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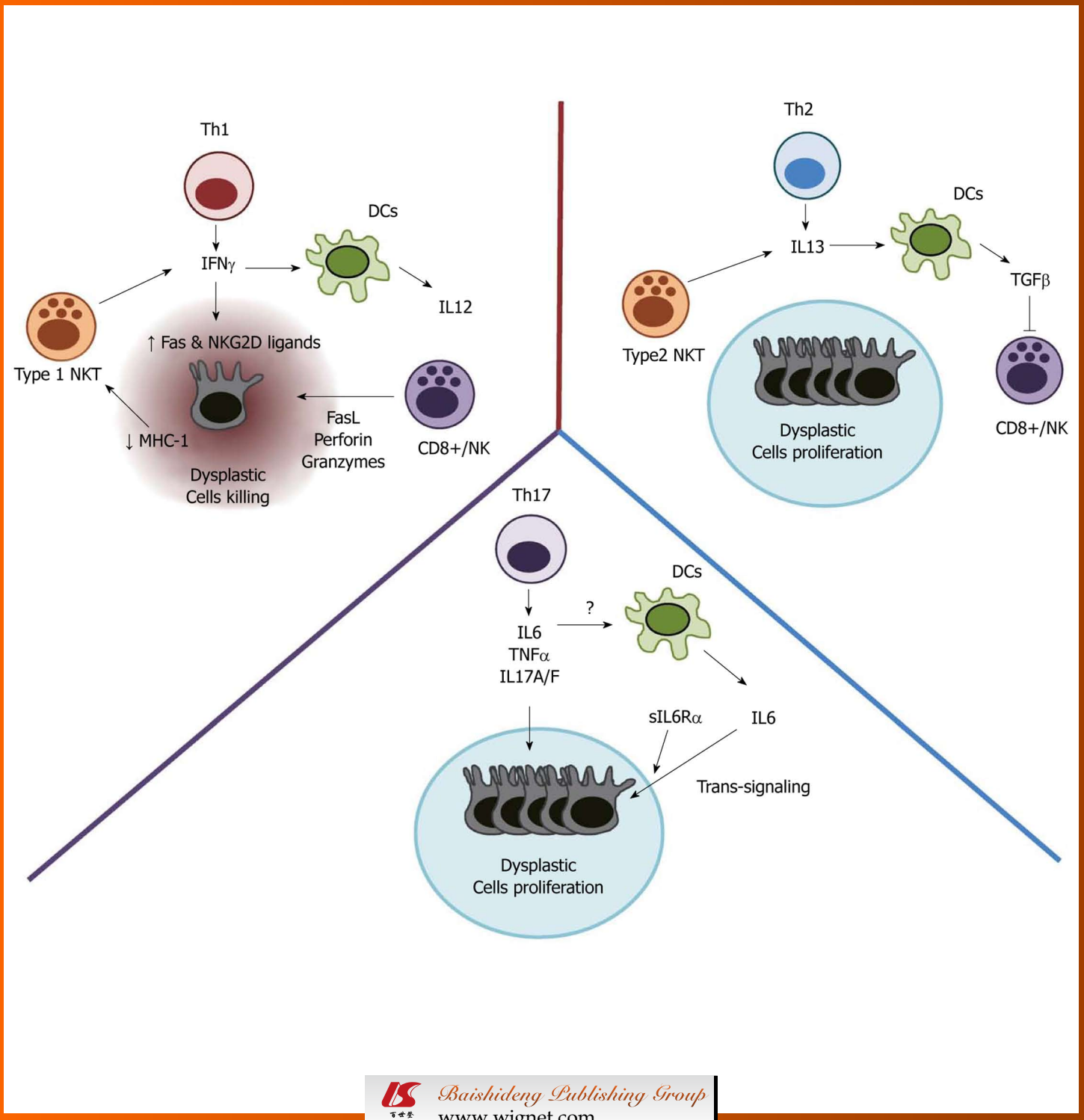

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EDITORIAL

Anti-angiogenesis in hepatocellular carcinoma treatment: Current evidence and future perspectives

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Abstract

Hepatocellular carcinoma (HCC) is among the most common cancer diseases worldwide. Arterial hypervascularisation is an essential step for HCC tumorigenesis and can be targeted by transarterial chemoembolization (TACE). This interventional method is the standard treatment for patients with intermediate stage HCC, but is also applied as "bridging" therapy for patients awaiting liver transplantation in many centers worldwide. Usually the devascularization effect induced by TACE is transient, consequently resulting in repeated cycles of TACE every 4-8 wk. Despite documented survival benefits, TACE can also induce the up-regulation of proangiogenic and growth factors, which might contribute to accelerated progression in patients with incomplete response. In 2007, sorafenib, a multi-tyrosine kinase and angiogenesis inhibitor, was approved as the first systemic treatment for advanced stage HCC. Other active targeted compounds, either inhibitors of angiogenesis and/or growth factors, are currently being investigated in numerous clinical trials. To overcome revascularisation or tumor progression under TACE treatment it seems therefore attractive to combine TACE with systemic targeted agents, which might theoretically block the effects of proangiogenic and growth factors. Over the last 12 mo, several retrospective or prospective cohort studies combining TACE and sorafenib have been published. Nevertheless, robust results of the efficacy and tolerability of such combination strategies as proven by randomized, controlled trials are awaited in the next two years.

Key words: Hepatocellular carcinoma; Sorafenib; Anti-angiogenesis; Transarterial chemoembolization

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INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) is rising with a world-wide annual incidence above 600 000[1]. Treatment of HCC is challenging because HCC mainly occurs within liver cirrhosis[1], and therapy options and prognosis are determined by tumor biology as well as impaired liver function. Several clinical staging systems have been proposed[2]. However, the most commonly used in Western countries is the Barcelona Clinic Liver Cancer (BCLC) system[3,4]. According to this algorithm, treatment is stratified according to tumor stage, liver function, and performance status. Intermediate stage HCC (BCLC stage B) without options for surgical treatment or ablation is treated by transarterial chemoembolization (TACE). TACE has been shown to expand median survival from 16 to 19-20 mo[5,6]. In patients with advanced (BCLC stage C) and especially end-stage HCC (BCLC stage D), survival depends not only on progression of

tumor disease but depends incremental on accompanying liver dysfunction, also. Without intervention, survival of patients with advanced HCC rarely exceeds 6 mo, and median survival in patients with end-stage HCC (BCLC stage D, Okuda stage Ⅲ, performance status 3-4) is commonly below 3-4 mo[4,7-9]. According to the modified BCLC system, the dual kinase inhibitor sorafenib is considered the standard of care for patients with advanced HCC[10]. However, the survival benefit is limited to approximately 3 mo, whereas disease stabilization can be achieved in 27%-78% as shown in prospective trials[11-14].

Typically, HCC is a hypervascularized tumor with characteristic early arterial enhancement during dynamic imaging, which is the rational for TACE. By TACE, however, mainly central vessels of a tumor nodule are occluded, while progression may occur via neovascularization in the tumor periphery. In theory, this might be prevented or at least attenuated by concomitant systemic treatment with anti-angiogenic agents (Figure 1).

ANGIOGENESIS IN PATHOGENESIS OF HEPATOCELLULAR CARCINOMA

Chronic hepatitis and hepatic fibrogenesis are closely connected to angiogenesis[15]. Different cytokines, growth factors, and metalloproteinases are involved in these processes. Vascular endothelial growth factor (VEGF) was shown to be crucially involved in angiogenesis as well as fibrogenesis[15]. Despite other factors, hepatic tissue hypoxia seems to be a relevant trigger for angiogenesis in necroinflammatory liver disease, especially by induction of VEGF, resulting in increasing arterial contribution to hepatic perfusion[16,17]. At this stage, the majority of neo-vessels originate from the portal vein, supporting short-circuits between the portal vein system and the hepatic veins[16,18]. Despite the predominant occurrence of HCC in liver cirrhosis rather than in non-cirrhotic liver disease[1], it is still unknown whether HCC arises from hepatic stem cells or from hepatocytes *via* malignant transformation. The latter concept is supported by the observation that development of HCC from dysplastic nodules has been described[19,20]. Arterial hypervascularization seems to be pathognomonic for established HCC, and HCC nodules larger than 2 cm regularly show arterial enhancement[21,22]. Therefore, neovascularization seems to be crucial for HCC tumorigenesis.

