[b]	0.119	0.024[a]	0.207
MAPK	0.186	0.597	0.230	0.188	0.182	0.001[b]
E-cadherin	0.028[a]	0.342	0.080	0.041[a]	0.223	0.004[b]
MSH2	0.964	0.006[b]	0.012[a]	0.061	0.001[b]	0.001[b]
ADCY-2	0.458	0.430	0.779	0.470	0.878	0.082
Shp2	0.020[a]	0.004[b]	0.035[a]	0.849	0.006[b]	0.006[b]
SPARCL1	0.002[b]	0.171	0.037[a]	0.021[a]	0.044[a]	0.014[a]

The relationship between the expression of candidate markers and some pathologic features was evaluated by Kruskal-Wallis test (SPSS Version 13.0 software) in immunohistochemistry analyses. All *P* values are two-sided ([a]$P < 0.05$, [b]$P < 0.01$). The pathologic features in the table were differentiation, bowel wall invasion (pT), lymph node metastasis (pN), distant metastasis (pM), TNM stage (TNM), post-surgery recurrence or metastasis (Post-surgery).

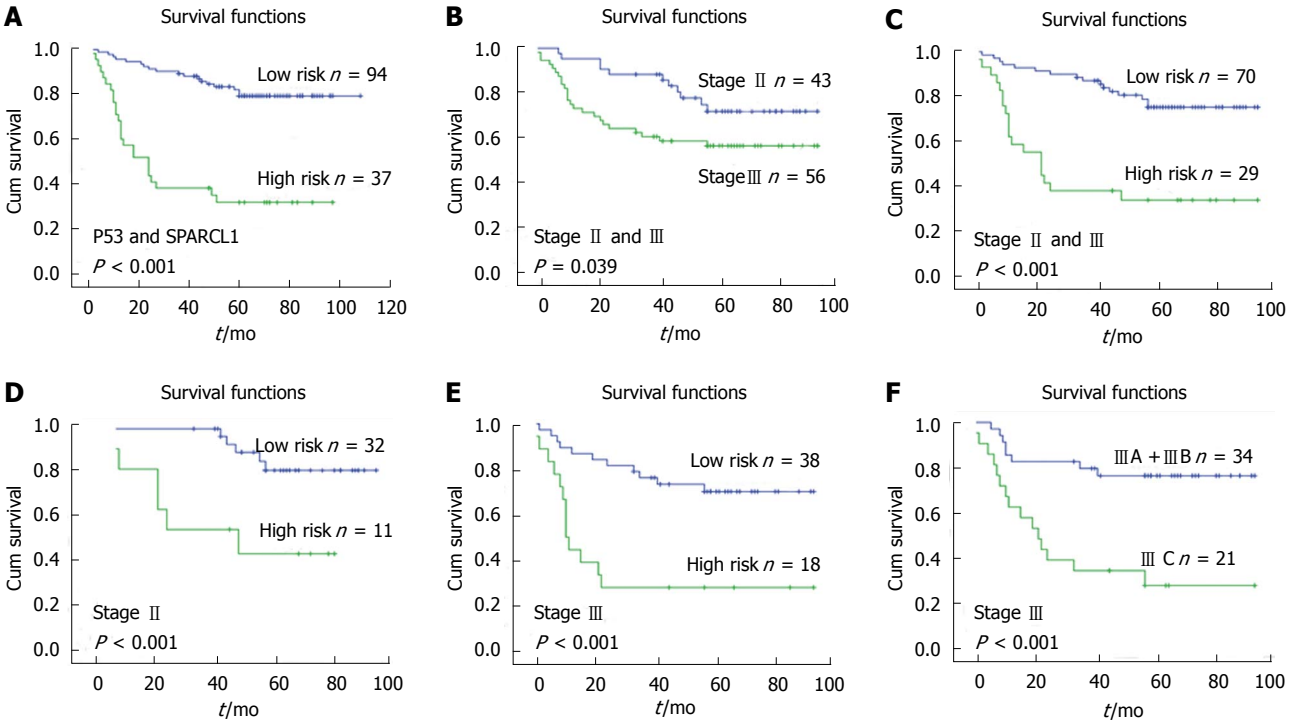

Figure 3 Prognostic value of p53/SPARCL1 model in colorectal cancer patients. According to the prediction result (PR) given by the p53/SPARCL1 model, patients could be divided into two groups: "high risk" (PR > 0) and "low risk" (PR < 0). A: 3-year survival of the "low risk" group was 88.30%, significantly higher at twice that of the "high risk" group, which was only 37.84% ($P < 0.001$). B: The 3-year survival of stage Ⅱ ($n = 43$) and Ⅲ ($n = 56$) patients was 88.40% *vs* 62.50% ($P = 0.039$), with an only 15.90% survival difference ($P = 0.039$); C: The same 99 stage Ⅱ/Ⅲ patients, when divided by the PR of the p53/SPARCL1 model: the 3-year survival of "low risk" ($n = 70$) and "high risk" ($n = 29$) group was 87.14% vs 37.93%, with a survival difference of 49.21% ($P < 0.001$), much more than the difference between stage Ⅱ and Ⅲ patients; D: According to the PR of the p53/SPARCL1 model, the 3-year survival of "low risk" and "high risk" patients at stage Ⅱ was 100% and 54.55%, respectively, with a significant difference of 45.45% ($P < 0.001$); E: At stage Ⅲ ($n = 56$), the 3-year survival was 78.95% of "low risk" patients and 27.78% of "high risk" patients, with a 51.17% higher survival rate ($P < 0.001$); F: At stage Ⅲ ($n = 56$), the 3-year survival was different between stage ⅢA/ⅢB ($n = 34$) and ⅢC ($n = 22$) patients: 82.36% *vs* 31.82% ($P < 0.001$).

Ⅲ patients.

Among the 99 stage Ⅱ/Ⅲ patients, 43 patients were of stage Ⅱ, all of whom were classified as stage ⅡA. According to the PR of the p53/SPARCL1 model, the 3-year survival rates of low risk and high risk stage Ⅱ patients were 100% and 54.55%, respectively. This 45.45% difference between the survival rates of low risk and high risk stage Ⅱ patients was significant ($P < 0.001$) (Figure 3D).

Among the 56 stage Ⅲ patients, the 3-year survival was 78.95% for low risk patients and 27.78% for high risk ones, and this 51.17% difference was statistically significant ($P < 0.001$) (Figure 3E). Similar survival difference was found between stage ⅢA/ⅢB ($n = 34$) and ⅢC ($n = 22$) patients, i.e. 82.36% *vs* 31.82% in 3-year survival rates ($P < 0.001$) (Figure 3F).

DISCUSSION

Which CRC patients should receive adjuvant chemotherapy after radical resection? Currently, it is a standard recommendation for stage Ⅲ but not stage Ⅱ patients. However, the 5-year survival rate of stage ⅡB (T4N0M0)

patients is even lower than stage ⅢA (T1-2N1M0)[9]. One explanation for this may be due to not dissecting enough lymph nodes during surgery. Another potential cause is that stage ⅡB tumors penetrate to the surface of the visceral peritoneum or directly invade the adjacent organs, which indicates that the biological behavior of the tumor is poor. Further, we do not know which of the stage Ⅱ patients are at high risk and should receive adjuvant chemotherapy to improve their survival. Therefore, better molecular tumor markers are urgently needed to predict which patients may potentially benefit from adjuvant chemotherapy.

In the network of cancer-related genes, pathways are the frame by which we can understand the network logically. In the present study, 14 candidate markers were selected based on the most frequently mutated genes and pathways listed in the study of Wood *et al*[4], and their expression levels in CRC specimens were detected by IHC, which is generally used for regular pathological detection. Among these 14 markers, seven markers (SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK) were significantly prognosis-related.

Shp2 is an essential component in several oncogene signaling pathways[10]. Here, we surprisingly found that Shp2 is a predictive marker for good prognosis, which is in stark contrast to previous studies indicating a role for Shp2 in promoting carcinogenesis in other cancers[11-13]. MSH2 is a vital mismatch repair gene. Patients with high MSH2 expression had better survival in CRC[14,15], and higher gene expression of MSH2 in responders to 5-fluorouracil-based chemotherapy indicates a predictive value of MSH2 in chemotherapy[16,17]. The MAPK signal pathway is associated with proliferation, survival and apoptosis of tumor cells and therefore plays a very important role in carcinogenesis[18,19]. E-cadherin, a member of the cadherins, is related to invasion and metastasis in many cancers[20]. Loss or low expression of E-cadherin is more frequent in CRC patients with liver metastasis[21], demonstrating that loss of E-cadherin is related to poor prognosis. ADCY is involved in the G-protein system-related GnRH signal pathway. The proliferation of rat pancreatic tumoral AR4-2J cells can be stimulated by pituitary ADCY-activating peptide through the ADCY pathway[22,23], which suggests that ADCY promotes the growth of tumor cells. Among these markers in the present study, Shp2, MSH2, MAPK and E-cadherin were significant markers for predicting good prognosis, but ADCY-2 was not.

P53 is an indispensible tumor suppressor that plays an important role in several carcinogenic processes. Previous studies suggest that p53 has an influence on the prognosis of patients in many cancers[24] including CRC, and is associated with tumor staging, multi-drug resistance, response to chemotherapy or radiotherapy, post-surgery recurrence and metastasis[25-30]. Recently, research has shown that mutant p53 proteins not only lose their tumor suppressive functions but may also gain new abilities that enhance tumorigenesis[31]. Indeed, the p53 mutation is linked with chemo-resistance and transformation to a more aggressive disease in many tumor types[32]. The p53 codon 72 polymorphism causes an increased risk for liver metastases in CRC patients positive for p53 overexpression[33]. In the present study, we found that a high expression of mutant p53 protein was associated with more frequent lymph node metastasis, advanced TNM stage and poor survival (Table 2), which is consistent with other reports.

SPARCL1, also known as hevin[34], belongs to the matricellular protein family. SPARCL1 is down-regulated in transformed prostate epithelial cell line P69SV40T[35,36], and tissues of metastatic prostate adenocarcinoma, non-small cell lung cancer, bladder and pancreatic ductal carcinoma, but up-regulated in liver cancer tissues[35-39]. Additional work by our group has revealed that SPARCL1 expression is significantly different between CRC specimens with and without liver metastasis (to be published). In the present study, the expression of SPARCL1 was not only significantly associated with histological differentiation and survival but also with distant and lymph node metastasis, suggesting that SPARCL1 is likely to be an important negative regulator in the progression or metastasis of CRC.

In the present study, SVM was utilized to analyze and establish prognostic models of CRC from the combinations of the 7 prognostic biomarkers mentioned above. For SVM, the right balance is struck between the accuracy attained on a particular training set and the "capacity" of the machine, i.e. the ability of the machine to learn any training set without error to achieve the best generalization ability. The remarkably robust performance of SVM with respect to sparse and noisy data has made it the system of choice in a number of applications. When used for classification, SVM separates a given set of binary labeled training data and can work in combination with the kernels technique for cases in which no linear separation is possible. The accuracy of our models was evaluated by 10-fold cross validation.

Ultimately, the combination of p53 and SPARCL1 was found to be the best prognostic model of those tested. Survival analysis proved that the prediction result of the p53/SPARCL1 model was a statistically significant prognostic factor for CRC patients in all stages or only stage Ⅱ/Ⅲ (Figure 3).

Other researchers have attempted to identify biomarkers to further stratify stage Ⅱ or stage Ⅲ patients. Prognostic advantages were found in patients with MSI-high tumors and stage Ⅱ and Ⅲ CRC patients treated with 5-fluorouracil-based adjuvant therapy[40,41]. In the PETACC-3 study, the prognostic value of MSI status was found to be more significant in patients with stage Ⅱ disease than in stage Ⅲ cases[42]. However, value of MSI status as a prognostic or predictive marker may be affected by mutations in other genes involved in cancer etiology, such as the BRAF gene[43]. Additionally, chromosome 18q loss of heterozygosity (LOH) has been associated with poor prognosis in stage Ⅱ and stage Ⅲ CRC patients in some studies[40,44] but not others[45,46]. Differences in the methodologies used possibly explained the contradictory

findings reported. In a large prospective study of patients with non-MSI-high CRC, 18q LOH was also not associated with patient survival, indicating that 18q LOH is not an independent survival predictive marker[47].

In our study, according to the p53/SPARCL1 model, the survival rate of low risk stage ⅡA patients was 45.45% higher than that of high risk ones. Moreover, low risk stage Ⅲ patients had a 51.17% higher 3-year survival rate than high risk ones ($P < 0.001$), the same as the survival difference between stage ⅢA/ⅢB and ⅢC. Therefore, the p53/SPARCL1 model established in this study can likely be used to supplement TNM staging, especially in stage Ⅱ and Ⅲ patients.

In conclusion, we discovered that SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK are significant prognostic markers in CRC. The p53/SPARCL1 model is of predictive use in discriminating patients with high or low risk, especially at stage Ⅱ and Ⅲ. Patients may benefit from accurate valuation and realistic treatment strategies for their disease with the help of potential prognostic markers. Larger scale studies and those involving multiple centers are planned to confirm clinic applicability of this prognosis model.

COMMENTS

Background

The incidence and mortality of colorectal cancer (CRC) are in the forefront of all cancers in China and western developed countries. More useful prognostic markers are urgently needed to provide evidence for the strategy of adjuvant therapy, especially for stage Ⅱ and Ⅲ patients.

Research frontiers

There are many ways to find useful markers in cancers. Cancer genomic research from the Vogelstein group has provided an enormous amount of information on genetic alterations in colorectal cancers. In this study, the authors set out to utilize this information to help in choosing their candidate markers. Moreover, the bioinformatics tool, which is used more and more to screen useful information from a large data set, was used here to further build prognostic models in CRC patients.

Innovations and breakthroughs

Some biomarkers have been identified as being related to the prognosis of CRC patients, including MSH2, E-cadherin and p53. However, this is the first study to report that SPARCL1, Shp2, ADCY-2 and MAPK are also potential prognostic markers in CRC. Furthermore, survival analysis proved that the p53/SPARCL1 model, established by the bioinformatics tool, could differentiate CRC patients with different prognoses in all stages or only stage Ⅱ/Ⅲ.