Consistently, increased expression of angiopoietin-1/-2 mRNA in tumor tissue was reported, suggesting a critical role of neo-vascularisation for HCC pathogenesis[23]. Moreover, augmented expression of VEGF was found in HCC, and higher serum VEGF levels were associated with poor prognosis of patients with HCC[24-29]. In contrast, a recent study showed that neither VEGF-A nor VEGFR were up-regulated in HCC tissue, and angiotensin-1/-2 expression were only modestly changed[30]. Of note, sinusoidal capillarization suggesting vascular remodeling was observed within the same study[30]. These inconsistent data further highlight that tumor angiogenesis is a complex process and most likely heterogeneous. The angiopoietin/VEGF system seems to play an important role in angiogenesis of HCC, but other, yet incompletely understood pathways may also be involved.

THERAPEUTIC INHIBITION OF ANGIOGENESIS IN HEPATOCELLULAR CARCINOMA

Inhibition of angiogenesis is an established and successful treatment strategy in a variety of malignant diseases. The liver is predominantly supplied by the portal venous system, whereas HCC nodules are characterized by typical arterial hypervascularization. This accounts for the rationale for use of hypervascularization as a diagnostic criterion as well as development of angiogenesis inhibition treatment strategies. In the absence of targeted agents, embolization of arterial tumor vessels was established in the 1980s. Currently, TACE is commonly used in patients with HCC BCLC stage 0/A as bridging therapy until liver transplantation and as non-curative therapy in patients with HCC BCLC stage B and C[6].

Indeed, TACE may lead to reduction of tumor vascularization and viable tumor volume[6]. Recently, this has also been confirmed for a modified TACE technique using doxorubicin eluting beads (DEB)[31]. Furthermore, VEGF levels as a surrogate marker for angiogenesis were shown to correlate with therapeutic outcome after TACE. Pretreatment VEGF levels were significantly higher in patients not responding to TACE compared to patients with disease stabilization. Moreover, pretreatment VEGF serum levels > 240 pg/mL were an independent prognostic factor for survival[32].

It has been suggested that tumor progression after TACE may be caused by activation of angiogenesis due to TACE-induced hypoxemia[33]. Plasma VEGF levels were shown to increase shortly after TACE, reaching a peak value one day after TACE[34-37]. Additionally, increase of plasma VEGF levels after TACE was correlated with the development of metastasis and a reduced progression free survival[35,37]. Unfortunately, reliable biomarkers predicting response to TACE are missing. Nevertheless, a median survival of 35 mo has been reported in patients with complete tumor response[38]. In this study low VEGF levels were associated with a longer survival, while higher VEGF levels were detectable in patients without tumor response. Of note, prior TACE was reported to induce angiogenesis in surgical specimens, whereas patients who underwent surgery without prior TACE had no induction of angiogenesis[39]. Whether the use of DEB-TACE, which can induce higher rates of tumor response, also leads to upregulation of proangiogenic factors is under debate[40,41].

Sorafenib, the first systemically agent approved for HCC, is a multikinase inhibitor with activity against VEGFR2, PDGFR, c-Kit receptors, b-RAF, and p38[42], signal transduction pathways which seem to be involved in pathogenesis of HCC[43]. However, there are limita-

Table 1 Efficacy of systemic targeted monotherapy in hepatocellular carcinoma according to current phase Ⅰ-Ⅲ studies

Author	Year	Phase	Investigational drug	*n*	RR	DS	PFS/TTP	PFS-6m	OS
O'Neil *et al*[59][1]	2009	Ⅱ	AZD 6244	16	0	37.5	NR	NR	NR
Malka *et al*[60]	2007	Ⅱ	Bevacizumab	30	12.5	54	3.5/NR	17	NR
Schwartz *et al*[61]	2006	Ⅱ	Bevacizumab	30	6.7	57	NR/6.4	NR	NR
Siegel *et al*[58]	2008	Ⅱ	Bevacizumab	46	13	NR	6.9/NR	NR	12.4
Raoul *et al*[62]	2009	Ⅱ	Brivanib	55	11	10	NR/2.8	NR	10
Gruenwald *et al*[63]	2007	Ⅱ	Cetuximab	27	0	44	2.0/1.9	22.2	NR
Zhu *et al*[64]	2007	Ⅱ	Cetuximab	30	0	17	1.4/NR	NR	9.6
Philip *et al*[65]	2005	Ⅱ	Erlotinib	38	9	50	3.2/NR	32	13
Thomas *et al*[66]	2007	Ⅱ	Erlotinib	40	0	43	3.1/NR	NR	6.25 (10.75)[2]
Blaszkowsky *et al*[67]	2010	Ⅲ	Everolimus	25	4	44	3.8/3.9	8%	8.4
O'Dwyer *et al*[68]	2006	Ⅱ	Gefitinib	31	3	22.5	2.8/NR	NR	6.5
Lin *et al*[69]	2008	Ⅱ	Imatinib	15	0	13.3	NR/NR	NR	NR
Ramanathan *et al*[70]	2006	Ⅱ	Lapatinib	37	5	35	2.3/NR	2.3	6.2
Rizell *et al*[71]	2008	Ⅱ	Sirolimus	21	4.8	23.8	NR/NR	NR	6.5
Abou-Alfa *et al*[12]	2006	Ⅱ	Sorafenib	137	2.2	33.6	NR/4.2	NR	9.2
Cheng *et al*[72]	2009	Ⅲ	Sorafenib	226 (150 treated)	3.3	54	NR/2.8	NR	6.5
Furuse *et al*[13]	2008	Ⅰ	Sorafenib	27	4	83	NR/4.9	46.2	15.6
Llovet *et al*[11]	2008	Ⅲ	Sorafenib	602 (299 treated)	2	71	NR/5.5	NR	10.7
Yau *et al*[14]	2009	Ⅱ	Sorafenib	51	8	18	3.0/NR	NR	5
Zhu *et al*[73]	2009	Ⅱ	Sunitinib	34	2.9	47	3.9/4.1	NR	9.8
Faivre *et al*[74]	2009	Ⅱ	Sunitinib	37	2.7	35	3.7/5.3	NR	8
Hoda *et al*[75]	2008	Ⅱ	Sunitinib	23	6	35	NR/NR	NR	NR
Koeberle *et al*[54]	2010	Ⅱ	Sunitinib	45	2	40	2.8/2.8	NR	9.3
Kanai *et al*[57]	2010	Ⅰ/Ⅱ	TSU-68	35	8.6	42.8	NR/2.1	NR	13.1

[1]Trial stopped; [2]Recorded from therapy start (recorded from diagnosis). DS: Disease stabilization (%); NR: Not reported; OS: Overall survival (mo); PFS/TTP: Progression free survival/time to progression (mo); PFS-6m: Progression free survival rate at 6 mo (%); RR: Response rate [complete + partial response (%)].