Applications

By finding significant prognostic markers in CRC, patients could be discriminated with high or low risk, especially in stage Ⅱ and Ⅲ. Patients may benefit from accurate valuation and realistic treatment strategies for their disease with the help of potential prognostic markers and models.

Terminology

The Support Vector Machine (SVM) classifier is a kind of bioinformatics tool, which is considered to be powerful for identifying the best discriminator from a large data set. Therefore, SVM applications are actively used in various areas from face recognition to genomics and SVM is also a powerful tool for analyzing multiple markers.

Peer review

This paper reports some novel findings on patient outcome with colorectal cancer. The array of genetic markers is extensive and worthy of publication.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.2037

World J Gastroenterol 2011 April 21; 17(15): 2037-2043
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

DEC1 nuclear expression: A marker of differentiation grade in hepatocellular carcinoma

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Supported by The National Natural Science Foundation of China, No. 81000869; the "Spring City Scholars" Construction Project of Jinan City (Q2-06), the Key Projects of Science and Technology of Jinan City, No. 200807027; and the Youth Science and Technology Star Project of Jinan City, No. 20080210
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Received: September 27, 2010 Revised: December 12, 2010
Accepted: December 19, 2010
Published online: April 21, 2011

Abstract

AIM: To investigate the expression patterns of human differentiated embryo chondrocyte 1 (DEC1) in hepatocellular carcinoma (HCC) and corresponding adjacent non-tumor and the normal liver tissues, the association between DEC1 expression and histopathological variables and the role of DEC1 in hepatocarcinogenesis.

METHODS: The expression of DEC1 was detected immunohistochemically in 176 paraffin-embedded sections from 63 patients with HCC and 50 subjects with normal liver tissues.

RESULTS: DEC1 protein was persistently expressed in the cytoplasm of hepatocytes in normal liver and HCC tissues. Compared with adjacent non-tumor liver tissues, HCC tissues showed high nuclear expression of DEC1 protein. However, high DEC1 nuclear expression was more frequently detected in well-differentiated (83.3%) than in moderately (27.3%) and poorly differentiated HCC (16.7%). Low DEC1 expression was associated with poor histological differentiation and malignancy progression. A correlation was found between the nuclear expression of DEC1 protein and histological differentiation (r = 0.376, P = 0.024).

CONCLUSION: DEC1 is expressed in the cytoplasm of hepatocytes and because nuclear DEC1 expression is decreased with decreasing differentiation status of HCC, nuclear DEC1 might be a marker of HCC differentiation.

Key words: Differentiated embryo chondrocyte 1; Hepatocellular carcinoma; Differentiation; Immunohistochemistry

Peer reviewer: Filip Braet, Associate Professor, Australian Key Centre for Microscopy and Microanalysis, Madsen Building (F09), The University of Sydney, Sydney NSW 2006, Australia

Shi XH, Zheng Y, Sun Q, Cui J, Liu QH, Qü F, Wang YS. DEC1 nuclear expression: A marker of differentiation grade in hepatocellular carcinoma. *World J Gastroenterol* 2011; 17(15): 2037-2043

Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/2037.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.2037

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major global health problem, with an estimated incidence of 500 000-1 000 000 cases and 600 000 deaths annually. It is the fifth most common cancer in the world and the third most common cause of cancer-related death[1]. The high morbidity and mortality of HCC is due to pre-existing primary chronic liver diseases, such as chronic viral hepatitis, aflatoxin B1, alcoholic liver disease and dysmetabolism, including hereditary haemochromatosis, obesity, diabetes and steatosis[2]. Each of these scenarios has its own genetic and epigenetic alterations, chromosomal aberrations, gene mutation and altered molecular pathways in the process of hepatocarcinogenesis[2,3]. Because of these varied background and heterogeneity, HCC is complex. Although dysregulation of signaling pathways such as Wnt/b-catenin, Ras, p14ARF/p53, p16INK4A/Rb, transforming growth factor-beta (TGF-beta) and PTEN/Akt has been reported in some HCC cases[4], the specific gene mutation(s) and exact molecular mechanism involved in hepatocarcinogenesis is not well known.

Human differentiated embryo chondrocyte 1 (DEC1), a basic helix-loop-helix (bHLH) transcription factor, has rat and mouse orthologs, named enhancer of split and hairy-related protein-2 (SHARP-2) and stimulation of retinoic acid 13 (Stra13), respectively[5-7]. The factors play important roles in regulation of gene expression in cell differentiation, proliferation, immune regulation and metabolism homeostatic control[8]. DEC1 is expressed ubiquitously in both embryonic and adult tissues with human and various extracellular stimuli such as growth factors, serum starvation, hypoxia, hormones, nutrients, cytokines, UV radiation, and infection, which regulate its expression[8,9]. The regulation of DEC1 is cell-type specific[5,9-11].

Several studies have described various DEC1 expression patterns in different tumor tissues, which suggest that it might contribute to oncogenesis. In human breast cancer, the overexpression of DEC1 contributes to a more aggressive phenotype[12]. The association between upregulation of DEC1 expression and differentiation of gastric cancer suggests its important role in the differentiation and progression of gastric cancer[13]. Linked to oncogenesis, DEC1 is highly expressed in colon carcinomas but not in the adjacent normal tissues[14]. It is involved in the UV signal transduction pathway and takes part in the process leading to skin cancer[15]. In combination with carbonic anhydrase-IX (CAIX) and carbonic anhydrase-XII (CAXII), DEC1 may help with a more accurate classification of all renal carcinomas[9]. However, in lung cancer, upregulated or downregulated DEC1 expression has been found[16,17]. The expression patterns and level of DEC1 protein in HCC have not been systematically investigated, and its potential role in hepatocarcinogenesis is unknown.

We aimed to investigate the expression of DEC1 in HCC. We evaluated the distribution and level of expression of DEC1 protein in 176 paraffin-embedded tissue sections from 63 patients with HCC and 50 subjects with normal liver tissues by immunohistochemistry. We also investigated the correlation of DEC1 expression with clinicopathological features and differentiation status of HCC to evaluate the functional characteristics of DEC1 in the development of HCC.

MATERIALS AND METHODS

Patients and samples

Three kinds of human liver sections (n = 176) were evaluated, including 126 HCC and adjacent non-tumor tissues from 63 patients with primary HCC, and 50 normal liver tissues from patients with hepatic hemangioma who underwent hepatectomy in Qianfoshan Hospital and Jinan Central Hospital, Shandong University, China. The 63 HCC patients included 52 males; the median age was 56 years (range, 35-77 years), and the 50 normal liver patients included 17 males. The formalin-fixed, paraffin-embedded tissue samples were retrospectively collected and randomly selected from the files of the Department of Pathology after the protocol was approved by the local research ethics committee. All HCC patients underwent surgery without prior radiotherapy or chemotherapy and other diseases such as viral hepatitis had been excluded in the hemangioma patients. All sections were reviewed independently by pathologists blinded to the clinicopathological characteristics, 63 HCC and 50 normal livers with the same pathological results were selected in our study. Among the 63 HCC samples, 18 were well differentiated, and 45 were moderately and poorly differentiated. We also collected data on sex, age, tumor size, hepatitis B virus infection, presence of cirrhosis, and α-fetoprotein (AFP) level in HCC.

Immunohistochemical staining

The tissues were fixed in 10% neutral buffered formalin for 12 h and routinely processed. Paraffin wax-embedded tissue blocks were cut into 4-μm-thick sections. Briefly, formalin-fixed, paraffin-embedded sections were heated at 60℃ for 60 min and placed into xylene to be deparaffinized and graded ethanol to be rehydrated, and then washed in phosphate-buffered saline (PBS). Antigen retrieval was performed in a prewarmed pressure cooker with a solution of antigen retrieval citrate buffer (pH 6.8) for 3 min. Following de-pressurization, cold water was poured into the cooker for 10 min, and then sections were rinsed well in warm water. Endogenous peroxide and oxidative compounds were quenched by incubation in 3% H_2O_2 in methanol for 10 min. Sections were washed 3 times with PBS, and incubated with rabbit polyclonal DEC1 antibody diluted in TBS-Tween20 (1:300 dilution)

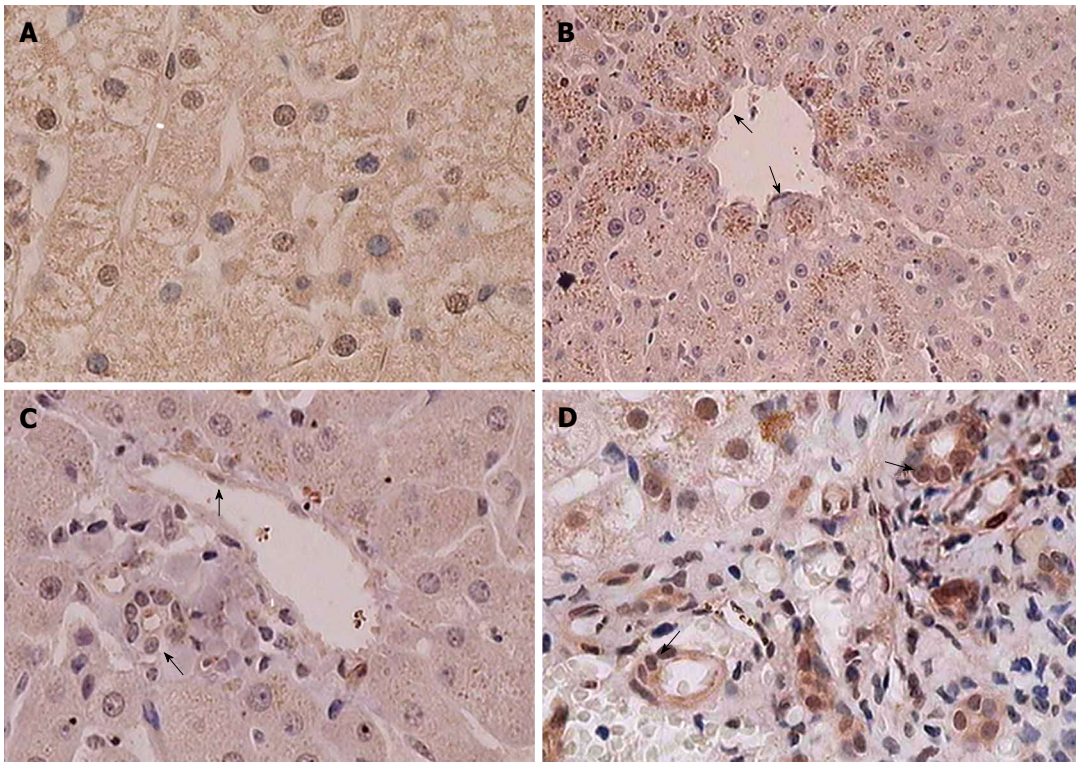

Figure 1 Differentiated embryo chondrocyte 1 expression in normal liver tissue by immunohistochemical staining. A: Normal liver tissues showing strong nuclear differentiated embryo chondrocyte 1 (DEC1) staining and diffuse cytoplasmic staining in hepatocytes, × 400; B: Strong granular staining of DEC1 in cytoplasm of hepatocytes around the central vein in the intact hepatic lobule, × 200; C: Absent granular staining and diffuse DEC1 staining pattern around portal area, × 400; D: Liver mesenchymal tissues, × 400. Endothelial cells (single arrow) and bile duct epithelial cells (double arrow) showing cytoplasm and/or nuclear DEC1 immunoreactivity.

(Bethyl Laboratories Inc, Montgomery, TX) overnight in a moist chamber at 4℃. After a final wash, secondary antibody (KIT-5010, Max Vision, Maixin.Bio, China) was applied, and TBS-Tween20 was used in all the dilutions and intervening rinses involved. Diaminobenzidine (DAB) was the chromogenic substrate. Sections were allowed to develop in DAB for 5 min, and then counterstained with hematoxylin. Slides were reviewed under microscope. Sections incubated without primary antibody were used as negative controls, and breast cancer sections were used as positive controls. Positive and negative controls were included in each run.

In hepatocytes, DEC1 protein was persistently expressed in all cytoplasms. We used a scoring standard for nuclear DEC1 expression according to our former paper[13]: negative expression, no nuclear staining; low expression, nuclear staining < 10% of cancer cells; and high expression, nuclear staining > 10% of cancer cells. The staining was evaluated by two independent observers.

Statistical analysis

Categorical variables were compared by χ^2 test or Fisher's exact test as appropriate. Spearman analysis was used to assess the correlation between DEC1 expression and tumor differentiation status. All statistical analyses were performed using SPSS v11 for Windows (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS

DEC1 expression in normal liver tissues

In normal liver tissues, DEC1 expression was diffuse in the cytoplasm of hepatocytes accompanied by a varying degree of nuclear immunoreactivity (Figure 1A). A strong granular pattern of DEC1 staining was seen within the cytoplasm of the hepatocytes around the central vein in the intact hepatic lobule, and this special staining strength became gradually weakened away from the central vein (Figure 1B). However, no granular cytoplasmic staining was seen around the portal area (Figure 1C). The two different cytoplasmic staining patterns may result from the special anatomic structure and the particular blood supplication of the hepatic lobule. The specific conditions around the central vein, such as hypoxia, low nutrition and acidity, could affect DEC1 expression status and lead to the granular pattern, and the diffusive expression pattern may be hypoxia independent. In addition, endothelial and bile duct epithelial cells showed nuclear and/or cytoplasmic DEC1 immunoreactivity (Figure 1A, B, D).

DEC1 expression in HCC and adjacent non-tumor tissues

Diffuse cytoplasm expression of DEC1 protein was observed in all 126 HCC and adjacent non-tumor liver tissues, except in 2 poorly differentiated HCC samples with weak cytoplasmic staining. Nuclear DEC1 expression was

Table 1 Differentiated embryo chondrocyte 1 nuclear expression in hepatocellular carcinoma and adjacent non-tumor tissues *n* (%)

	Negative	Positive
HCC (63)	27 (42.9)	36 (57.1)[a]
Adjacent non-tumor tissues (63)	46 (73.0)	17 (27.0)

[a]$P < 0.05$ *vs* adjacent non-tumor tissues. HCC: Hepatocellular carcinoma.

detected in 36 (57.1%) of 63 samples of primary HCC, with 26 (41.3%) samples showing high nuclear expression (Table 1). Nuclear DEC1 expression was observed in only 17 (27.0%) of the 63 matched adjacent non-tumor tissues. Compared with HCC tissue, adjacent non-tumor tissues showed reduced nuclear DEC1 expression ($P = 0.001$). The proportion of positive DEC1 nuclear staining was higher in normal (66.7%) than in HCC tissues, but was not significantly different ($P = 0.650$). In LO-2 cells (normal hepatocytes), nuclear DEC1 expression was higher than in HepG-2 cells (data not shown). Compared with the normal control, adjacent non-tumor tissues showed a significantly low positive staining rate ($P = 0.002$), which may result from extrusion around the tumor or the response of hepatocytes to extracellular stimulation from the tumor. In addition, in HCC tissues with negative nuclear staining, only two cases showed positive nuclear staining in the corresponding adjacent non-tumor tissues. Therefore, the specific microenvironment around the HCC tumor and the present chronic liver disease such as viral hepatitis may affect DEC1 expression in adjacent non-tumor tissues.

Clinical significance of DEC1 nuclear expression in HCC

For the 36 cases with positive DEC1 nuclear staining, the proportion of DEC1 staining in well, moderately and poorly differentiated HCC tissues was 94.4% (17/18), 42.4% (14/33) and 41.7% (5/12), respectively. Negative DEC1 nuclear protein staining was associated with high histological HCC grade (Table 2). Well-differentiated tissue was significantly different from moderate and poorly differentiated tissues ($P < 0.001$). Factors such as sex ($P = 0.886$), age ($P = 0.383$), tumor size ($P = 0.571$), hepatitis B virus infection ($P = 0.842$) and cirrhosis ($P = 0.616$) had no significant association with DEC1 nuclear expression.

Nuclear DEC1 expression in HCC tissues is correlated with tumor differentiation

As a transcription factor, DEC1 plays its role in the nucleus. We found strong nuclear-positive staining and high nuclear DEC1 expression in well-differentiated HCC samples. In poorly differentiated tumors with large and pleomorphic cells, DEC1 nuclear expression was weak, even negative. We investigated the nuclear immunoreactivity of DEC1 related to differentiation status (Figure 2A-C). The proportion of high DEC1 nuclear expression in well, moderately and poorly differentiated HCC tissues was 83.3%, 27.3% and 16.7%, respectively (Figure 2D).

Table 2 Correlation between nuclear differentiated embryo chondrocyte 1 protein expression and clinicopathologic features of hepatocellular carcinoma

Clinicopathologic features	*n*	Nuclear DEC1 protein expression		*P* value
		Negative	Positive	
Sex				
Male	52	23	29	0.886[1]
Female	11	4	7	
Age (yr)				
< 56	31	15	16	0.383
⩾ 56	32	12	20	
Tumor size (cm)				
⩽ 5	28	11	17	0.571
> 5	30	14	16	
Pathological grade				
Grade (Ⅰ)	18	1	17	0.000
Grade (Ⅱ-Ⅲ)	45	26	19	
Venous infiltration				
Absent	9	2	7	0.070[2]
Present	10	7	3	
No. tumor nodules				
< 3	49	22	27	0.781[1]
⩾ 3	9	3	6	
Hepatitis B virus infection				
Yes	51	22	29	0.842[1]
No	3	2	1	
Cirrhosis				
Yes	47	21	26	0.616
No	16	6	10	
α-fetoprotein level (ng/mL)				
> 20	40	18	22	0.713
⩽ 20	20	8	12	

[1]Corrected χ^2 analysis; [2]Fisher's exact test.

We found a correlation between nuclear DEC1 protein expression and histological differentiation in HCC ($r = 0.376$, $P = 0.024$) (Table 3).

DISCUSSION

HCC is a complex and heterogeneous cancer, and the key drivers are not well known[3,4]. Human DEC1 and the homologs rat SHARP-2 and mouse Stra13 belong to the bHLH family[8]. In many tumor-derived cell lines and tumor tissues, DEC1 mRNA levels are upregulated[9,18]. Its protein is also widely expressed with restricted patterns in many tissues[9,10]. Nevertheless, the distribution and role of DEC1 in carcinogenesis and in the differentiation of human HCC are unknown. A previous study showed 5 of 6 cases of HCC with positive cytoplasmic and nuclear staining[9]. However, the number of samples in this study was too small. In the current research, we studied the expression of DEC1 in 176 samples of HCC and normal liver tissues by immunohistochemistry.

We found that DEC1 protein was persistently expressed in the cytoplasm of both normal and malignant hepatocytes, with varying degrees of nucleic immunoreactivity and two staining patterns, granular and diffusive, in the cytoplasm of hepatocytes. Compared with the adjacent non-tumor tissues, HCC tissue showed predominant

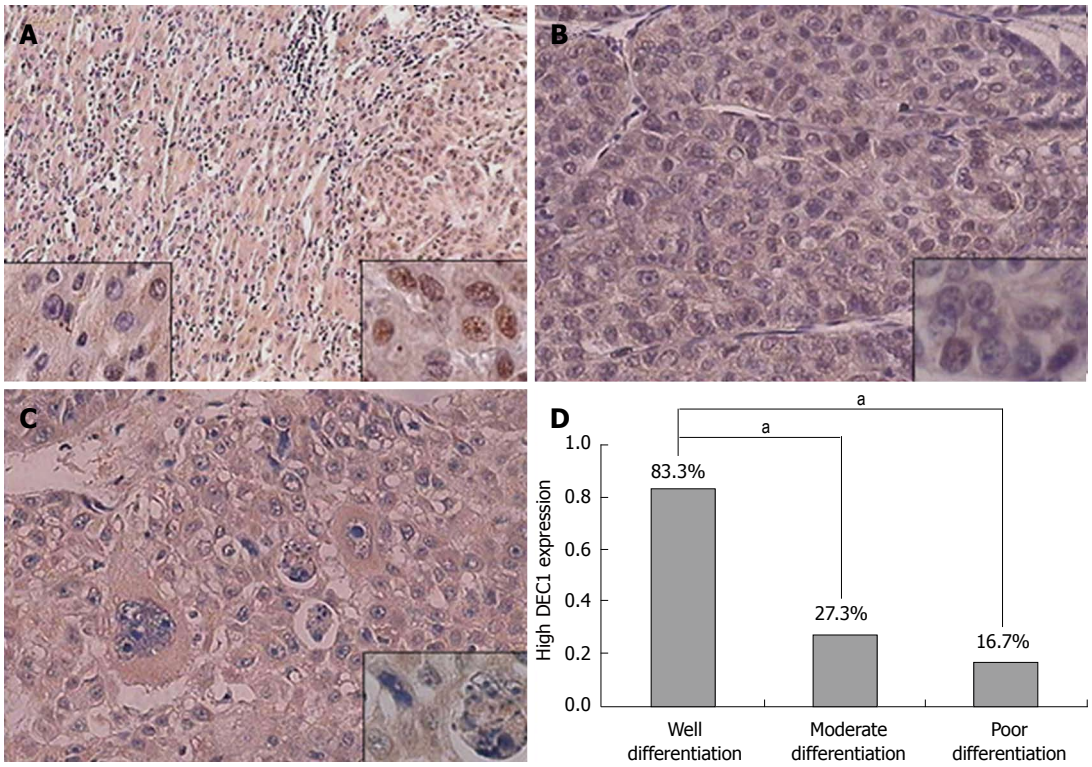

Figure 2 Differentiated embryo chondrocyte 1 expression in hepatocellular carcinoma by immunohistochemical staining. A: Well-differentiated hepatocellular carcinoma (HCC), × 100. Intense nuclear staining in HCC (right inset), and weak nuclear staining in the corresponding adjacent non-tumor tissues (left inset), with no significant difference in cytoplasmic staining; B: Moderately-differentiated HCC, × 200; C: Poorly-differentiated HCC, × 200. Inserted figure (at high magnification) showing differentiated embryo chondrocyte 1 (DEC1) staining in the cytoplasm and nucleus, × 400; D: Quantitation of DEC1 nuclear expression in HCC by different differentiation status. [a]$P < 0.05$.

Table 3 Correlation between differentiated embryo chondrocyte 1 nuclear expression and hepatocellular carcinoma differentiation

Tumor differentiation (cases)	DEC1 nuclear expression[1]		*r* value	*P* value
	Low	High		
Well (n = 17)	2	15	0.376	0.024
Moderate (n = 14)	5	9		
Poor (n = 5)	3	2		

[1]Number of cases. DEC1: Differentiated embryo chondrocyte 1.

DEC1 nuclear protein expression, but the nuclear expression of DEC1 was decreased from well to moderately and poorly differentiated HCC. It seems that low DEC1 expression is associated with poor histological differentiation and malignancy progression in HCC. However, the mechanism of this distribution of DEC1 expression in HCC needs further investigations.

The intracellular distribution of DEC1 protein shows cell specificity: in Hela cells, DEC1 is equally present in both the nucleus and cytoplasm; in HepG2 cells, DEC1 is located mostly in the cytoplasm; and in the 786-0 cell line, the nuclear expression of DEC1 is predominant[9]. In the present study, DEC1 protein was persistently expressed in almost all cytoplasms of hepatocytes, with a varying degree of nuclear immunoreactivity. This expression pattern implies that DEC1 may take part in the normal vital functions of the liver such as metabolism and detoxification. DEC1-positive granular staining gradually weakened outward from the central vein in the cytoplasm of hepatocytes. The mechanism underlying this phenomenon might be the hypoxic environment around the central vein. Hypoxia-induced factor-1α (HIF-1α), the most important hypoxia effecter, can upregulate the expression of DEC1 by binding hypoxia response element (HRE) on *DEC1* gene promoter[19]. We also found that the DEC1 expression level in HCC was higher than in normal liver tissues. The oxygen tension in HCC was similar to that in normal liver tissues, so the overexpressed HIF-1α in HCC was independent of hypoxia[20]. Thus, the diffuse staining pattern of DEC1 in normal liver tissues and HCC was independent of hypoxia, and the granular staining pattern of DEC1 may represent a low oxygen concentration. Whether the different patterns of staining imply a different function of DEC1 in the cytoplasm of hepatocytes awaits further investigations. Additional studies are required to unravel the mechanism by which DEC1 performs its function in cytoplasm. Furthermore, the subcellular localization of DEC1 may be helpful in identifying the primary origin of certain tissues and cells.

DEC1 usually acts as a transcription repressor, but it also can function as a transcriptional activator under particular circumstances. It has been shown that signal transducers and activators of transcription 3 (STAT3), which

are essential for the carcinogenesis of HCC, are a protein partner of DEC1[21,22]. DEC1 can bind with phosphorylated STAT3β and/or STAT3α isoforms, thus activating the downstream transcription from STAT-dependent cis-elements[22]. For example, co-expression of STAT3α or STAT3β with DEC1 can modify the transcription status of Fas, which can regulate cell survival and apoptosis[22]. DEC1, together with STAT3, is involved in complex regulation of STAT downstream transcriptional targets. Thus, inappropriate co-activation of DEC1 and STAT3 may lead to oncogenic transformation in hepatocytes. Through this network, DEC1 may contribute to the regulation of critical processes of cell survival and growth in hepatocellular carcinogenesis.

Unlike other bHLH proteins, such as c-Myc and ID, which exhibit intrinsically growth-promoting activity, DEC1 can cause proliferation inhibition and differentiation promotion. In NIH 3T3 cells, induced Stra13 strongly represses the expression of the cell proliferation-associated gene c-Myc by interacting with the basal transcription factor TFIIB[18]. The proximal promoter region of ID1 gene contains several potential DEC1 responsive elements by which DEC1 can inhibit ID1 expression, thus promoting cell differentiation[23]. DEC1 has been involved in various differentiation processes, such as neurogenesis[6,7], chondrogenesis[5] and myogenesis[24]. The differentiation inducer hydroxyurea can markedly induce the expression of DEC1[13]. The promotion or inhibition function of DEC1 in cell differentiation is cell-type specific and differs in different original tissues. Overexpression of Stra13 inhibits mesodermal but promotes neuronal differentiation in P19 cells[6]. In rats, the expression of sharp-2 mRNA was slightly higher in the well-differentiated than in the poorly differentiated malignant hepatoma cell lines[25]. Similarly, in our study, high DEC1 nuclear expression was more frequently detected in well-differentiated HCC than in poorly differentiated HCC. Thereby, DEC1 may serve as a marker for the degree of HCC differentiation. As a transcription repressor, DEC1 can regulate expression of many other transcription factors and maintain the homeostasis of the tissues and cells[8]. The reduced expression of DEC1 in poorly differentiated HCC might lead to exacerbation through impairing the homeostasis of the liver.

Epidemiological studies indicated that metabolic disturbance, especially abnormal lipid and glucose metabolism, is a major characteristic of the cause of HCC[4]. Liver X receptor (LXR), together with sterol-regulatory-element-binding protein 1c (SREBP1c) and fatty acid synthase (FAS), enhances the development of HCC caused by chronic viral infection[26]. And insulin resistance increases the risk of HCC[27]. Recent reports have proved that DEC1 was closely associated with LXR, SREBP1c, FAS and insulin[28-31]. However, further researches are needed about the relationship between the function of DEC1 interacted with these genes in liver metabolism and the differentiation grade of HCC.

In summary, we demonstrated DEC1 protein expression patterns in the liver and characterized the relationship between nuclear DEC1 protein expression and histological differentiation of HCC. DEC1 might be a marker of HCC differentiation. However, DEC1 expression can be regulated through several pathways to contribute to its specific functions in different tissues. In the liver, DEC1 may be situated at the crossroads of a complex transcriptional network that is able to modulate metabolism, energy, physiological function and tumor genesis. Dysregulation of *DEC1* gene causes alteration of homeostasis in tissues and cells, leading to abnormal cell proliferation, differentiation and death as well as subsequent development of cancer in a cell type-dependent manner.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the common fatal cancers worldwide. The aggressive phenotype and resistance to therapy makes HCC particularly dangerous. Recent reports indicate that the transcription factor differentiated embryo chondrocyte 1 (DEC1) contributes to tumorigenesis. The expression of DEC1 and its role in human HCC are unknown. DEC1 may take part in the vital metabolism function of liver and in the procession of hepatocarcinogenesis.

Research frontiers

DEC1 is a new transcriptional factor with helix-loop-helix (HLH) domains. It plays an important role in the maintenance of the homeostatus of metabolism and energy, oncogenesis, cell growth and apoptosis, immune balance and circadian rhythm.