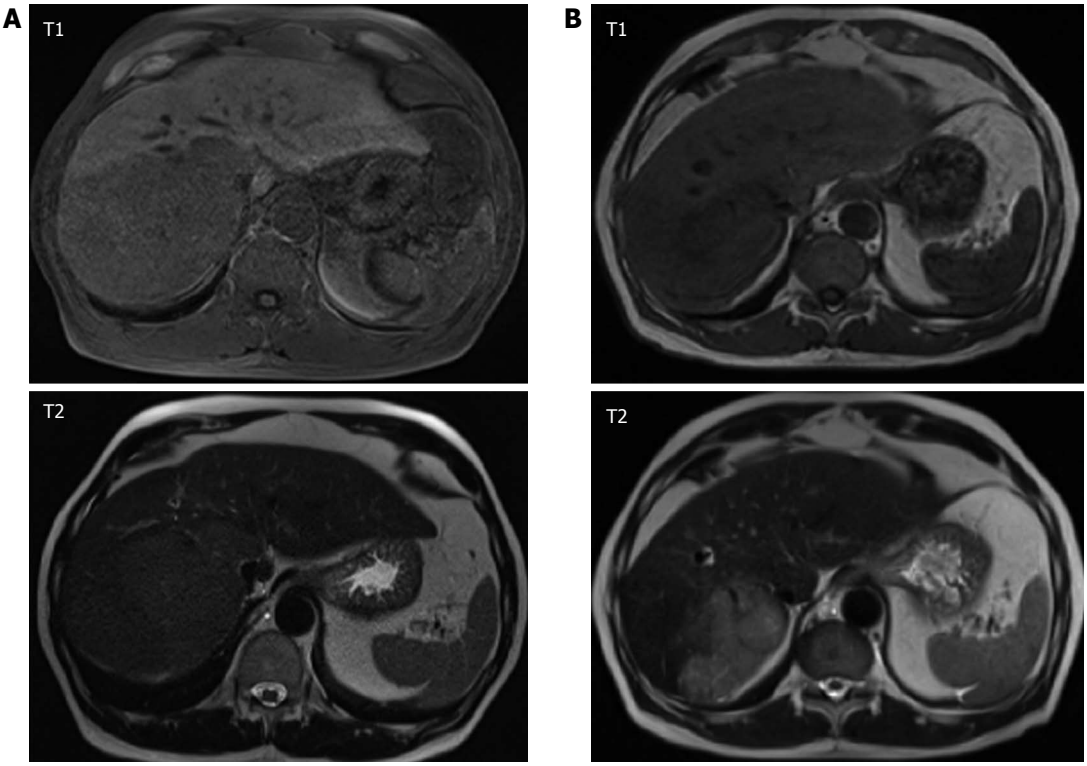

Figure 1 Dynamic gadolinium-enhanced magnetic resonance imaging (MRI; T1, T2 weighting), in a 67 year old patient with hepatocellular carcinoma evolved from liver cirrhosis due to hemochromatosis (A) before initiation of anti-angiogenic therapy and (B) after 70 d or three cycles of transarterial chemoembolization and continuous administration of sorafenib, respectively. Patient showed partial response according to RECIST criteria. Serum alfa-fetoprotein level decreased from 276 to 115 ng/mL.

tions on the therapy with sorafenib, founded on restricted efficacy and potential side effects, mainly fatigue, diarrhea and hand-food syndrome. In comparison to TACE valid predictive biomarkers are missing, also[11].

Table 2 Efficacy of combination therapy with systemic acting agents and targeted therapy in hepatocellular carcinoma according to current phase Ⅰ-Ⅱ studies

Author	Year	Phase	Investigational drug	*n*	RR	DS	PFS/TTP	PFS-6m (%)	OS
Sun *et al*[76]	2007	Ⅱ	Bevacizumab/CapOx	30	11	78	4.5/NR	40	NR
Thomas *et al*[55]	2009	Ⅱ	Bevacizumab/erlotinib	40	25	42.5	9.0/NR	NR	15.7
Hsu *et al*[77]	2008	Ⅱ	Bevacizumab/capecitabine	45	9	42	4.1/NR	NR	10.7
Zhu *et al*[78]	2006	Ⅱ	Bevacizumab/GemOX	33	20	27	5.3/NR	NR	9.6
Berlin *et al*[79]	2008	Ⅱ	Bortezomib/doxorubicin	39	2.3	25.6	2.4/NR	NR	5.7
Asnacios *et al*[80][1]	2008	Ⅱ	Cetuximab/GemOx	45	20	40	4.7/NR	NR	9.5
Louafi *et al*[81][1]	2007	Ⅱ	Cetuximab/GemOx	35	24	4.5	NR/NR	40	9.2
Knox *et al*[82][2]	2008	Ⅱ	G3139/doxorubicin	17	0	35	NR/1.8	17.2	5.4
Abou-Alfa *et al*[83][3]	2010	Ⅱ	Sorafenib/doxorubicin	96	4	77	6.9/8.6	2.7	13.7
Richly *et al*[84]	2009	Ⅰ	Sorafenib/doxorubicin	18	6.3	69	4.0[4]/NR	NR	NR

[1]Overlap of patient cohorts cannot be excluded from information provided in the abstracts; [2]Trial stopped due to lack of efficacy; [3]Trial stopped due to superiority of sorafenib; [4]Calculated from a median duration of disease control rate (combined endpoint for complete and partial response as well as stable disease) of 17.4 wk. CapOx: Capecitabine and oxaliplatin; DS: Disease stabilization (%); GemOx: Gemcitabine and oxaliplatin; NR: Not reported; OS: Overall survival (mo); PFS/TTP: Progression free survival/time to progression (mo); PFS-6m: Progression free survival rate at 6 mo (%); RR: Response rate [complete + partial response (%)].

Table 3 Efficacy of sorafenib and transarterial chemoembolization in hepatocellular carcinoma (sequential therapy not included) according to current phase Ⅰ-Ⅱ studies

Author	Year	Phase	Investigational drug	*n*	RR	DS	PFS/TTP	OS
Chow *et al*[45]	2010	Ⅱ	Sorafenib + SIRT	35	31.4	77.1	NR/NR	10.8
Chung *et al*[47][1]	2010	Ⅱ	Sorafenib + TACE	50	NR[2]	96	NR/NR	NR
Dufour *et al*[48]	2010	Ⅰ	Sorafenib + TACE	14	NR[3]	NR[3]	NR[3]	NR[3]
Erhardt *et al*[46][1]	2009	Ⅱ	Sorafenib + TACE	44	NR[4]	63.6	8.0/16.1	11.7
Reyes *et al*[49][1]	2009	Ⅱ	Sorafenib + DEB-TACE	50	NR[5]	NR	NR/NR	NR

[1]Interim analysis; [2]20/50 patients received 2 cycles of transarterial chemoembolization (TACE), only, and 18 of these 20 patients achieved complete response compared to 2 patients with progressive disease. 30/50 patients received more than 2 cycles of TACE and achieved partial response or stable disease; [3]Primary objective of this prospective trial was evaluation of safety and tolerability of a continuous regimen of sorafenib combined with TACE; [4]According to 31 patients who received at least 1 cycle of TACE, 2/31 (6.5%) showed complete response, 15/31 (48.4%) showed partial response, and 11/31 (35.5%) showed stable disease. PFS, TTP, and OS are given for all 44 patients enrolled at time point of interim analysis; [5]Patients who completed DEB-TACE showed 100% objective tumor response and 100% partial response or stable disease according to EASL or RECIST criteria, respectively. DEB-TACE: (Drug eluting beads)-transarterial chemoembolization; NR: Not reported; DS: Disease stabilization (%); OS: Overall survival (mo); PFS/TTP: Progression free survival/time to progression (mo); PFS-6m: Progression free survival rate at 6 mo (%); RR: Response rate [complete + partial response (%)]; SIRT: Selective internal radio therapy.

STRATEGIES FOR COMBINATION OF TACE AND TARGETED AGENTS IN HCC

Combination of local and systemic inhibition of angiogenesis seems to be a consequential step to improve outcome in intermediate and advanced stage HCC[44]. Tolerability of combination therapy with sorafenib and conventional TACE as well as DEB-TACE was shown within different trials[45-49]. Currently, the combination of conventional TACE and sorafenib as well as combination of sorafenib and DEB-TACE (SPACE trial) is being evaluated in phase Ⅱ and Ⅲ trials[50]. Moreover, sorafenib was combined with selective internal radiation therapy within a multicenter phase Ⅱ study showing good efficacy in patients with advanced HCC but without extra-hepatic metastasis[45]. So far, no increased toxicity has been reported. The combination of brivanib, a dual VEGFR and fibroblast growth factor inhibitor[51], and TACE is currently evaluated within the multicenter phase Ⅲ BRISK TA Study.

Another interesting approach could be the inhibition of VEGF driven angiogenesis by targeting VEGF with siRNA as shown in a proof-of-concept study recently[52]. Furthermore, promising results were reported for other agents alone or in combination with TACE, e.g. tegafur/uracil, the multi-tyrosine kinase inhibitor TSU-68, sunitinib, erlotinib, and the VEGF antibody bevacizumab[53-57]. However, none of these agents is approved for HCC. Of these, bevacizumab is the currently most commonly clinical used VEGF inhibitor in a variety of malignant entities. However, despite encouraging results in earlier trials, even as single agent treatment, bleeding complications were reported in up to 11% of patients treated with bevacizumab[58]. For the combination of bevacizumab with TACE, severe bleeding and septic complications have been reported in 25% of patients, and the AVATACE-1 trial investigating TACE in combination with bevacizumab has been terminated due to safety concerns in the treatment arm, which does not justify a further clinical development of bevacizumab in this indication. This highlights that large phase Ⅲ trials are required for new agents in HCC, which seems challenging given the increasing number of phase Ⅰ and Ⅱ studies addressing HCC in the last years (Tables 1-3).

In summary, inhibition of angiogenesis in HCC seems a very promising approach for future treatment

of HCC. Multimodal approaches with combination of local and systemic therapy may further improve survival in intermediate and advanced stage HCC.