Innovations and breakthroughs

This is the first study to describe the expression of DEC1 in HCC and normal liver tissues. In this study, an inverse relationship was found between the nuclear DEC1 protein expression and the histological grade of HCC. DEC1 might be a marker for HCC differentiation. Based on all these results, DEC1 may be situated at the crossroads of a complex transcriptional network that is able to modulate metabolism, energy, physiological function and tumor genesis in the liver.

Applications

By understanding the distribution of DEC1 protein expression and the function of this bHLH molecule, this study may represent a future strategy for therapeutic intervention in patients with HCC.

Terminology

Dec1 and Hif are transcriptional factors with HLH domains. They are important in oncogenesis. Sterol-regulatory-element-binding protein 1c (SREBP1c), fatty acid synthase (FAS) and liver X receptor (LXR) play vital functions in metabolism and tumor genesis in the liver. All these proteins take part in the maintenance of the homeostatus of metabolism and energy, cell growth and apoptosis, oncogenesis.

Peer review

The morbidity and mortality of HCC are high in China. In the procession of hepatocarcinogenesis, there are already pre-existing chronic liver diseases. In this paper, the authors demonstrated for the first time that DEC1 protein expression patterns in normal liver tissue and HCC systematically. It is important for further studying the development of HCC and the function of DEC1.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.2044

World J Gastroenterol 2011 April 21; 17(15): 2044-2048
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Apoptotic bone marrow CD34+ cells in cirrhotic patients

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Supported by National Natural Science Foundation of China, No.30670961
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Received: August 13, 2010 Revised: December 9, 2010
Accepted: December 16, 2010
Published online: April 21, 2011

Abstract

AIM: To access the frequency and level of apoptotic CD34+ cells isolated from the marrow fluid of patients with post-hepatitis cirrhosis.

METHODS: The frequency of bone marrow CD34+ cells and apoptotic bone marrow CD34+ cells in 31 in-patients with post-hepatitis cirrhosis (cirrhosis group), and 15 out-patients without liver or blood disorders (control group) was calculated by flow cytometry. Parameters were collected to evaluate liver functions of patients in cirrhosis group.

RESULTS: The percentage of normal bone marrow CD34+ cells was 6.30% ± 2.48% and 1.87% ± 0.53% (t = 3.906, P < 0.01) while that of apoptotic marrow CD34+ cells was 15.00% ± 15.81% and 5.73% ± 1.57% (t = 2.367, P < 0.05) in cirrhosis and control groups, respectively. The percentage of apoptotic marrow CD34+ cells was 6.25% ± 3.30% and 20.92 ± 18.5% (t = 2.409, P < 0.05) in Child-Pugh A and Child-Pugh B + C cirrhotic patients, respectively. The percentage of late apoptotic marrow CD34+ cells was positively correlated with the total bilirubin and aspartate aminotransferase serum levels in patients with cirrhosis.

CONCLUSION: The status of CD34+ marrow cells in cirrhotic patients may suggest that the ability of hematopoietic progenitor cells to transform into mature blood cells is impaired.

Key words: Cirrhosis; CD34; Hematopoietic stem cells/Hematopoietic progenitor cells; Apoptosis

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Dang SS, Wang WJ, Gao N, Wang SD, Li M, Liu LY, Sun MZ, Dong T. Apoptotic bone marrow CD34+ cells in cirrhotic patients. *World J Gastroenterol* 2011; 17(15): 2044-2048 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/2044.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.2044

INTRODUCTION

Cirrhosis represents the final stage for a wide variety of chronic liver diseases. One of its main clinical manifestations in its decompensatory stage is cytopenia. The causes have been generally recognized as hypersplenism and abnormal bone marrow[1]. However, the more detailed mechanism underlying cytopenia in cirrhotic patients is not clear. An understanding of its mechanism underlying loss of peripheral blood cells would shade further insight

Table 1 Characteristics of patients in cirrhosis and control groups

Parameters	Control	Child-Pugh A	Child-Pugh B	Child-Pugh C	*P* value (cirrhosis *vs* control)
Demographic data					
Number	15	12	11	8	
Male/female	9/6	6/6	6/5	5/3	
Age	42 (32-53)	39 (35-50)	46 (32-60)	48 (29-60)	NS
Lab tests					
WBC ($\times 10^9$)	7.05 ± 2.21	4.34 ± 2.01	2.93 ± 0.50	3.62 ± 1.78	< 0.01
RBC ($\times 10^{12}$)	3.53 ± 0.60	3.67 ± 0.61	3.93 ± 0.75	3.34 ± 0.57	NS
PLT ($\times 10^9$)	210 ± 105	141 ± 114	58 ± 42	54 ± 27	< 0.01
ALT(IU/L)	16 ± 14	55 ± 49	87 ± 48	61 ± 31	< 0.01
AST(IU/L)	20 ± 11	50 ± 33	73 ± 24	106 ± 63	< 0.01
Albumin (g/L)	43 ± 5	40 ± 6	35 ± 3	30 ± 6	< 0.01
TBIL (μmol/L)	10.3 ± 3.5	15.8 ± 7.6	40.7 ± 23.0	78.4 ± 57.8	< 0.01
γ-GT(IU/L)	15 ± 12	64 ± 63	143 ± 171	100 ± 108	< 0.01

TBIL: Total bilirubin; NS: No significance.

into the progress of cirrhosis and its treatment.

Peripheral blood cells are derived from hematopoietic stem cells (HSC). HSC are derived from mesenchymal cells located in wall of yolk sac during the embryonic stage. After establishment of embryonic blood circulation, HSC spread to the liver and hematopoiesis is initiated in the 6th week of embryonic stage and reoccurs in the spleen when hematopoiesis in the liver is decelerated. After birth, the production of blood cells in the liver and spleen dwindles, and almost halts altogether[2]. HSC settle mainly in the bone marrow and produce blood cells for life. As yet, very few studies are available on the phenotypic change of HSC located in bone marrow of cirrhotic patients and its impact on the production of peripheral blood.

CD34, a well known marker for HSC, is a type of phosphoglycoprotein belonging to type 1 trans-membrane protein, helps HSC/hematopoietic progenitor cells (HPC) to adhere to marrow stroma, inhibits differentiation of hematopoietic cells, stimulates formation of HPC, and is involved in intracellular signal transduction, *etc*. The number of CD34+ cells including hematopoietic cells at several stages is heterogeneous and can differentiate to all blood cell lineages. The CD34 molecule gradually disappears when HSC/HPC differentiate into mature blood cells[3].

In this study, the percentage of apoptotic CD34+ bone marrow cells in patients with post-hepatitis cirrhosis at different stages was calculated by flow cytometry.

MATERIALS AND METHODS

Study subjects

Thirty-one patients with post-hepatitis cirrhosis, admitted to Department of Infectious Diseases, Second Affiliated Hospital of Xi'an Jiaotong University from November 2008 to April 2009, were divided into three groups according to the Child-Pugh classification. The patients did not receive treatment with interferon and only 6 patients were treated with lamivudine or adefovir intermittently before they were enrolled in this study. The control group consisted of 15 patients attending the Outpatient Department of Hematology in our hospital. Bone marrow smears showed that they had no liver and hematological disorders. Laboratory tests were performed to evaluate their liver functions. The clinical details of cirrhosis and control groups are shown in Table 1. Bone marrow was aspirated from each patient. Mononuclear cells (MNC) were isolated from 4 mL freshly-heparinized marrow fluid by Isopaque-Ficoll (Bioer, Hangzhou, China) gradient centrifugation. The study was approved by the local ethical committee, and informed consent was obtained from the patients.

Flow cytometry evaluation

After Ficoll gradient separation, the MNC were washed with 100 mL of phosphate-buffered saline (PBS), and 10^6 cells were stained with anti-CD34-FITC MAb (Biosynthesis, Beijing, China) for 30 min at 4℃. Percentage of CD34+ cells was assayed with a Keygen annexin V apoptosis detection kit containing annexin V-PE, propidium iodide (PI; Sigma, US) solution and annexin V binding buffer. CD34+ cells were analyzed to determine the early annexin V+/PI- or late annexin V+/PI+ apoptotic phase.

The MNC were stained again with anti-CD34-FITC MAb, annexin V-PE (Keygen, Nanjing, China) and PI, washed again with PBS and re-suspended in 100 mL of annexin V-PE for 15 min at room temperature. Another 200 mL of binding buffer and 5 mL of PI solution were added and 800 000 cells were obtained by flow cytometry (BD, USA)[4].

Statistical analysis

Statistical analysis was conducted using the SPSS 13.0 statistical package. Difference in independent variables of apoptotic CD34+ cells was detected by *t*-test and one way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Percentage of bone marrow CD34+ cells in cirrhotic patients

Marrow CD34+ cells were gated from the side scatter height (SSC-H)/CD34 FITC dot plot according to the Milan protocol (Figure 1).

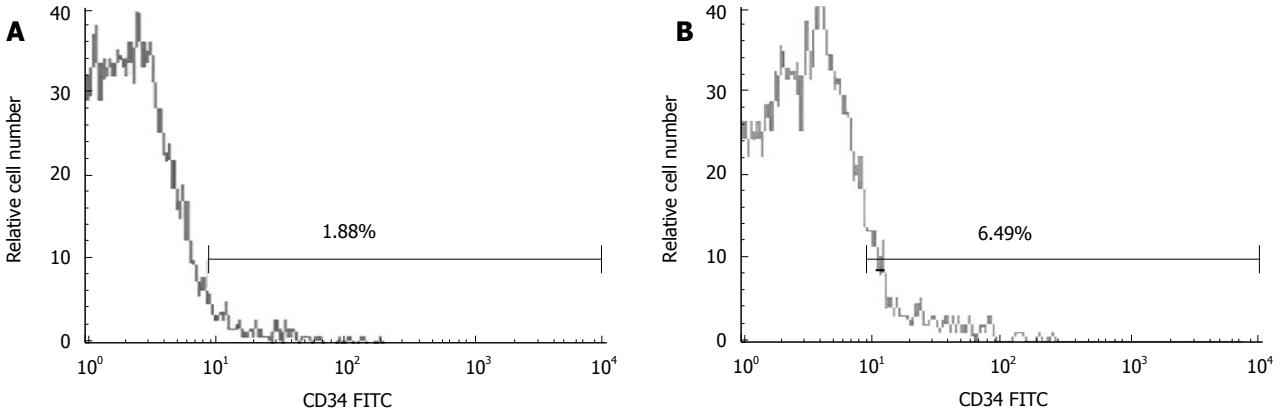

Figure 1 Flow cytometry showing the percentage of CD34+ cells in controls (A) and cirrhotic patients (B).

Table 2 Correlation between early, late and total CD34+ cells and laboratory parameters

Parameters	Early apoptotic cells		Late apoptotic cells		Total CD34+ cells	
	r	*P*	*r*	*P*	*r*	*P*
Age	-0.124	0.625	0.225	0.385	0.048	0.839
RBC	0.024	0.924	-0.181	0.487	-0.107	0.653
WBC	-0.152	0.548	0.244	0.345	0.322	0.167
PLT	-0.216	0.389	0.195	0.452	0.218	0.355
TBIL	-0.186	0.490	0.717	0.003[b]	0.314	0.205
ALT	-0.015	0.957	0.256	0.358	0.206	0.412
AST	-0.043	0.874	0.663	0.007[b]	0.146	0.564
γ-GT	-0.117	0.667	0.136	0.630	0.471	0.048[a]
Albumin	-0.283	0.270	-0.033	0.905	0.465	0.045[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* early apoptotic cells.

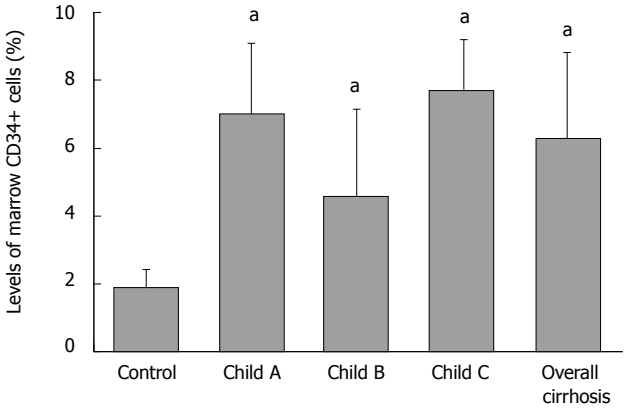

Figure 2 Percentage of bone marrow CD34+ cells in two groups. [a]$P < 0.01$ *vs* control control.

The percentage of normal bone marrow CD34+ cells (out of mononuclear cells -CD34+/MNC) was 6.30% ± 2.48% and 1.87% ± 0.53% (t = 3.906, $P < 0.01$), respectively, in cirrhosis and control groups, while that of CD34+/MNC was 7.01% ± 2.1%, 4.58% ± 2.56%, and 7.72% ± 1.49% (F = 3.586), respectively, in Child-Pugh A-C cirrhosis patients (Figure 2).

Percentage of apoptotic bone marrow CD34+ cells in cirrhotic patients

The percentage of bone marrow CD34+ cells was determined by FACS analysis with annexin V/PI staining (Figure 3). The percentage of early apoptotic bone marrow CD34+ cells (out of total marrow CD34+ cells) was 6.60% ± 5.83% and 3.88% ± 1.22% (t = 0.912), respectively, while that of late apoptotic bone marrow CD34+ cells was 9.24% ± 13.67% and 1.86% ± 0.86% (t = 2.207, $P < 0.05$), respectively, in cirrhosis and control groups. The total percentage of apoptotic bone marrow CD34+ (out of total marrow CD34+ cells) cells was 15.00% ± 15.81% and 5.73% ± 1.57% (t = 2.367, $P < 0.05$), respectively, in cirrhosis and control groups.

The total percentage of apoptotic bone marrow CD34+ cells (out of total marrow CD34+ cells) was 6.25% ± 3.30% and 20.92% ± 18.5% (t = -2.409, $P < 0.05$), respectively, in early (Child-Pugh A) and late stage (Child-Pugh B+C) cirrhotic patients (Figure 4).

Correlation between apoptotic CD34+ bone marrow cells and reduced bilitubin and aminotransferase serum levels

The correlation between apoptotic CD34+ bone marrow cells and reduced bilitubin and aminotransferase serum levels was assessed (Table 2), which showed that the early apoptotic CD34+ bone marrow cells were positively correlated with the serum levels of γ-GT and albumin, while the late apoptotic CD34+ bone marrow cells were negatively correlated with the serum levels of total bilirubin and aspartate aminotransferase (AST) in cirrhotic patients.

DISCUSSION

HSC, the ancestors of different blood cells, are multipotent and self-renewable with a great ability to proliferate, and can differentiate into HPC which then differentiate into red blood cells, white blood cells and platelets. HPC, derived from HSC, are not able to renew. The number of HPC is retained by proliferation which expands different mature blood cells[5].

In this study, the expression of CD34 antigen in bone

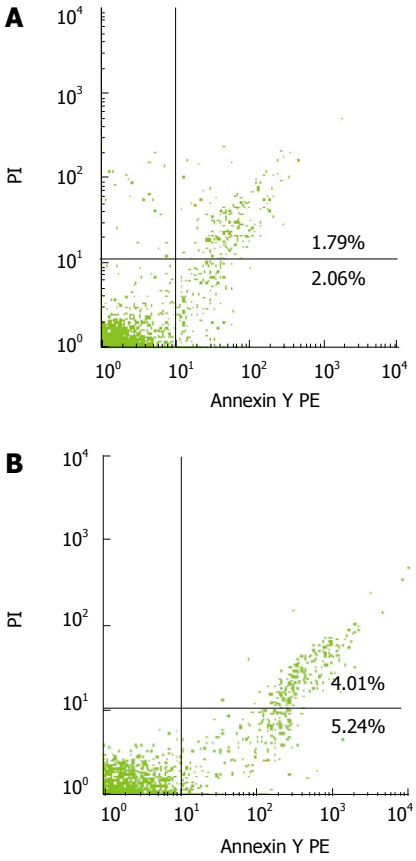

Figure 3 Scatter plot showing percentages of early and late apoptotic bone marrow CD34+ cells in control group (A) and cirrhosis group (B).

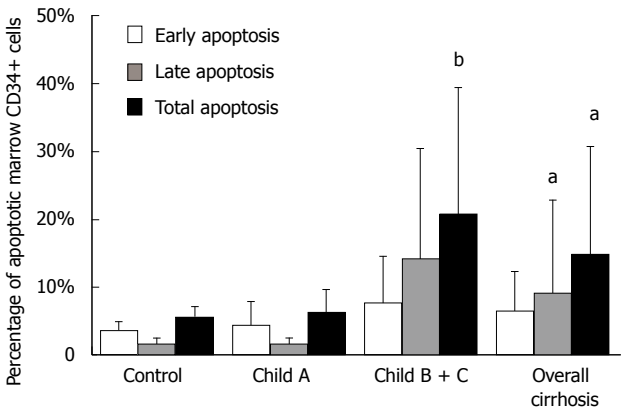

Figure 4 Percentage of apoptotic bone marrow CD34+ cells in controls and Child A, Child B + C cirrhotic patients. [a]*P* < 0.05 *vs* controls, [b]*P* < 0.05 *vs* Child A patients.

marrows of 31 patients with post-hepatitis cirrhosis was detected by flow cytometry. The percentage of bone marrow CD34+ cells was significantly higher in cirrhosis group than in control group, indicating that bone marrow CD34+ cells are activated in patients with post-hepatitis cirrhosis. HSC/HPC have a compensatory proliferation capacity for cytopenia in cirrhotic patients and enhance their ability to differentiate into mature blood cells, in order to make up the loss of mature blood cells because of hypersplenism, which is significantly different from that for aplastic anemia, in which cytopenia is caused by abnormal HSC/HPC[6].

In this study, the percentage of apoptotic bone marrow CD34+ cells was significantly different in cirrhosis and control groups, and between early and late stage cirrhosis. The percentage of apoptotic CD34+ cells was significantly higher in cirrhosis group than in control group. The percentage of apoptotic bone marrow CD34+ cells (20.92% ± 18.5%) was very high in late stage cirrhotic patients, and almost comparable between early stage cirrhotic patients and controls (6.25% ± 3.30% *vs* 5.73% ± 1.57%), suggesting that the compensatory ability of HSC/HPC to proliferate is impaired, thus limiting their ability to differentiate into mature blood cells, which may be the cause of cytopenia in late stage cirrhotic patients. This could also explain why the peripheral blood cell count in early stage cirrhotic patients remains normal and the peripheral blood cell count is decreased in late stage cirrhotic patients, although the number of bone marrow CD34+ HPC is still high[7]. Our previous study showed that the Fas expression level is significantly higher in rats with carbon tetrachloride-induced cirrhosis than in normal rats[8]. It is also known that the serum TNF-α and IFN-γ levels are higher in cirrhotic patients, thus up-regulating the expression of Fas, leading to cell apoptosis. We therefore speculate that the TNF-α and IFN-γ/Fas pathways may contribute to the apoptosis of bone marrow HSC/HPC[9-13].

Finally, the correlation between parameters used to evaluate liver functions and apoptotic HSC/HPC was analyzed. The late apoptotic CD34+ bone marrow cells were positively correlated with the serum levels of total bilirubin and AST, indicating that that the impaired function of bone marrow CD34+ cells is correlated with late stage cirrhosis. The establishment of such a correlation can be used in evaluating hematopoiesis in bone marrow of cirrhotic patients.

It has been shown that hypersplenism is the main cause of cytopenia in cirrhotic patients. Decreased production of thrombopoietin in liver may also contribute to thrombocytopenia[14,15]. In this study, the number of HSC/HPC was increased and become more active in cirrhotic patients leading to compensatory effects of peripheral cytopenia. However, as cirrhosis progresses and apoptosis of HSC/HPC increases, hematopoiesis in bone marrow is impaired, eventually leading to disordered homeostasis and poor prognosis, in addition to liver failure and hypersplenism, in end-stage cirrhotic patients. The subjects involved in this study had hepatitis B or hepatitis C-associated cirrhosis. The virological impact on activation of bone marrow CD34 cells and functional impairment therefore merits further investigation.

In conclusion, the percentage of CD34+ bone marrow cells is high in cirrhotic patients, and increased apoptotic CD34+ HSC/HPC are correlated with late stage cirrhosis and total bilirubin and AST serum level.

COMMENTS

Background

Peripheral cytopenia is quite common in cirrhotic patients and the percentage of apoptotic CD34+ cells in bone marrow of cirrhotic patients was calculated.

Research frontiers

The relation between bone marrow CD34+ cells and liver cells has been reported. Bone marrow CD34+ cells can transform into hepatocytes *in vitro* and *in vivo*. Meanwhile, autologous or umbilical stem cell transplantation seems to be a promising new therapy for cirrhosis.

Innovations and breakthroughs

Bone marrow CD34+ cells were studied in cirrhotic patients. In this study, the percentage of bone marrow CD34+ cells was high in cirrhotic patients and that the increased apoptotic CD34+ HSC/HPC are correlated with late stage cirrhosis and the total bilirubin and AST serum level.

Applications

The findings in the study can partly explain why peripheral cytopenia occurs in patients with post-hepatitis cirrhosis.

Terminology

HSC: Multipotent stem cells that increase different blood cells including myeloid and lymphoid lineages and are defined by their ability to replenish different blood cells and their ability to self-renew.

Peer review

This is an interesting manuscript describing the role of CD 34+ cells, a well known marker for hematopoietic stem cells, in cirrhotic patients. The flow cytometry evaluation is adequate. The statistical analysis has well been conducted.

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S- Editor Sun H **L- Editor** Wang XL **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.2049

World J Gastroenterol 2011 April 21; 17(15): 2049-2053
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

A case-control study on the relationship between salt intake and salty taste and risk of gastric cancer

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Received: December 9, 2010 Revised: January 5, 2011
Accepted: January 12, 2011
Published online: April 21, 2011

Abstract

AIM: To investigate the relationship between salt intake and salty taste and risk of gastric cancer.

METHODS: A 1:2 matched hospital based case-control study including 300 patients with gastric cancer and 600 cancer-free subjects as controls. Subjects were interviewed with a structured questionnaire containing 80 items, which elicited information on dietary, lifestyle habits, smoking and drinking histories. Subjects were tested for salt taste sensitivity threshold (STST) using concentrated saline solutions (0.22-58.4 g/L). Conditional logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (95% CI).

RESULTS: Alcohol and tobacco consumption increased the risk of gastric cancer [OR (95% CI) was 2.27 (1.27-4.04) for alcohol and 2.41 (1.51-3.87) for tobacco]. A protective effect was observed in frequent consumption of fresh vegetable and fruit [OR (95% CI) was 0.92 (0.58-0.98) for fresh vegetable and 0.87 (0.67-0.93) for fruit]. Strong association was found between STST $\geqslant$ 5 and gastric cancer [OR = 5.71 (3.18-6.72)]. Increased STST score was significantly associated with salted food intake and salty taste preference ($P < 0.05$).

CONCLUSION: A high STST score is strongly associated with gastric cancer risk. STST can be used to evaluate an inherited characteristic of salt preference, and it is a simple index to verify the salt intake in clinic.

Key words: Gastric cancer; Salt taste sensitivity threshold; Salt taste preference

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Yang WG, Chen CB, Wang ZX, Liu YP, Wen XY, Zhang SF, Sun TW. A case-control study on the relationship between salt intake and salty taste and risk of gastric cancer. *World J Gastroenterol* 2011; 17(15): 2049-2053 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/2049.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.2049

INTRODUCTION

Gastric cancer is the fourth most common cancer in the world, and is the second most common cause of death

from cancer[1]. Its incidence shows wide geographical variation, but almost two-thirds of the cases are from developing countries, including 42% from China. Although gastric cancer is decreasing in most populations, the absolute number of cases is predicted to increase up to the year 2050 due to the aging of the population[2]. In China, gastric cancer is the third most common cancer, with an age-standardized incidence of 37.1 and 17.4 cases per 100000 person-years in men and women, respectively[3]. Therefore, prevention of gastric cancer is one of the most important cancer control strategies both in China and around the world.

High intake of salt is hypothesized to be a cause of cancer and an important cause of gastric cancer[4,5]. Evidence has proved that a high salt intake damages the gastric mucosa producing atrophy and intestinal metaplasia[6,7]. In addition, a high salt diet has been shown to have a synergistic interaction with gastric carcinogens[8,9]. In animal experiments, the co-administration of a high dietary salt intake enhances both the initiation and promotion of gastric cancer induced by carcinogenic N-nitroso compounds[10]. It has been shown that a high intake of salted food is associated with increased risk for gastric cancer. But a recent meta-analysis showed a weak association between salt and gastric cancer[4], and the relationship between quantity of salt intake and gastric cancer is hard to estimate. Most studies evaluated the salt preference only by the subjective feeling of subjects, and salty taste sensitivity test has the capacity to identify the flavor of salt, and its threshold can influence salt appetite or salt food preference[11]. This method can be used to assess the association between salt preference and gastric cancer. The purpose of this study was to analyze the relationship between salt intake, salty taste and risk of gastric cancer..

MATERIALS AND METHODS

A hospital-based case-control study was carried out in the Shanyin People's Hospital in Shayin of Shanxi. The study included 300 patients aged 40-75 years who had histologically confirmed diagnosis of gastric cancer from January 2006 to July 2010. Hospital-based controls were individually matched to cases by gender and age (± 5 years). Controls were patients selected from the Surgical Department, Plastic Surgery Department, ENT Department and Department of Gynecology. Ratio of cases to controls was 1:2. Totally, there were 600 controls who were non-cancer or cancer-free subjects.

A self-administered structured questionnaire consisting of 80 items was used in the study. It included questions about demographic information, dietary and lifestyle habits, smoking and drinking history and so on. Face to face interview was made for all subjects by trained interviewers. A completed questionnaire was obtained from 900 subjects. Cancer patients were asked of habits a year before the disease diagnosed. After interviewing for questionnaires, the salt taste sensitivity test was performed in all the subjects. The salt taste sensitivity threshold (STST) was measured using NaCl solutions on the tip of the tongue with a dropper. Five drops of the test solution were dripped on the tongue. Ten seconds after closing the mouth, the cases and controls who tasted the usual food were perceived. The solutions were offered in increasing concentrations. Between the tests, the subjects were asked to wash their mouths with distilled water at a 30-s interval during the successive tests. The concentrations of each test NaCl solution were classified into ten grades from 0.22 g/L to 58.4 g/L, and the STST value for salt recognition in normal individuals was 0.015 mol/L of NaCl (0.9 g/L) (Table 1).

Table 1 Concentration of sodium chloride *n* (%)

STST score	NaCl concentration		Cases (*n* = 300)	Controls (*n* = 600)
	g/L	mol/L		
1	0.22	0.004	5 (2)	12 (2)
2	0.45	0.008	7 (2)	48 (8)
3	0.90	0.015	24 (8)	108 (18)
4	1.80	0.030	43 (14)	174 (29)
5	3.60	0.060	57 (19)	132 (22)
6	7.30	0.120	76 (25)	60 (10)
7	14.60	0.150	54 (18)	42 (7)
8	29.20	0.500	31 (10)	18 (3)
9	58.40	1	3 (1)	6 (1)

STST: Salt taste sensitivity threshold.

Questions included the frequency of intake of various food. For diet preference, the subjects chose one of the following frequencies: < 3 times/wk and ≥ 3 times/wk. Salty food preference was classified into not salty, medium, and salty. Cigarette smoking was measured in pack-years (number of cigarette smoking per day/20 × smoking time in years) and divided into two categories: smokers who consumed < 40 packs/year and ≥ 40 packs/year or more; alcohol consumption was calculated according to the amount of alcohol consumed per day in grams. The subjects were classified into two categories: drinkers who consumed less than 22.8 g alcohol per day and ≥ 22.8 alcohol per day.

The ethics committee of each collaborating institution reviewed and approved the study, and informed consent was obtained from all the participants.

Statistical analysis

The conditional logistic regression was used to calculate odds ratios (ORs), and corresponding 95% confidence intervals (CI) for gastric cancer in relation to exposure of interest. Two models were examined: (1) none-adjusted; (2) age, sex, smoking, drinking, fresh fruit and fresh vegetables adjusted. Tests for trend were computed by fitting conditional logistic regression model to ordinal values representing levels of exposure. All reported trend test significance levels (*P* values) were two-sided[12]. The relationship between STST score and lifestyle and dietary factors was evaluated by Chi-square test. The coherence of STST score with salty taste preference was detected by Anova test. All the calculations were performed by statistical package version 9, STATA 9, College Station, TX.