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REVIEW

Pediatric nonalcoholic fatty liver disease, metabolic syndrome and cardiovascular risk

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Abstract

Nonalcoholic fatty liver disease (NAFLD) encompasses a range of liver histology severity and outcomes in the absence of chronic alcohol use. The mildest form is simple steatosis in which triglycerides accumulate within hepatocytes. A more advanced form of NAFLD, nonalcoholic steatohepatitis, includes inflammation and liver cell injury, progressive to cryptogenic cirrhosis. NAFLD has become the most common cause of chronic liver disease in children and adolescents. The recent rise in the prevalence rates of overweight and obesity likely explains the NAFLD epidemic worldwide. NAFLD is strongly associated with abdominal obesity, type 2 diabetes, and dyslipidemia, and most patients have evidence of insulin resistance. Thus, NAFLD shares many features of the metabolic syndrome (MetS), a highly atherogenic condition, and this has stimulated interest in the possible role of NAFLD in the development of atherosclerosis. Accumulating evidence suggests that NAFLD is associated with a significantly greater overall mortality than in the general population, as well as with increased prevalence of cardiovascular disease (CVD), independently of classical atherosclerotic risk factors. Yet, several studies including the pediatric population have reported independent associations between NAFLD and impaired flow-mediated vasodilatation and increased carotid artery intimal medial thickness-two reliable markers of subclinical atherosclerosis-after adjusting for cardiovascular risk factors and MetS. Therefore, the rising prevalence of obesity-related MetS and NAFLD in childhood may lead to a parallel increase in adverse cardiovascular outcomes. In children, the cardiovascular system remains plastic and damage-reversible if early and appropriate interventions are established effectively. Therapeutic goals for NAFLD should address nutrition, physical activity, and avoidance of smoking to prevent not only end-stage liver disease but also CVD.

Key words: Nonalcoholic fatty liver disease; Metabolic syndrome; Cardiovascular risk; Children

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INTRODUCTION

Over the last two decades, the rise in the prevalence rates of overweight and obesity may explain the emergence of nonalcoholic fatty liver disease (NAFLD) as the leading

cause of liver disease in pediatric populations worldwide[1]. NAFLD comprises a disease spectrum ranging from simple steatosis to steatohepatitis (NASH), with varying degrees of inflammation and fibrosis, progressing to end-stage liver disease with cirrhosis and hepatocellular carcinoma[2,3]. NAFLD affects from 2.6% to 9.8% of children and adolescents, and this figure increases up to 74% among obese individuals[4-8]. NAFLD is strongly associated with obesity, insulin resistance, hypertension, and dyslipidemia, and is now regarded as the liver manifestation of the metabolic syndrome (MetS)[9], a highly atherogenic condition. When compared to control subjects who do not have steatosis, patients with NAFLD have a higher prevalence of atherosclerosis, as shown by increased carotid wall intimal thickness, increased numbers of atherosclerotic plaques, and increased plasma markers of endothelial dysfunction, that are independent of obesity and other established risk factors[10-13]. Consistent with these observations natural history studies have reported that the increased age-related mortality observed in patients with NAFLD is attributable to cardiovascular as well as liver-related deaths[14-17].

Pathologic studies have shown that atherosclerosis is an early process beginning in childhood, with fatty streaks observed in the aorta and the coronary and carotid arteries in children and adolescents[18,19]. There is a positive correlation between the extent of early atherosclerotic lesions in the aorta and the coronary and carotid arteries and cardiovascular risk factors, including obesity, dyslipidemia, hypertension, and diabetes[20-22]. Yet the exposure to cardiovascular risk factors of children and adolescents is independently associated with an increased carotid atherosclerosis in early to middle adulthood[23,24]. Thus, the possible impact of NAFLD on cardiovascular disease (CVD) deserves particular attention in view of the implications for screening/surveillance strategies in the growing number of children and adolescents with NAFLD. In the present review, we examine the current evidence on the association between NAFLD and atherosclerosis in the pediatric population, discuss briefly the possible biological mechanisms linking NAFLD and early vascular changes, and address the approach to treatment of NAFLD to prevent not only end-stage liver disease but also CVD.

NAFLD AND THE METABOLIC SYNDROME

NAFLD is closely associated with abdominal obesity, atherogenic dyslipidemia, hypertension, insulin resistance and impaired glucose tolerance, which are all features of the MetS. Approximately 90% of patients with NAFLD have at least one of the features of MetS, and about 33% meet the complete diagnosis, placing NAFLD as the hepatic representation of MetS[25]. The relationship of NAFLD with MetS features has been confirmed in adults in several studies[9,26-29]. Evidence for a relationship between MetS and NAFLD in children is also emerging[30-32]. The Korean National and Nutrition Examination Survey found that participants aged 10-19 years who presented with three or more risk factors for MetS, had an odds ratio (OR) of 6.2 (95% CI, 2.3-16.8) for an elevated serum alanine aminotransferase (ALT), which they used as an indicator of fatty liver[30]. A single center study from Italy reported MetS to be present in 65.8% of children (3-18 years) with biopsy-proven NAFLD and found grade of fibrosis to be the only histological feature significantly associated with MetS on univariate analysis[31]. A case-control study comparing 150 overweight children with biopsy-proven NAFLD to 150 age-, sex-, and obesity-matched children without evidence of NAFLD, found that, after adjustment for age, sex, race, ethnicity, and hyperinsulinemia, children with MetS had an OR of 5.0 (95% CI, 2.6-9.7) for NAFLD compared with children without MetS[32]. This is the most compelling data to support a significant relationship between NAFLD and MetS, not explicable merely by the coexistence of overweight or obesity in these two conditions, and lend support to the hypothesis that fat accumulation in the liver has an important role in the pathogenesis of other obesity-related comorbidities[33].

NAFLD AND CARDIOVASCULAR DISEASE

Increases in morbidity and mortality from CVD are probably among the most important clinical features associated with NAFLD[13]. Published studies have shown that mortality among patients with NAFLD is higher than that in the general population, mainly due to concomitant CVD and liver dysfunction[14-17]. Using the resources of the Rochester Epidemiology Project, Adams *et al*[14] conducted a population-based cohort study to examine the natural history of patients diagnosed with NAFLD on the basis of imaging studies (83%) or liver biopsy (17%). Mean (SD) follow-up was 7.6 (4.0) years culminating in 3192 persons/years follow-up. Death occurred in 12.6% of patients and was most commonly due to malignancy and ischemic heart disease, which were also the two most common causes of death in the Minnesota general population of the same age and sex. Liver disease was also an important contributor of death among patients with NAFLD, being the third most common cause and accounting for 13% of all deaths. In contrast, "chronic liver disease and cirrhosis" was the 13th leading cause of death among the Minnesota general population, accounting for less than 1% of all deaths[14]. This implies that the increased overall mortality rate among NAFLD patients compared with the general population was at least in part due to complications of NAFLD. In a cohort study involving 129 consecutively enrolled patients diagnosed with biopsy-proven NAFLD, Ekstedt *et al*[15] compared survival and causes of death with a matched reference population. Mean follow-up (SD) was 13.7 (1.3) years. Mortality was not increased in patients with steatosis. In contrast, survival of patients with NASH was significantly reduced. A comparison of the causes of death of patients with NASH with those of the corresponding reference population showed it was significantly more common for patients with NASH to die from liver-related causes (2.8% *vs* 0.2%) and from cardiovascular disease

(15.5% *vs* 7.5%). No significant differences in causes of death were found between non-NASH patients and the corresponding reference population[15]. In a cohort study involving 173 patients retrospectively identified as having a diagnosis of biopsy-proven NAFLD, Rafiq *et al*[16] showed that after a median follow-up of 18.5 years, patients with histologic NASH had significantly higher liver-related mortality than the non-NASH NAFLD cohort (17.5% *vs* 2.7%). The most common causes of death were coronary artery disease, malignancy, and liver-related death. In a very recent study involving a cohort of 118 subjects with NAFLD who underwent liver biopsy because of elevated liver enzymes, Söderberg *et al*[17] confirmed that, after a 28-year follow-up, overall survival was reduced in subjects with NASH, whereas bland steatosis with or without severe fibrosis was not associated with any increase in mortality risk in comparison with the general population. The main causes of death among patients with NAFLD were CVD, followed by extrahepatic cancers and hepatic diseases. All these data provide evidence of an increased risk for cardiovascular mortality in patients with NASH. However, most studies which examined the natural history of NAFLD were retrospective cohort studies with relatively small numbers of patients with histologically proven NAFLD who were seen at tertiary referral centers - features that limit the generalizability of the findings to a community-based practice where patients may have a milder disease. Indeed, among people with NAFLD, those who are referred to hepatologists may have a more advanced liver disease than those detected in the community or population based screening but are not referred. Therefore, the magnitude of mortality risk in NAFLD depends on the setting and method of ascertainment. Future longitudinal studies with larger and less selected cohorts of patients are needed to identify through reliable, noninvasive means the true impact of the wide spectrum of NAFLD in the general population on the long-term overall and cardiovascular mortality.