Table 2 Odds ratio and 95% CIs for lifestyle- and diet-related factors and gastric cancer *n* (%)

Characteristics	Cases (*n* = 300)	Controls (*n* = 600)	OR (95% CI)	*P* value
Age (yr)	52.1 ± 5.4	52.4 ± 4.9		> 0.05
Male	214 (23.7)	428 (71.3)		
Education				0.12
Literate	96 (32.0)	162 (27.0)	0.79 (0.59-1.06)	
Smoking	156 (52.0)	276 (46.0)	2.27 (1.27-4.04)	< 0.001
Drinking	72 (24.0)	134 (22.3)	2.41 (1.51-3.87)	< 0.001
Fresh vegetable				
< 3 times/wk	99 (33.0)	282 (47.0)	-	< 0.001
⩾ 3 times/wk	201 (67.0)	318 (53.0)	0.92 (0.58-0.98)	
Fresh fruit				
< 3 times/wk	144 (48.0)	219 (36.5)	-	< 0.001
⩾ 3 times/wk	156 (52.0)	381 (63.5)	0.87 (0.67-0.93)	
Salted food(meat and fishes, pickled vegetable)				
< 3 times/wk	120 (40.0)	298 (49.7)	-	
⩾ 3 times/wk	180 (60.0)	302 (50.3)	1.54 (1.15-2.93)	< 0.05
STST score	5.52 ± 1.26	4.4 ± 0.91	1.66 (1.48-1.85)	< 0.001

STST: Salt taste sensitivity threshold. OR: Odds ratio.

Table 3 Relationship between salt taste sensitivity threshold score and lifestyle factors

	n (%)	STST score (mean ± SD)	*P*
Age (yr)			
< 50	224 (24.9)	4.79 ± 1.25	0.76
50-64	543 (60.3)	5.10 ± 0.83	
⩾ 65	133 (14.8)	5.08 ± 1.56	
Smoking (packs/yr)			
< 40	660 (73.3)	4.88 ± 1.02	0.93
⩾ 40	240 (26.7)	5.24 ± 1.51	
Drinking (g/d)			
< 22.8	517 (57.4)	5.10 ± 1.02	0.10
⩾ 22.8	383 (42.6)	5.19 ± 1.12	
Fresh vegetable(times/wk)			
< 3	241 (26.8)	5.14 ± 1.08	0.18
⩾ 3	659 (73.2)	5.07 ± 0.97	
Fresh fruit (times/wk)			
< 3	363 (40.3)	5.18 ± 1.41	0.11
⩾ 3	537 (59.7)	5.07 ± 1.15	
Salted food intake (times/wk)			
< 3	418 (46.4)	4.68 ± 1.32	
⩾ 3	482 (53.6)	5.47 ± 0.98	< 0.001

STST: Salt taste sensitivity threshold.

RESULTS

The characteristics of the subjects are listed in Table 2. Among the 300 cases and 600 controls, 71% were males, and their mean age was 52.1 and 52.4 years, respectively. There was a significant difference in educational level between cases and controls. Smokers and drinkers showed an increased risk of developing gastric cancer with OR (95% CI) = 2.27 (1.27-4.04) and 2.41 (1.51-3.87), respectively. In contrast, consumed protective effect was found in those who took 3 times/wk of fresh vegetable and fruit, OR (95% CI) = 0.92 (0.58-0.98) and 0.87 (0.67-0.93), respectively. The mean STST score of cases was significantly higher than that of controls.

We analyzed the association between STST score and other risk factors. The results showed that STST score was increased with age and duration of smoking and drinking, but no significant association was found (Table 3). STST score was significantly increased with a higher salted food intake.

The mean STST score of all subjects was 4.8 ± 1.1, and the median NaCl concentration was 3.6 g/L (3.6-7.3) or 0.06 (0.06-0.12) mol/L corresponding to a score of 5 (Table 2). There were more patients with STST ⩾ 5 than the controls (Table 4). We defined the STST cut-point as 5 (3.6 g/L or 0.06 mol/L). Subjects with STST ⩾ 5 had 5.71 times greater risk of gastric cancer than those with STST < 5 (Table 4).

The relationship between the salty taste preference and STST score indicated that the salty taste preference was significantly associated with STST score ($P < 0.001$) (Table 5), which means that the subjects with a higher STST score was more likely to prefer a salty taste.

DISCUSSION

The present hospital based case-control study indicated that high consumption of smoking, drinking and salty taste preference elevated the risk of gastric cancer, and that the gastric cancer was associated with a higher STST score. The STST score of 3.6 g/L (0.03 mol/L) was independently associated with a high risk of gastric cancer with the OR (95% CI) of 5.71 (3.18-6.72).

STST is a personal characteristic of an individual and is a useful index to evaluate the salty preference[13]. STST test was used for predicting hypertension in previous studies[14,15], and it indicated that hypertensive individuals were more salt sensitive than the normal individuals[16]. Our study proved that subjects with a higher STST score were more likely to have salty taste preference and a high intake of salty food (Table 4), and it further indicated that STST is a helpful test to evaluate the salty taste preference and

Table 4 Odds ratio and 95% CIs for salt-related factors and gastric cancer *n* (%)

Salt factors	Cases	Controls	OR1 (95% CI)	*P*	OR2 (95% CI)	*P* value
Salted taste preference						
Not salty	118 (39.3)	301 (50.2)	-	-	-	-
Medium	162 (54)	265 (44.2)	1.12 (0.79-1.89)	$P = 0.45$	1.34 (0.92-2.67)	$P = 0.08$
Salty	20 (6.6)	34 (5.7)	1.33 (1.02-1.75)	< 0.05	1.94 (1.37-4.76)	< 0.05
STST⩾ 5	221 (73.7)	258 (43)	4.03 (2.87-5.65)	< 0.001	5.71 (3.18-6.72)	< 0.001

OR1 for salt related factors and gastric cancer was none-adjusted; OR2 was adjusted for age, sex, smoking, drinking, fresh fruit and fresh vegetables; STST: Salt taste sensitivity threshold. OR: Odds ratio.

Table 5 Relationship between salt taste sensitivity threshold score and salt intake *n* (%)

Salty taste preference	STST score						*P*
	1-2	3-4	5-6	7-8	9	Total	
Dislike	60 (6.7)	233 (25.9)	98 (10.9)	26 (2.9)	2 (0.2)	419 (46.6)	$\chi^2 = 220.1$
Not prefer	11 (1.2)	104 (11.6)	206 (22.9)	104 (11.6)	2 (0.2)	427 (47.4)	$P < 0.001$
Like	1 (0.1)	12 (1.3)	21 (2.3)	15 (1.7)	5 (0.6)	54 (6.0)	
Total	72 (8.0)	349 (38.8)	325 (36.1)	145 (16.1)	9 (1.0)	900 (100)	

STST: Salt taste sensitivity threshold.

salt intake. The 24 h urinary excretion of salt was used previously as an objective method for salt intake measurement[17,18], and epidemiologic studies indicated that gastric cancer mortality is weakly or non-significantly correlated with dietary salt measured by the 24 h urinary salt excretion. This method is impracticable for a large-scale population study and case-control study because it can only reflect the situation of 24 h salt intake. However, STST test is simpler, cheaper and more acceptable than the 24 h urinary salt excretion, and patients can identify the taste before diagnosis.

In our study, significantly increased risk for gastric cancer was observed among those with a high STST score. Age, histories of smoking and drinking, and consumption of fruit and vegetables did not show any significant interactions with STST for gastric cancer. STST ⩾ 5 showed a higher risk for gastric cancer in our study, and the possible explanation may be that the high STST is associated with a high intake of salty food such as salted meat or fish (Table 3). Previous studies indicated that ingestion of salt could induce gastritis and co-administration with N-methyl-N-nitro-N-nitrosoguanidine could enhance the effect of gastric carcinogens[9,19], and high intragastric salt concentration could destroy the mucosal barrier, leading to inflammation and damage such as diffuse erosion and degeneration. The induced proliferous change might enhance the effect of food-derived carcinogens.

There are various methods for measuring salt intake and salt preference. Most studies only use the frequency of salt consumption and self-reported salty taste preference, but these methods could not objectively reflect the real situation of the subjects. The mean salt intake varies among different populations, and salt consumption levels which were considered high in one study might be considered low in another one[19], Salt can be derived from different food species, so it is difficult to calculate the total salt consumption from all food. Self-reported salty taste preference is a method often biased by subject's feelings. Therefore, the methods of measuring salt intake from food consumption and self-reported salty taste preference could induce measurement bias and confounding bias. In contrast, STST test is a simple method which is related to salt intake and consumption, and it could indirectly reflect the objective salty preference and avoid the measurement bias.

Several limitations of this study should be considered. Firstly, the STST test was not conducted before the cancer occurred, and the patients may change their salt preference after clinical symptom appearance, so there is recall bias in our study. But as the recall bias could not be avoided in every case-control study, we used the method of STST and the habits were defined to a year before the disease was diagnosed. Secondly, the cases and controls were selected from a hospital, which may have selection bias. We selected controls from the Surgical Department, Plastic Surgery Department, ENT Department and Department of Gynecology. Thirdly, we did not detect the *Helicobacter pylori* (*H. pylori*) infection in both the cases and controls, and *H. pylori* could bias the effect of STST score in gastric cancer, but the *H. pylori* could not reverse the result of STST score in gastric cancer due to the high OR. We will detect the *H. pylori* infection in the future studies. Fourthly, the objective method of measuring salt intake is the 24 h urinary salt excretion, but we did not use it to verify STST in our study. Because 24 h urinary salt excretion could not reflect the previous salt habit, we could only used salt preference questionnaire to verify it. Further cohort studies on the relationship between STST and 24 h urinary salt excretion are needed. Finally, there may be variability in the taste of each subject, and it may induce measurement bias. In order to avoid the bias, we offered the same concentration of NaCl solution to subjects after they chose the usual taste concentration.

To summarize, this study suggests that a high STST score is strongly associated with gastric cancer risk. STST

may be used as a test to evaluate an inherited characteristic of salt preference, and a useful index to verify the salt intake in clinic. However, the role of STST has to be further studied to answer the questions raised from the present study.

COMMENTS

Background

A high intake of salt is hypothesized to be a cause of cancer and an important cause of gastric cancer. But the salt intake and salt preference are hard to measure. salt taste sensitivity threshold (STST) test was once used to predict hypertension, and this study used it to measure the salt intake in an attempt to explore its association with gastric cancer.

Research frontiers

Salt taste sensitivity is independently associated with gastric cancer, and it is proved to be a better index to reflect the salt preference in this study. This method could help identify the risk population of gastric cancer.

Innovations and breakthroughs

This study for the first time explored the relationship between salt taste sensitivity and risk of gastric cancer. A high STST score was found to be strongly associated with gastric cancer risk, and STST score could also reflect the salt preference and salt intake of the subjects, and it may be used for predicting the risk population of gastric cancer.

Applications

STST test is a cheap and fast examination to evaluate an inherited characteristic of salt preference, and it is a simple method to verify the salt intake in clinic.

Peer review

This is an interesting study, and the data suggest that STST may be a potentially useful tool to screen patients at risk of developing gastric cancer.

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S- Editor Tian L **L- Editor** Ma IY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.2054

World J Gastroenterol 2011 April 21; 17(15): 2054-2057
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Laparoscopic repair of hiatal hernia with mesenterioaxial volvulus of the stomach

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Received: August 9, 2010 Revised: September 27, 2010
Accepted: October 4, 2010
Published online: April 21, 2011

Abstract

Although mesenterioaxial gastric volvulus is an uncommon entity characterized by rotation at the transverse axis of the stomach, laparoscopic repair procedures have still been controversial. We reported a case of mesenterioaxial intrathoracic gastric volvulus, which was successfully treated with laparoscopic repair of the diaphragmatic hiatal defect using a polytetrafluoroethylene mesh associated with Toupet fundoplication. A 70-year-old Japanese woman was admitted to our hospital because of sudden onset of upper abdominal pain. An upper gastrointestinal series revealed an incarcerated intrathoracic mesenterioaxial volvulus of the distal portion of the stomach and the duodenum. The complete laparoscopic approach was used to repair the volvulus. The laparoscopic procedures involved the repair of the hiatal hernia using polytetrafluoroethylene mesh and Toupet fundoplication. This case highlights the feasibility and effectiveness of the laparoscopic procedure, and laparoscopic repair of the hiatal defect using a polytetrafluoroethylene mesh associated with Toupet fundoplication may be useful for preventing postoperative recurrence of hiatal hernia, volvulus, and gastroesophageal reflux.

Key words: Gastric volvulus; Mesenterioaxial form; Hiatal hernia; Mesh repair; Laparoscopic procedure

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Inaba K, Sakurai Y, Isogaki J, Komori Y, Uyama I. Laparoscopic repair of hiatal hernia with mesenterioaxial volvulus of the stomach. *World J Gastroenterol* 2011; 17(15): 2054-2057 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/2054.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.2054

INTRODUCTION

Intrathoracic gastric volvulus is an uncommon disease entity characterized by an enlarged esophageal hiatus and weakened gastrosplenic and gastrocolic ligaments[1]. Although gastric volvulus possibly occurs in all ages, it often occurs over the fourth decade of life. If undiagnosed, it can lead to ulceration, perforation, hemorrhage or ischemia of the incarcerated gastrointestinal tract[2]. Two types of gastric volvulus have been recognized: an organoaxial form and a mesenterioaxial form[3,4]. The mesenterioaxial form is characterized by rotation at the transverse axis, extending from the middle of the greater curvature to the porta hepatis.

We recently experienced a case of intrathoracic mesenterioaxial volvulus of the distal portion of the stomach and duodenum, which was successfully treated with laparoscopic repair of the hiatal defect using a polytetrafluoroethylene mesh associated with Toupet fundoplication. The laparoscopic technique employed is described and its therapeutic implications, mesh-related complications, and long-term clinical outcome are discussed.