Data on the prognosis and clinical complications of NAFLD in children remain scant[3]. Although coronary artery disease and stroke usually occur in middle and late age, autopsy studies have shown that the atherosclerotic process in the vascular wall begins in childhood and is accelerated in the presence of risk factors[18-24]. Given the large number of children affected, it is imperative that we establish a better understanding of the natural history of pediatric NAFLD in terms of the progression of liver disease as well as its complications (including long-term cardiovascular risk profile). Feldstein *et al*[34] recently reported the first longitudinal study describing the long-term survival of children with NAFLD who underwent a follow-up of up to 20 years. That study demonstrated that NAFLD in children is a disease of progressive potential. Some children presented with cirrhosis, others progressed to advanced fibrosis or cirrhosis during follow-up, and some developed end-stage liver disease with the consequent need for liver transplantation. Feldstein *et al*[34] also showed that NAFLD in children is associated with significantly shorter long-term survival than the expected survival in the general population of the same age and sex. Children with NAFLD had a 13.8-fold higher risk of dying or requiring liver transplantation than the general population of the same age and sex. The recorded deaths were not liver-related.

Recent epidemiological studies in adult subjects have also demonstrated that NAFLD is associated with an increased risk of incident CVD that is independent of the risk conferred by traditional risk factors and components of the MetS[35-42]. Yet, several studies (including the pediatric population) have reported independent associations between NAFLD and impaired flow-mediated vasodilatation (FMD) and increased carotid-artery intimal medial thickness (cIMT) - two reliable markers of subclinical atherosclerosis - after adjusting for cardiovascular risk factors and MetS[10,12,43-47].

NAFLD AND MARKERS OF SUBCLINICAL ATHEROSCLEROSIS IN CHILDREN

The relation between obesity and atherosclerosis development has been evaluated in many pediatric studies[48], but few studies focused on the relation between NAFLD and atherosclerosis (Table 1)[32,47,49-56]. In an autopsy study involving 817 children (aged 2 to 19 years) who died of external causes (accident, homicide, suicide) from 1993 to 2003, Schwimmer *et al*[49] showed that the prevalence of atherosclerosis was increased by a factor of 2 among those with NAFLD. Atherosclerosis was assessed as absent, mild (aorta only), moderate (coronary artery streaks/plaques), or severe (coronary artery narrowing). Fatty liver was present in 15% of the children. For the entire cohort, mild atherosclerosis was present in 21% and moderate to severe atherosclerosis in 2%. Atherosclerosis was significantly more common in children with fatty liver than those without the disease (30% *vs* 19%, $P < 0.001$). Body mass index (BMI) was not independently correlated to the presence of atherosclerosis, but fatty liver status and BMI did interact significantly ($P < 0.01$). Consequently, for obese subjects the odds of having atherosclerosis was more than 6 times higher in children with fatty liver than those without[49].

Despite this, there are currently few data regarding the possible association between liver histopathologic changes and atherogenic risk in children[32,52,56]. In the Bogalusa heart study in children, investigators found that the extent to which the intimal surface was covered with atherosclerotic lesions was significantly associated with elevation of concentrations of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), triglycerides (TG), and lower concentration of high-density lipoprotein cholesterol (HDL-c). Ratios of cholesterol ester-rich lipoprotein level (TC/HDL-c and LDL-c/HDL-c) are well-established predictors of C VD[57]. More recently, the TG/HDL-c ratio has been shown to be a strong predictor of MetS and CVD[58,59]. In a case-control study, Schwimmer *et al*[32] showed that children with a biopsy-proven NAFLD had a significantly higher fasting glucose, insulin, TC, LDL-c,

Table 1 Published studies on the association between nonalcoholic fatty liver disease and markers of atherosclerosis in the pediatric population

Authors	Study population and sample size (No.)	Diagnosis	Outcomes	Main results
Schwimmer *et al*[49]	Children (817) who died of external causes (accident, homicide, suicide) from 1993 to 2003. Fatty liver was present in 15% of the children	Autoptic liver biopsy	Atherosclerosis was assessed as absent, mild (aorta only), moderate (coronary artery streaks/plaques), or severe (coronary artery narrowing)	For the entire cohort, mild atherosclerosis was present in 21% and moderate to severe atherosclerosis in 2%. Atherosclerosis was significantly more common in children with fatty liver than those without the disease (30% *vs* 19%; $P < 0.001$)
Schwimmer *et al*[32]	Overweight children with (150) and without (150) NAFLD matched for gender, age, and severity of obesity	Liver biopsy	Prevalence of cardiovascular risk factors (abdominal obesity, dyslipidemia, hypertension, insulin resistance, and glucose abnormalities)	NAFLD was strongly associated with multiple cardiovascular risk factors independently of both BMI and hyperinsulinemia
Pacifico *et al*[50]	Obese children with (29) and without (33) NAFLD; Healthy lean children (30)	Liver ultrasound	cIMT	cIMT was significantly higher in obese children with NAFLD compared with obese children without NAFLD and control group. Yet, the severity of fatty liver was associated with cIMT independently of anthropometric and metabolic features
Demircioglu *et al*[51]	Obese children with mild (32), moderate-severe (22) NAFLD, and without NAFLD (26); Healthy lean subjects (30) matched for age and gender	Liver ultrasound	cIMT	cIMT measured at left sites of common carotid artery, carotid bulb, and internal carotid artery was significantly higher in obese children compared with controls. Moreover, there was an increase in the mean cIMT of each segment with the increase in steatosis grade
Kelishadi *et al*[54]	Obese adolescents with (25) and without (25) components of MetS; Normal weight adolescents with (25) and without (25) components of MetS	Liver ultrasound and elevated ALT	cIMT	cIMT was significantly associated with insulin resistance and NAFLD
Manco *et al*[52]	Overweight and obese children with (31) and without (49) NAFLD matched for gender, age, and BMI	Liver biopsy	cIMT	cIMT was similar in cases and controls on the right side but significantly higher on the left site. There was no association between cIMT and severity of steatosis as well as fibrosis, and NAFLD activity score
Caserta *et al*[53]	Randomly selected adolescents (642) of whom 30.5% and 13.5% were, respectively, overweigth and obese. Overall prevalence of NAFLD, 12.5%	Liver ultrasound	cIMT	NAFLD, BMI (or waist circumference) and systolic blood pressure were independently associated with increased cIMT
Nobili *et al*[56]	Children with NAFLD (118)	Liver biopsy	Atherogenic lipid profile (TG/HDL-c, TC/HDL-c, and LDL-c/HDL-c ratios)	The severity of liver injury was strongly associated with a more atherogenic lipid profile, independently of BMI, insulin resistance, and presence of MetS
Pacifico *et al*[47]	Obese children with (100) and without (150) NAFLD; Healthy lean children (150)	Liver ultrasound and elevated ALT	cIMT and FMD	Obese children had more functional and morphologic vascular changes than healthy lean controls, regardless of liver involvement. However, obese children with NAFLD had significantly decreased FMD response and increased cIMT compared to obese children without NAFLD independently of other cardiovascular risk factors and MetS
Weghuber *et al*[55]	Obese children with (14) and without (14) NAFLD	Proton MR spectroscopy	FMD	FMD was comparable between the two groups

NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; cIMT: Carotid intima-media thickness; TG: Triglycerides; HDL-c: High-density lipoprotein cholesterol; TC: Total cholesterol; LDL-c: Low--density lipoprotein cholesterol; ALT: Alanine aminotransferase; FMD: Flow mediated dilation; MetS: Metabolic syndrome; MR: Magnetic resonance.

TG, systolic and diastolic blood pressure than age-, sex-, and BMI-matched peers without NAFLD. These data confirm that fat accumulation in the liver may play a more important role than obesity itself in determining the risk for "weight-related" metabolic comorbidities[33]. Thus, the authors concluded that NAFLD may serve as a marker to stratify the cardiovascular risk of overweight and obese children and adolescents[32]. Furthermore, in a study involving 118 consecutive children with biopsy-proven NAFLD undergoing extensive metabolic profiling, Nobili and colleagues found that the NAFLD activity and fibrosis scores had a significant positive correlation with TG/HDL-c, TC/HDL-c, and LDL-c/HDL-c ratios[56]. After adjusting for potential confounders including BMI, homeostatic model assessment index, impaired glucose tolerance, and presence of MetS, both NAFLD activity

score and stage of fibrosis remained independent predictors of an atherogenic lipid profile. The lipid ratios were found to be markedly higher in children with established NASH compared with those patients with simple steatosis or borderline disease, indicating that severity of liver injury in children with NAFLD is strongly associated with increased atherogenic risk[56].

Recent improvements in imaging technology have identified early vascular changes that can be assessed by the use of ultrasonography. These early changes include impairment of FMD, arterial stiffness, and increased cIMT. The measurement of FMD and cIMT by high-resolution ultrasound is increasingly used for cardiovascular risk evaluation in young individuals with obesity, MetS or its components, and pre-diabetes[23,60,61]. Pacifico *et al*[50] first showed that the severity of ultrasonographically diagnosed NAFLD in obese children was significantly associated with carotid atherosclerosis, independently of anthropometric and metabolic features. Demircioglu *et al*[51], in a subsequent study, also found an association between ultrasonographically detected NAFLD and cIMT measured at sites of the common carotid artery, carotid bulb and internal carotid artery. In addition, there was an increase in the mean of cIMT of each segment with the increase in hepatosteatosis grade[51]. Kelishadi *et al*[54] also demonstrated that cIMT was significantly associated with insulin resistance and NAFLD, suggesting that the liver and the vessels share common mediators. This is in contrast to the case-control study by Manco *et al*[52] including a mixed population of overweight and mildly obese children of whom 31 had biopsy-proven NAFLD, whereas 49 had no ultrasound evidence of NAFLD and no abnormal levels of aminotransferases. Although cIMT was statistically significantly higher on the left side in NAFLD cases, the authors concluded that this difference was unlikely to be clinically relevant because of the substantial overlap of cIMT values between cases and controls. Also, there were no differences in the frequency of MetS components between the groups. Finally, there was no association between histologic severity of NAFLD and cIMT[52]. However, a recent study by Patton *et al*[30] showed the potential power of MetS as a prognostic indicator of disease severity in NAFLD. Of the MetS features, central obesity and insulin resistance were most consistently associated with NAFLD histology[30].

The association between NAFLD and carotid atherosclerosis has also been determined in a large, randomly selected adolescent population from Reggio Calabria, a town in southern Italy[53]. The authors found that NAFLD, BMI, waist circumference, and systolic blood pressure were independent markers of increased cIMT. Likewise, in a very recent study with a large sample size it has been shown that obese children with NAFLD have a significantly lower FMD response and increased cIMT compared to obese children without NAFLD independently of other cardiovascular risk factors and MetS, and that obese children exhibit more functional and morphologic vascular changes than healthy lean controls, regardless of liver involvement[47]. The larger number of subjects in that study may in part account for the associations the authors were able to identify between NAFLD and functional vascular changes, in contrast to the study by Weghuber *et al*[55]. in which a very small sample of obese children with and without NAFLD had a similar FMD response.

Although longitudinal studies are needed to clarify the extent to which pediatric NAFLD and its severity influence long-term cardiovascular outcomes in the general population, overall the above cross-sectional findings suggest that childhood NAFLD is associated with early atherosclerosis.

POSSIBLE BIOLOGICAL MECHANISMS LINKING NAFLD AND ACCELERATED ATHEROSCLEROSIS

The biologic mechanisms by which NAFLD contributes to accelerated atherosclerosis, independently of other risk factors, are still poorly understood. Increased visceral adipose tissue and insulin resistance are the undisputed major contributors to NAFLD, MetS, and atherosclerosis[9,62,63]. The adipose tissue inflammation with consequent release of multiple proinflammatory molecules is one of the earliest steps in the chain of events involved in the development of insulin resistance and atherosclerosis, in particular in obese and overweight persons[64-66]. While insulin resistance promotes fatty acid accumulation in the liver, the latter causes hepatic insulin resistance characterized by a lack of suppression of endogenous liver glucose production. Therefore, NAFLD might act as a stimulus for further increased whole-body insulin resistance and dyslipidemia (with a characteristic overproduction of triglyceride- and cholesterol-rich remnant particles), leading to accelerated atherosclerosis[33]. It is also conceivable that other atherogenic mechanisms could be involved in patients with NAFLD including enhanced oxidative stress and chronic, subclinical inflammation, which are thought to be causal factors in the progression from simple steatosis to more advanced forms of NAFLD[27-29,62]. Indeed, patients with NAFLD frequently have higher plasma markers of oxidative stress and inflammation, at least partially derived from the diseased liver, as well as decreased adiponectin concentrations, an adipose-secreted cytokine with antiatherogenic properties[11,67-69].

Recent research has suggested a role for increased fructose consumption as a risk factor for NAFLD[70,71]. Strong evidence exists that fructose consumption may promote hepatic de novo lipogenesis and intrahepatic lipids, inhibition of mitochondrial β-oxidation of long-chain fatty acids, triglyceride formation and steatosis[72]. In addition, compared to glucose consumption, sustained dietary fructose has been reported to significantly increase plasma concentrations of fasting small dense LDL-c, oxidized LDL-c, and postprandial remnant-like-particle-triglyceride and -cholesterol in overweight and obese subjects[73]. These changes may be associated with increased risk of CVD[74,75].

Finally, NAFLD could be linked to accelerated athero-

genesis through the presence of abnormal lipoprotein metabolism, especially during the post-prandial phase[76,77]. Apolipoprotein (APO) B is a large protein involved in the transport of triglycerides and cholesterol from the liver to peripheral tissues. Diminished synthesis of APO B, a rate-determining step in the very low density lipoproteins (VLDL) assembly, would impair the ability of the hepatocyte to export triglycerides and cholesterol esters. Impaired VLDL secretion would also result in increased levels of atherogenic triglyceride- and cholesterol-rich remnant particles. Recent studies have suggested a genetic basis for abnormal lipoprotein metabolism in patients with NAFLD. Two single-nucleotide polymorphisms in the gene encoding APOC3 may be associated with hypertriglyceridemia[78-80]. APOC3 variants C-482T and T-455C lead to increased plasma concentrations of APOC3, which in turn inhibit lipoprotein lipase and triglyceride clearance, thus conferring a predisposition to both fasting and postprandial hypertriglyceridemia due to an increase in chylomicron-remnant particles[81].

THERAPEUTIC APPROACH

The only accepted therapy for pediatric NAFLD is lifestyle modification with diet and physical exercise. The close association of NAFLD with MetS and obesity in children provides the rationale for the therapeutic role of weight reduction in the treatment of fatty liver disease. Fortunately, this approach may also be beneficial in improving cardiovascular risk profile.

Weight-loss oriented lifestyle interventions in the overweight pediatric population have been shown to increase glucose tolerance and improve the MetS risk factors[82]. In children with presumed NAFLD, several studies demonstrate a normalization of serum ALT associated with weight loss[5,83,84]. However, the relative efficacy of weight loss and degree of weight loss needed to induce histologic improvement in pediatric NAFLD is unknown. Studies in adults with NAFLD suggest that weight loss also leads to significant improvement in liver histology. In particular, a weight loss greater than 5% has been associated with significant improvement in liver histology[85]. There is only one clinical trial using liver histology as the primary end point in children and adolescents with NAFLD[83]. The study demonstrated that 2 years of lifestyle intervention with a diet tailored on individual caloric requirement and increased physical activity was associated with a mean weight loss of approximately 5 kg, resulting in a significant improvement in liver histology as well as in insulin resistance, serum levels of aminotransferases, and lipid levels. No information exists on recommending any type of diet. A low-carbohydrate diet has been shown to lead to a reduction in serum ALT and fatty liver content in adult patients[86]. A randomized controlled study in obese adolescents has demonstrated that a diet based on a reduced glycemic load is more effective than a low fat diet in achieving weight loss[87], but similar data are not available in children with NAFLD. Current data on the role of a low fructose diet in children with NAFLD are inconclusive[71]. A 6-month pilot study in children with NAFLD showed that a low fructose diet was associated with a significant decrease in oxidized LDL-c[88]. However, no effect on serum ALT concentrations was found. N-3 long-chain polyunsaturated fatty acids (LCPUFA) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been reported to control some of the metabolic stigmata of obesity[89]. Dietary N-3 LCPUFA lower blood triglycerides and have anti-inflammatory as well as insulin-sensitizing effects[89]. A recent randomised clinical trial has shown that the supplementation of DHA improves liver steatosis and insulin sensitivity in children with NAFLD[90]. Their role in prevention of CVD is also emerging[91]. At this time, however, the available information is insufficient to derive dietary intake recommendations for EPA and DHA[92]. Finally, diet duration and amount of weight loss have not been definitively assessed in children[83,93,94].