CASE REPORT

A 70-year-old Japanese woman had been diagnosed with hiatal hernia for several years by her home doctor. Because the patient had complained of no specific symptoms related to the hiatal hernia, no treatment had been undertaken during that period. She had no other past history of any diseases except for a significant kyphosis. She was admitted to our hospital because of the sudden onset of epigastralgia and nausea. Physical examination at admission revealed no abdominal tenderness suggesting diffuse peritonitis. A chest X-ray film showed a large-sized air pocket associated with air-fluid level in the chest, which was likely to be a dilated stomach incarcerated into the intrathoracic cavity (Figure 1). She was dehydrated and vomited during the placement of a nasogastric tube which resulted in aspiration pneumonia. She then presented with acute prerenal failure as a complication, and further treatment was required. Total parenteral nutrition was performed for the management of the intermittent abdominal pain and nausea. The patient responded to intravenous fluid management and the administration of antibiotics. The patient soon recovered from the critically-ill condition and further examinations were performed for precise diagnosis. An upper gastrointestinal series revealed an incarceration of the distal portion of the stomach and the duodenum into the thoracic cavity (Figure 2). An abdominal computed tomography scan also showed an incarcerated upper gastrointestinal tract (Figure 3). These findings were compatible with a mesenterioaxial volvulus of the stomach and duodenum incarcerated into the thoracic cavity through an esophageal hiatus. A laparoscopic approach for the repair of hiatal hernia was selected for complete repair.

During surgery, pneumoperitoneum was established at an intraabdominal pressure of 12 mmHg. A total of 4 ports were placed, 3 ports of 5 mm diameter in the right and left upper abdomen and left upper abdomen and 1 port of 12 mm diameter in the right middle abdomen. The endoscopic port was placed in the umbilicus. The laparoscopic view during the operation showed a hiatal hernia of the sliding type and also showed that the distal portion of the stomach and the proximal portion of the duodenum were rotated at its transverse axis of the upper gastrointestinal tract, and were incarcerated through the porta of the left crus of the diaphragm. The incarcerated distal stomach and proximal duodenum through the esophageal hiatus was covered with the hernia sac. Because there was no adhesion of the herniated content with the surrounding tissues, the incarcerated stomach and duodenum were deliberately pulled out from the thoracic cavity through a relatively large-sized paraesophageal hiatal defect (Figure 4A). The hernia sac and crus of the diaphragm were then dissected. The lower esophagus was also mobilized. The hiatal defect was closed and was reinforced by using a polytetrafluoroethylene (PTFE) mesh (Figure 4B). Toupet fundoplication was then added to prevent gastroesophageal reflux. The fundic wrap was sutured with the right crus and left-sided diaphragm with interrupted sutures. No intra- and/or postoperative complications were observed. Postoperative upper gastrointestinal series performed 5 d after surgery revealed appropriate arrangement of the upper gastrointestinal tract and no sign of recurrence of the hiatal hernia (Figure 5).

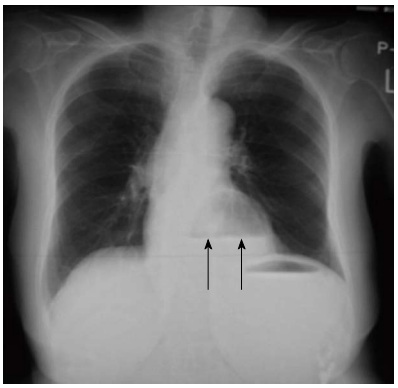

Figure 1 A chest X-ray film at admission, revealing air in the dilated stomach associated with air-fluid level (arrows), which was dislocated into the chest.

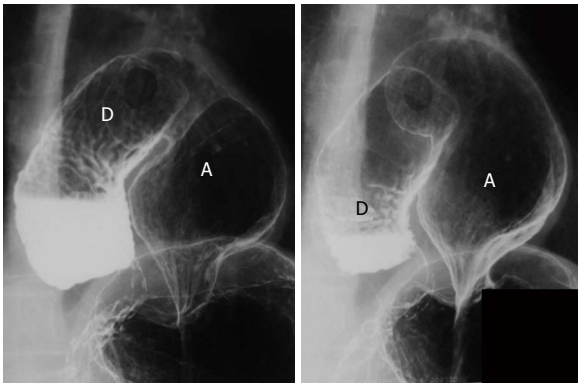

Figure 2 An upper gastrointestinal series performed preoperatively, revealing dislocated distal portion of the stomach (A) and duodenum (D) into the thoracic cavity rotated in the mesenteric axis, which corresponded to a mesenterioaxial volvulus of the upper gastrointestinal tract.

The postoperative course was uneventful and the patient was discharged from the hospital 15 d after surgery. The patient was in good condition, and complained of no symptoms suggesting the recurrence of hiatal hernia and/or intrathoracic gastric volvulus for 4 years after surgery. The patient has undergone annual check-ups using upper gastrointestinal endoscopic examinations for 4 years after surgery and no recurrence has been found to date.

DISCUSSION

Although hiatal hernia is commonly encountered, hiatal hernia with mesenterioaxial intrathoracic gastric volvulus is extremely uncommon. Two types of gastric volvulus have been recognized: an organoaxial form and a mesenterioaxial form[3,4]. The organoaxial type is a common type of gastric volvulus, in which the stomach rotates on a vertical axis. This type of gastric volvulus is usually caused by eventuation, diaphragmatic hernia, pyloric obstruction, adhesions, or enlarged esophageal hiatus. This is also described as an intrathoracic stomach or upside down stomach. Another type is the mesenterioaxial form. In this form, the stomach

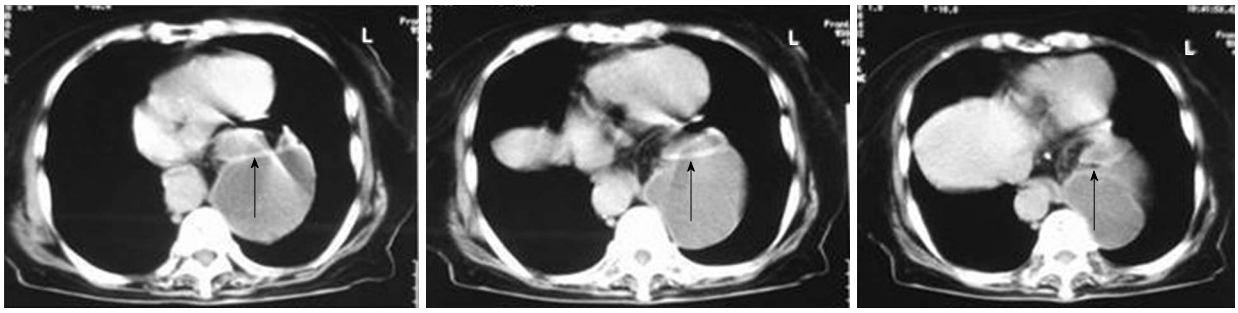

Figure 3 Abdominal computed tomography scan. An incarcerated upper gastrointestinal tract in the thoracic cavity (arrows). The gastrointestinal tract dislocated in the thoracic cavity was significantly dilated, where fluid had accumulated.

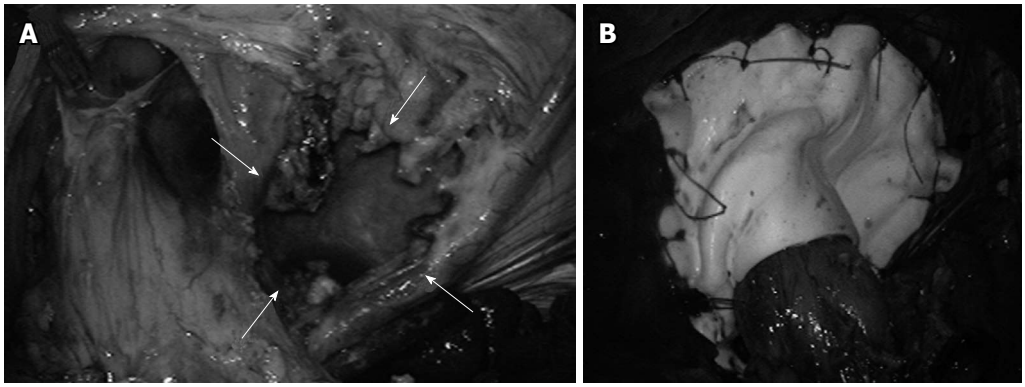

Figure 4 Laparoscopic view after initial reduction of the stomach from the chest into the abdomen. Sliding hernia and parahiatal hernia. A: A paraesophageal aperture (arrows) was large-sized and was located at the left side of the left crus of the esophageal hiatus; B: Hernia orifice was closed using the mesh reinforcement.

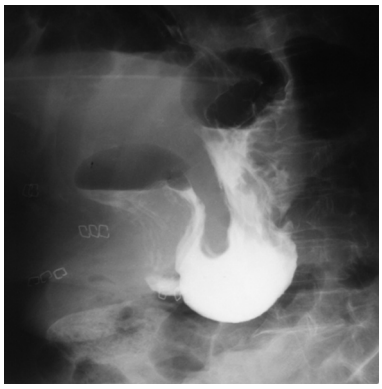

Figure 5 A postoperative upper gastrointestinal series performed at 5 d after surgery, showing the normal intraperitoneal location of the stomach and duodenum.

rotates on a horizontal axis, which extends from the middle of the greater curvature to the porta hepatis. The rotated stomach is located in the chest from the hiatal defect. Mesenterioaxial volvulus is even more uncommon and represents about 29% of all torsions occur in the stomach. Of mesenterioaxial volvulus, the idiopathic pattern was 37%[3].

In the present case, the patient had gastrointestinal obstruction, dehydration and acute prerenal failure caused by the volvulus of the stomach. Elective surgery was performed after recovering from these complications. In fact, emergency surgery is occasionally required for cases with acute gastric obstruction associated with strangulation due to gastric volvulus[5]. If there is no gastrointestinal ischemia and/or necrosis requiring emergency surgery, placement of a nasogastric tube may be useful for the effective decompression of the stomach, which may allow a reduction of the volvulus and thereby enabling elective surgery.

Treatment of gastric volvulus has classically involved reduction of the stomach, gastropexies in its normal intra-abdominal position, and correction of the associated diaphragmatic defect[4]. The laparoscopic repair of intrathoracic gastric volvulus and large paraesophageal hernias has been proved to be feasible and safe[4,6-9]. Previously published reports have confirmed the significant benefits of the minimally-invasive approach in these often elderly and/or debilitated patients[7]. Therefore, laparoscopic repair of gastric volvulus is best suited as a minimally-invasive approach as long as the precise repair of hiatal aperture and reinforcement are added.

In the present case, Toupet fundoplication was added to prevent gastroesophageal reflux because a simple reduction of the volvulus associated with gastropexy has been shown to be unsatisfactory[10]. A concomitant antireflux procedure has been recommended[8]. The fundoplication is mandatory as it prevents reflux because of the extensive dissection at the hiatus, and provides a good anchor for the repair. This approach is considered to be safe and effective, and it also provides for rapid recovery from the operation.

In the present case, neither mesh-related complications

nor recurrence of hiatal hernia was found for 4 years after surgery. It has been reported that primary laparoscopic hiatal hernia for paraesophageal hernia is associated with up to a 42% recurrence rate[11,12]. The most important reason is the absence of strong fascia at the hiatal aperture leading to suture pullout[13]. We decided to use synthetic mesh reinforcement for the crural repair, because direct suturing of the aperture of paraesophageal defect was difficult due to the size and the absence of strong fascia. Although mesh repair improved the recurrence rate[14], the use of mesh for the repair of hiatal hernia has still been controversial because mesh-related complications have frequently reported. Several clinical studies with significant patient numbers have demonstrated the safety and effectiveness of synthetic mesh reinforcement for the repair of hiatal hernia[14,15,16]. An improved recurrence rate has been reported in cases with the use of mesh for the tension free crural repair[17]. However, some surgeons have pointed out the risk of erosion into the esophageal or gastric lumen caused by placing the mesh[15,11,18]. Because several types of non-absorbable mesh have recently been used for the repair of hiatal hernia[9,19], further evaluation of clinical outcome may be required. Stadlhuber *et al*[17] analyzed 28 cases of mesh-related complications in patients undergoing laparoscopic or open hiatal closure. They demonstrated that 23 cases required reoperation, and surprisingly 7 patients required esophagectomy. Among the patients who required reoperation, PTFE was used in 12 cases for the hiatal repair. A total of 10 patients had mesh intraluminal erosion, 2 patients had dense hiatal fibrosis. Although there is no apparent relationship between mesh type and configuration with the complications, they suggested that it may be due both to the technical aspects of mesh placement and to the type of mesh material used[17]. Nonetheless, they concluded that complications related to synthetic mesh placement at the esophageal hiatus are more common than previously reported and that further studies are needed to determine the best method and type of mesh for implantation.

In summary, we experienced a case with mesenterioaxial volvulus of the stomach and duodenum associated with hiatal hernia, which was successfully treated with complete laparoscopic repair of the large-sized hiatal defect using a PTPE mesh associated with Toupet fundoplication. These laparoscopic procedures are safe and useful to obtain short-term as well as long-term clinical outcomes of patients with a large-sized hiatal hernia. Further accumulation of cases is required to precisely determine the best method and type of mesh for reinforcement and long-term clinical outcome.

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S- Editor Sun H **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.2058

World J Gastroenterol 2011 April 21; 17(15): 2058-2060
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Treatment of advanced rectal cancer after renal transplantation

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Received: January 6, 2011 Revised: February 15, 2011
Accepted: February 22, 2011
Published online: April 21, 2011

Abstract

Renal transplantation is a standard procedure for end-stage renal disease today. Due to immunosuppressive drugs and increasing survival time after renal transplantation, patients with transplanted kidneys carry an increased risk of developing malignant tumors. In this case report, 3 patients with advanced rectal cancer after renal transplantation for renal failure were treated with anterior resection or abdominoperineal resection plus total mesorectal excision, followed by adjuvant chemotherapy. One patient eventually died of metastasized cancer 31 mo after therapy, although his organ grafts functioned well until his death. The other 2 patients were well during the 8 and 21 mo follow-up periods after rectal resection. We therefore strongly argue that patients with advanced rectal cancer should receive standard oncology treatment, including operation and adjuvant treatment after renal transplantation. Colorectal cancer screening in such patients appears justified.