A general consensus exists about the key role of physical activity and its synergic effect when combined to diet modifications. Increasing energy expenditure is an additional way to reducing daily calories. Liver biopsy has shown improvement of histologic features in children with NAFLD who were engaged in a moderate daily exercise program (45 min/d aerobic physical exercise) associated to dietary changes[83].

Owing to the likely role of insulin resistance and oxidative stress in the development and progression of NAFLD, most studies on pharmacological treatment have focused on the use of metformin or antioxidants. These drugs have been found to be effective in pilot studies[5]. Recently, in a multicenter, randomized, placebo-controlled clinical trial of treatment with metformin, vitamin E, or placebo for 96 wk in 173 nondiabetic children with histologically confirmed NAFLD, the Nonalcoholic Steatohepatitis Clinical Research Network found that compared with placebo, neither vitamin E nor metformin was associated with a sustained reduction in serum ALT[95,96]. Compared to placebo, vitamin E significantly improved hepatocellular ballooning and NAFLD activity score and, in the subset of children with NASH at baseline, significantly increased resolution of NASH. Metformin had no significant effect on any secondary histologic outcome[96]. Neither vitamin E nor metformin had significant effects on fibrosis, lobular inflammation, or portal inflammation scores. No significant differences in safety were reported between groups[96]. However, the likelihood that vitamin E would need to be taken indefinitely[97] underlines the importance of long-term prospective studies involving patients with NASH to assess the effect of vitamin E on liver-related and cardiovascular mortality[98].

Given the role of obesity in the pathogenesis of NAFLD, bariatric surgery has been proposed as a potential treatment strategy. In obese adult patients, bariatric surgery has been shown to induce weight loss, ameliorate cardiovascular risk factors, resolve hepatic steatosis and, in most studies, inflammation[99-101]. NASH was improved in the majority of affected patients and was intimately associ-

ated with insulin resistance over both the short and long term[101]. The issue of NAFLD and bariatric surgery in children is complex[71]. Though adolescents are increasingly undergoing surgical treatment of obesity, the guidelines for eligibility are not standardized[102]. In addition, children with a clinical diagnosis of NAFLD are different from those typically undergoing bariatric surgery[103]. Children with a clinical diagnosis of NAFLD tend to be younger and less obese than adolescents undergoing surgical treatment of obesity[103]. Although several studies report resolution or improvement of comorbidities after bariatric surgery in adolescents[104,105], liver outcome data are needed. In a series of 41 adolescents, Nadler *et al*[105] reported improvement in liver function enzymes 1 to 2 years after surgery.

CONCLUSION

The current body of evidence suggests that NAFLD is associated with a significantly greater overall mortality than in the general population, as well as with increased CVD prevalence, independently of classical atherosclerotic risk factors. These observations raise the possibility that NAFLD may be not only a marker but also an early mediator of atherosclerosis.

Children with NAFLD may also be at a higher risk for atherosclerosis. Therefore, the rising prevalence of obesity-related MetS and NAFLD in childhood may lead to a parallel increase in adverse cardiovascular outcomes. In children, the cardiovascular system remains plastic and damage-reversible if early and appropriate interventions are established effectively. Therapeutic goals for NAFLD should address nutrition, physical activity and avoidance of smoking to prevent not only end-stage liver disease but also CVD.

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REVIEW

Intestinal inflammation and colorectal cancer: A double-edged sword?

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Abstract

Chronic inflammation is thought to be the leading cause of many human cancers including colorectal cancer (CRC). Accordingly, epidemiologic and clinical studies indicate that patients affected by ulcerative colitis and Crohn's disease, the two major forms of inflammatory bowel disease, have an increased risk of developing CRC. In recent years, the role of immune cells and their products have been shown to be pivotal in initiation and progression of colitis-associated CRC. On the other hand, activation of the immune system has been shown to cause dysplastic cell elimination and cancer suppression in other settings. Clinical and experimental data herein reviewed, while confirming chronic inflammation as a risk factor for colon carcinogenesis, do not completely rule out the possibility that under certain conditions the chronic activation of the mucosal immune system might protect from colonic dysplasia.

Key words: Colorectal cancer; Inflammation; T cells; Cytokines; Immunosurveillance

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INTRODUCTION

Chronic inflammation is thought to be the leading cause of many human cancers including colorectal cancer (CRC). Ulcerative colitis (UC) and Crohn's disease (CD), the two major forms of inflammatory bowel disease (IBD), are associated with an increased risk of developing colitis-associated colorectal cancer (CAC). The risk of CRC in UC patients is 2% after 10 years, 8% after 20 years and 18% after 30 years of active disease[1]. Although more recently other studies have estimated a lower risk in this class of patients, the overall incidence rate ratio for developing CRC calculated in UC patients was found by Bernstein *et al*[2] to be 2.75 [95% confidence interval (95% CI), 1.91-3.97] compared to the general population.

While the relationship between UC and CRC is well established, the risk associated with CD has been unclear until recently. Indeed, the heterogeneous nature of CD which can involve any part of the gut in a non-continuous way, with many patients having no colonic involvement, makes it difficult to estimate the actual risk of developing CRC in these patients. A milestone Swedish study demonstrated a relative risk of CRC of 5.6 for CD patients with exclusive localization in the colon and 3.2 for patients with ileo-colitis[3]. In contrast, patients with exclusive ileal localization of the disease had no increased risk. A meta-analysis of CRC risk in CD revealed an overall relative risk of 2.5 (95% CI, 1.3-4.7)[4]. In the subset of patients with exclusive colonic

localization, the risk was 4.5 (95% CI, 1.3-14.9) while the risk in patients with ileal disease was not significantly increased. Interestingly, when comparing Crohn's colitis with UC of similar extent, the relative risk of developing CRC is similar between the two groups.

Several risk factors concur to determine the probability of developing CRC in single patients. The observation that the cumulative risk increases over the years indicates that disease duration does play a role[1,3]. In addition, the extension of the disease has been shown to increase the risk of CAC; this being 1.7 in patients with ulcerative proctitis, 2.8 in those with left-sided colitis and 14.8 in patients with extensive colitis[3]. Also, the severity of inflammation independently correlates with the risk of developing CAC[5]. The same independent risk factors have been linked to the risk of developing CRC in CD. In addition, in CD patients, perianal disease, bypasses and strictures might be sites of increased risk of neoplastic transformation[6-8].

Overall, these data indicate that chronic inflammation of the colon such as that observed during either UC or CD increases the risk of developing CRC. However, the mechanisms involved in this process are still poorly understood. The current opinion regarding the pathogenesis of IBD is that, in genetically susceptible individuals, there is an overreaction of the immune system toward antigens of the gut microbiota leading to chronic inflammation[9]. UC and CD are characterized by different immune responses. While UC is caused by an atypical T helper (Th)2-mediated immune response characterized by high levels of IL-5 (but not IL-4) and IL-13, in CD there is a prevalent activation of Th1 cells with high expression of TNF-α and IFN-γ[10-13]. More recently, a new subset of IL-17-producing T helper cells, the Th17 cells, has been shown to play a role in the pathogenesis of CD[14,15]. Finally, in addition to CD4+ T cells, CD8+ T cells, natural killer, natural killer T cells and regulatory T cells have also been implicated in the pathogenesis of IBD[16-18].

Given the role played by these cell subsets and by the cytokines they express in the induction and maintenance of gut inflammation, their role has also been investigated in the pathogenesis of CAC. Here we review some of the recent data that implicate immune cells and inflammatory cytokines in the pathogenesis of CAC.