Key words: Rectal cancer; Renal transplantation; End-stage renal disease; Treatment; Screening

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Liu HY, Liang XB, Li YP, Feng Y, Liu DB, Wang WD. Treatment of advanced rectal cancer after renal transplantation. *World J Gastroenterol* 2011; 17(15): 2058-2060 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/2058.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.2058

INTRODUCTION

Renal transplantation, commonly performed for end-stage renal disease (ESRD), is an alternative to dialysis. An increased incidence of malignancy in transplant recipients is well recognized, which may be related to impaired immunosurveillance, direct neoplastic action of immunosuppressive agents, oncogenic viruses such as Epstein-Bar virus or cytomegalovirus, and chronic antigenic stimulation, uremia, or genetic predisposition[1].

There is evidence that renal transplant recipients are approximately three times more likely to develop cancer than the general population[2,3]. Their risks vary in different tumors. The risk of Kaposi's sarcoma is the highest (200 times increased risk) followed by that of non-melanocytic and melanocytic skin cancer (9-20 times increased risk)[2,3]. The risk other solid-organ cancers, such as colorectal cancer, is increased by approximately 2-3 times higher in renal transplant recipients than in general population[3,4]. Cancers occurring in transplanted patients are generally de novo and mainly diagnosed after the third year with an increase after 10 years[5]. The mean onset time of colorectal malignancies is 10.4 years[1].

The prognosis of renal transplant recipients with advanced-stage cancer is extremely poor. Currently, posttransplant malignancies are an important cause of mortality and the leading reason of death within the next 20 years[6]. Immunosuppression and late diagnosis have been implicated[7]. We report 3 cases of advanced rectal cancer after renal transplantation for renal failure.

CASE REPORT

In the past 5 years, 3 male patients at a mean age of 55.3 years who developed rectal cancer (RC) after renal transplantation were diagnosed and treated in Shanxi Cancer Hospital (Taiyuan, China). The mean elapsed time from renal transplantation to development of RC was 6.5 years. The 3 patients who underwent anterior resection (AR) or abdominoperineal resection (APR) plus total mesorectal excision (TME) had an uneventful postoperative course. The clinical data about each patient are listed in Table 1.

Case 1

A 68-year-old man who underwent renal transplantation for ESRD due to hydropigenous nephritis in 1993 at the age of 54 years. He received a second left renal transplantation in 2002 for graft failure followed by immunosuppressive therapy. In 2007, he underwent colonoscopy for rectal bleeding and dyschezia, which revealed a 5.0 cm mass at the upper rectum with 80% luminal occlusion. Biopsy showed a well-differentiated adenocarcinoma. Laboratory tests showed that his preoperative CEA level was 26.20 μg/L and his cellular immune function was low. The patient underwent AR with TME. Pathologic examination revealed a 5.0 cm moderately-differentiated adenocarcinoma with invasion through the serous membrane. Of the removed 7 lymph nodes, 1 was positive ($pT_4N_1M_0$). The postoperative course was uneventful. Following the operation, the patient did not receive any chemotherapy and radiotherapy due to his refusal. In November 2009, he received 3 cycles of Xeloda after liver and lung metastasis was discovered. The patient died in March, 2010.

Case 2

A 44-year-old man who received immunosuppressive therapy for ESRD in 2004 after renal transplantation. In 2009, he underwent colonoscopy for heme-positive stools, which revealed a 4 cm mass at the rectum with 60% luminal occlusion. Biopsy showed an adenocarcinoma of the rectum. Laboratory tests showed that his preoperative CEA level was 0.04 μg/L and his cellular immune function was low. The patient underwent APR with TME. Pathologic examination revealed a 4.0 cm poorly-differentiated adenocarcinoma with invasion of the adjacent perirectal fatty tissues. Eight lymph nodes were found with no malignant lymph node involved ($pT_3N_0M_0$). The patient was recovered uneventfully. He received a course of Xeloda and was well during the 21-mo follow-up period.

Case 3

A 54-year-old man with chronic pyelonephritis who underwent renal transplantation for ESRD in 2009. After the operation, he received immunosuppressive therapy. In 2010, 6 mo after renal transplantation, the patient presented with diarrhea and rectal bleeding. Colonoscopy showed a rectal mass with 70% luminal occlusion and a pedunculated polyp in sigmoid colon which was excised by electrocautery. Histology of the rectal mass showed a well-differentiated adenocarcinoma of the rectum while histology of the polyp suggested a tubular adenoma. Laboratory tests showed that his preoperative CEA level was 1.05 μg/L and his cellular immune function was low. The patient underwent APR with TME. Pathologic examination revealed a 5 cm moderately-differentiated adenocarcinoma and partial mucinous adenocarcinoma with invasion of the pericolonic adipose tissue. Thirteen lymph nodes were found with no lymph node metastasis ($pT_3N_0M_0$). Cancer emboli were identified in vessels of the mass. He received a course of Xeloda and recovered uneventfully during the 8-mo follow-up period.

DISCUSSION

It has been shown that the incidence of cancer is significantly higher in patients who underwent renal transplantation than in those who did not undergo renal transplantation[2,3]. For example, the risk of colorectal cancer is increased by approximately 2-3 times higher in patients than in general population[3,4]. However, some of the tumors may arise in renal recipients without any relation with renal transplantation, because they might have already presented at the time of renal transplantation but not detected. As a general rule, tumors detected within the first 12 mo after renal transplantation are considered pre-existed. Such patients should be excluded from the "*de novo*" group.

In our study, the 3 RC patients who underwent renal transplantation were males, and the onset interval from renal transplantation was 14 years, 5 years and 0.5 year, respectively. One patient was diagnosed with RC within the first 12 mo after renal transplantation, and pathological stage was $pT_3N_0M_0$ (advanced cancer). The other 2 patients were diagnosed with post-transplantation RC with a mean onset interval of 9.5 years. The 3 patients had radical AR and APR with TME.

It is well known that immunosuppressive treatment increases the incidence of cancers, which is supported by the fact that the incidence of tumors is higher in patients treated with immunosuppressants following renal transplantation due to chronic renal failure than in normal population. The causes for this difference might be explained by the immunological abnormalities induced by immunosuppressants[8-10]. Therefore, screening and early diagnosis of tumors are essential both before and after renal transplantation, which means that tumors, if existed, should be detected, thus unnecessary renal transplantation can be avoided. Furthermore, annual tumor screening after renal transplantation should be conducted so that treatment can be commenced at an early stage of malignancy. In our study, the patient who was diagnosed with advanced RC within 6 mo after renal transplantation had no tumor screening before renal transplantation. It is likely that he developed RC while he was on dialysis and waiting for renal transplantation.

In general, the prognosis of transplant recipients who develop malignancy following immunotherapy are poor due to delayed diagnosis[6,8,11-14]. Most cancers are at advanced stages when they are diagnosed, and usually progress rapidly with more than 50% of such patients died within the first year of diagnosis[15]. The average survival time is proximally 25.8 mo[15]. Evidence from National Cancer Institute Surveillance Epidemiology and End Results Database suggests

Table 1 Parameters of patients with rectal cancer after renal transplantation

Age (yr)	Elapsed time (yr)	Location	Grade	pTNM	Operation	Screening	Outcome
68	14	Rectum	Moderate	$pT_4N_1M_0$	AR + TME	No	Died after 31 mo
44	5	Rectum	Poor	$pT_3N_0M_0$	APR+TME	No	Alive after 21 mo
54	0.5	Rectum	Moderate to poor	$pT_3N_0M_0$	APR + TME	No	Alive after 8 mo

that transplant patients develop colorectal cancer at a younger age (58 *vs* 70 years, $P < 0.001$) and have a worse 5-year survival rate than the general population (overall, 44% *vs* 62%, $P < 0.001$; Dukes A and B, 74% *vs* 90%, $P < 0.001$; Dukes C, 20% *vs* 66%, $P < 0.001$; and Dukes D, 0% *vs* 9%, $P = 0.08$) mainly due to chronic immunosuppression which results in a more aggressive tumor biology[16]. RC patients who underwent renal transplantation usually develop more advanced (AJCC stage > Ⅱ) colon cancer with a worse disease-specific survival rate (all stages) than those who did not undergo renal transplantation. Multivariate analyses showed that renal transplantation is a negative risk factor for survival, and cancer stage at diagnosis is the most profound negative survival predictor[17], indicating that colorectal cancers in transplant recipients are biologically more aggressive, thus resulting to a worse prognosis in such patients than in general population. Moreover, Ho *et al*[7] also highlighted that immunosuppression and late diagnosis should be blamed for the poor prognosis of colorectal cancer patients after renal transplantation. In the present study, of the 3 patients with advanced rectal cancer, 1 died of multiple liver and lung metastases 31 mo after operation, indicating that frequent colorectal cancer screening should be warranted after renal transplantation.

Zittel *et al*[18] reported a case of a 48-year-old patient who developed advanced RC 6.5 years after pancreas-kidney-transplantation for type I diabetes. The patient received neo-adjuvant radio and chemotherapy followed by low anterior rectal resection with total mesorectal excision. Within the next thirteen months, he underwent consecutive resections for a solitary hepatic metastasis, a solitary pulmonary metastasis and a chest wall metastasis. The patient eventually died of metastasis 32 mo after the initial therapy although the organ grafts functioned well until his death, suggesting that although a higher degree of morbidity might be encountered, transplantation patients should receive standard oncology treatment, including neo-adjuvant therapy, if their general condition is good and the organ graft functions well.

In conclusion, early prevention, detection and treatment of malignancies after renal transplantation are the important management strategies for improving the survival time and quality of life of cancer patients because malignancies develop more frequently in cancer patients after renal transplantation than in general population. Surgical resection is still the first choice of treatment which is a safe procedure when indicated. However, if the patients have an advanced disease (local or metastatic), the standard oncology treatment, including neo-adjuvant treatment can be used.

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S- Editor Tian L **L- Editor** Wang XL **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i15.2061

World J Gastroenterol 2011 April 21; 17(15): 2061-2062
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Pancreatic hyperechogenicity on endoscopic ultrasound examination

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Received: November 15, 2010 Revised: February 15, 2010
Accepted: December 22,2010
Published online: April 21, 2011

Abstract

There is an ongoing discussion on how to diagnose a hyperechogenic pancreas and what is the clinical significance of diffusely hyperechogenic pancreas. Computerized tomography and magnetic resonance imaging are the more appropriate methods to diagnose pancreatic hyperechogenicity when compared with transcutaneous or endoscopic ultrasound examination. More importantly, pancreatic hyperechogenicity may not be a certain indicator of pancreatic fat infiltration. Even if it is true, we do not know the clinical significances of pancreatic fat accumulation. Some suggested that excess fat in the pancreas is associated with chronic pancreatitis. However, several histological studies on human alcoholic chronic pancreatitis did not prove the presence of fatty pancreas in such cases. Thus, except for aging, it is very rare to have truly steatotic pancreas in the absence of certain human diseases.

Key words: Hyperechogenic pancreas; Fatty pancreas; Endoscopic ultrasound; Aging; Chronic pancreatitis

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Ustundag Y, Ceylan G, Hekimoglu K. Pancreatic hyperechogenicity on endoscopic ultrasound examination. *World J Gastroenterol* 2011; 17(15): 2061-2062 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i15/2061.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i15.2061

TO THE EDITOR

We read with interest the article by Choi *et al*[1] entitled "Associated factors for a hyperechogenic pancreas (HP) on endoscopic ultrasound examination". The authors investigated the risk factors for hyperechogenic pancreas on endoscopic ultrasound (EUS). Their study group included 53 cases of HP and the control group consisted of 79 cases having various indications for endosonographic examination with normal pancreas echogenicity on EUS. They noted that HP was significantly associated with fatty liver, male gender, age older than 60 years, hypertension and visceral adipose tissue area (cm^2).

Pancreatic fat, readily observed on EUS, is only suspected when an overt HP is noted. However, as the authors in their study noted, mild hyperechogenic pancreas with respect to liver is a normal finding on ultrasound examination. Since the quantitative analysis of pancreatic parenchymal echogenicity was not conducted in their study, how could the authors be sure that pancreatic echogenicity they saw can be "hyper"? The authors also indicated the limitations of their study as the absence of direct determination of the pancreatic fat and visceral fat in pancreatic tissue. It would be unethical to get pancreatic biopsy samples. However, they could estimate the pres-

ence of pancreatic steatosis with the help of computerized tomography (CT) imaging which was already done to estimate the visceral adipose tissue area in all cases in their study. CT can be very helpful for the diagnosis and quantification of the existence of pancreatic steatosis[2].

We also do not know the clinical consequences of pancreatic steatosis as yet. Some epidemiologic data suggest that obesity is a risk factor for pancreatic cancer development[3] and that obese patients develop more severe pancreatitis than lean individuals[4]. Furthermore, postoperative fistula develops more commonly in obese subjects than in lean individuals[5]. However, in the absence of regular alcohol consumption, the obese patients with increased visceral adiposity are not accepted as having increased risk for chronic pancreatitis. We know that diffusely increased parenchymal echogenicity has not been suggested to be a EUS finding associated either with early or with late stage chronic pancreatic inflammation. Unlike the current evidence for the association between fatty liver and steatohepatitis, there is no similar evidence as yet to suggest that steatotic pancreas progresses to pancreatohepatitis and then to chronic pancreatitis. Indeed, pancreatic hyperechogenicity may not be a certain indicator of pancreatic fat infiltration. The belief that hyperechogenicity of the pancreas indicates the presence of fat in this organ has now been largely abandoned[6]. Moreover, several histological studies on human alcoholic chronic pancreatitis did not support the presence of fatty pancreas[7-9].

Thus, it would not be appropriate to diagnose diffuse HP solely on EUS, but CT or Magnetic resonance imaging imaging would be more reliable for such a diagnosis. More importantly, we need to clarify what is the clinical importance of HP on EUS. We are even not sure that HP represents pancreatic steatosis. Even if it does, we do not know the clinical consequences of pancreatic steatosis.

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S- Editor Sun H **L- Editor** Wang XL **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
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www.wjgnet.com

World J Gastroenterol 2011 April 28; 17(16): 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 April 21; 17(15): 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

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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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www.wjgnet.com

World J Gastroenterol 2011 April 21; 17(15): 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, 2009 Impact Factor: 2.092 (33/65 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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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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