INFLAMMATION AND TUMOR INITIATION: A DOUBLE-EDGED SWORD

Chronic inflammation is thought to induce dysplasia by inducing DNA modifications in intestinal epithelial cells. Indeed, chronic accumulation of activated immune cells such as neutrophils, macrophages and dendritic cells is accompanied by the release of oxygen and nitrogen reactive species, which are known to induce genomic mutations[19,20]. Moreover, chronic inflammation is associated with DNA methylation and histone modification[21-23]. All these processes have been associated with the altered expression of genes involved in carcinogenesis such as *p53, APC, K-ras* and *Bcl-2*[24]. Once initiated, dysplastic cells are subjected to the effect of cell-derived growth factors and cytokines which contribute to tumor growth. However, lines of evidence have also indicated that, under certain conditions, immune cell subsets and cytokines fight to maintain dysplastic cells in check thus preventing tumor progression. A change in the immune response and/or an adaptation to the selective pressure of the immune system, referred to as immune-editing, at a certain point will select dysplastic cell clones able to grow sustained by the presence of growth factors and proinflammatory cytokines released in the surrounding microenvironment, thus changing the role of the immune system from a negative regulator of tumor growth to cancer promoter[25]. Although most of the data sustaining this mechanism derive from models of sporadic cancer, it is possible that a similar alteration of the balance between immune system and dysplastic cells might also occur during long-standing intestinal inflammation.

CD4+ T cells and colitis-associated carcinogenesis

Whether T cells are required for the development of colitis-associated CRC is an open question. In the azoxymethane/dextran sulphate sodium (AOM/DSS) experimental model of CAC, RAG1-deficient mice that do not have B and T cells did not develop tumors even in the presence of colitis[26]. These results indicate that lymphocytes are required to promote tumor growth in the context of colitis. However, it is worth considering that an enhanced activity of natural killer (NK) cells, which are still present in RAG1-/- mice, might be responsible for tumor protection in these mice. Indeed, depletion of suppressive subsets of T cells (i.e. regulatory T cells) has been shown to increase NK cell activity and tumor rejection[27-29]. Experiments with RAG1-/-//γ-chain-/- double knockout mice which lack B, T and NK cells would help to address this issue.

With regard to T helper cell subsets, the role of Th1 and Th2 cells in CAC has been shown by Osawa *et al*[30]. The authors compared CAC development in IL4-/- and IFN-γ-/- deficient mice which have a biased Th2 and Th1 immune response, respectively. Interestingly, Th1-biased IFN-γ-/- mice developed more tumors than wild type. Since in these mice there was high expression of IL-4 and IL-5, the authors concluded that Th2-derived cytokines promote tumor growth. Indeed, a Th2 response has been correlated with progression of experimental and human sporadic CRC[31,32] while a Th1 response has been associated with a better prognosis[33]. Moreover, the Th2-related cytokines IL-4 and IL-13 have been shown to induce the upregulation of activation-induced cytidine deaminase (AID), an enzyme involved in DNA mutation in epithelial cells *in vitro*[34]. Accordingly, AID levels are highly expressed in tumor samples from UC patients. The higher susceptibility to develop CAC shown by IFN-γ-/- mice might be related to a decreased immunosurveillance[34]. Indeed, IFN-γ has been shown to be involved in the activation of cytotoxic T cells and NK cells which play a central role in the antitumor immune response[35,36].

The initial observation that UC patients have a higher risk of CAC in comparison to CD fits well with the con-

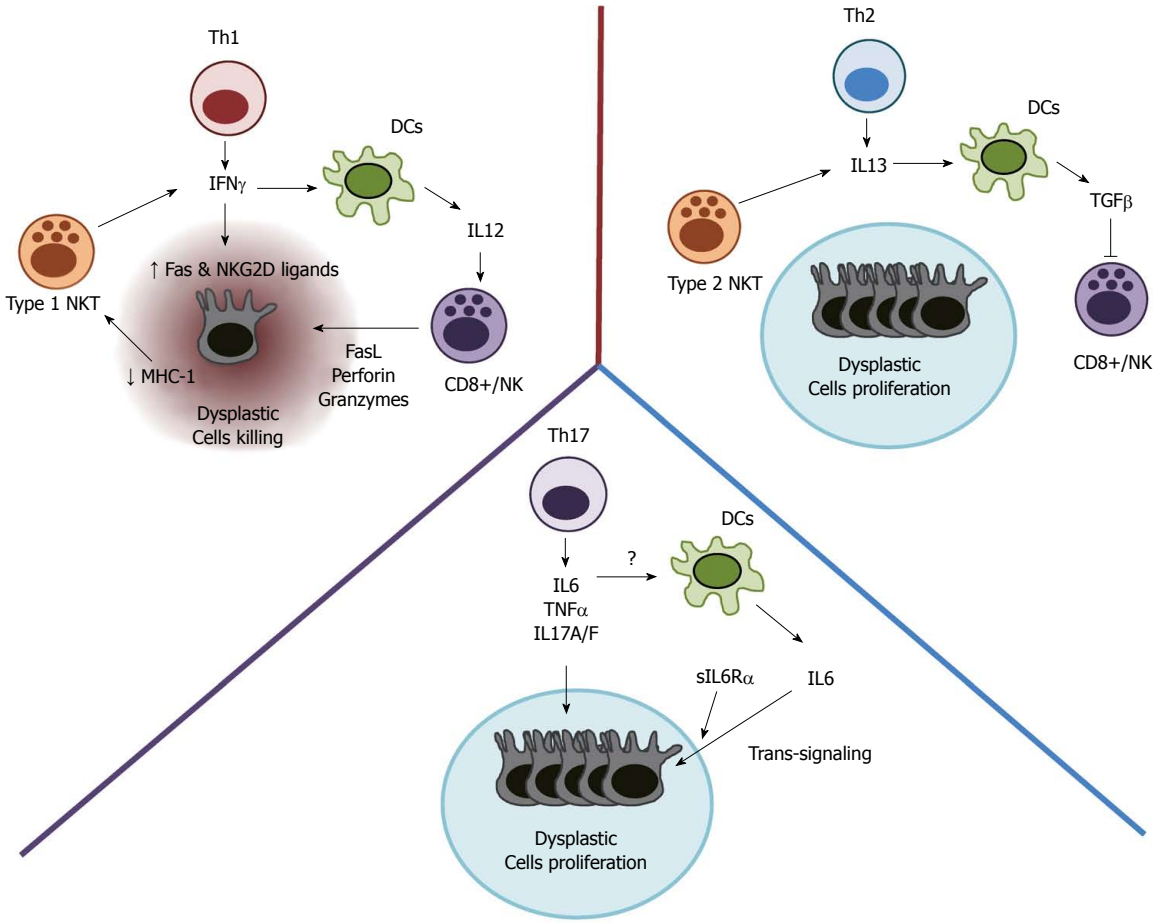

Figure 1 Different T helper-mediated immune responses might be associated with distinct effects on dysplastic cell survival. While Th2 and Th17 immune responses might promote dysplastic cell proliferation and tumor growth, Th1 cells could induce cell death thus preventing tumor progression. Th: T helper; NK: Natural killer; NKT: Natural killer T cells; IL: Interleukin; TGF-β: Transforming growth factor-β; DC: Dendritic cells; MHC: Major histocompatibility complex; Fas: Tumor necrosis factor receptor superfamily, member 6; NKG2D: Killer cell lectin-like receptor subfamily K, member 1; TNF-α: Tumor necrosis factor-α; sIL6R-α: Soluble IL-6 receptor-α.

cept that the Th2 immune response, observed in UC, promotes cancer development while the CD-associated Th1 response is protective. However, as mentioned above, the initially observed lower incidence of CAC in CD in comparison to UC is considered to be due to the methodological approach used in these studies. An alternative explanation for this apparent contradiction could derive from recent advances in the pathogenesis of CD. CD has been long thought to be a Th1-mediated immune disease. This concept derives from initial studies on the role of IL-12, a cytokine involved in the differentiation of Th1 cells[13,37]. In these studies, neutralization of p40, a subunit of IL-12, was effective in preventing gut inflammation both in experimental models and in humans[38-40]. However, we now know that p40 is shared by another cytokine, IL-23, which is a heterodimer composed of p40 and p19. IL-23 has been shown to be involved in the maintenance of Th17 cells, a novel class of T helper cells[41]. Accumulating evidence suggests that Th17 cells might play a role in the pathogenesis of CD. Gain of function polymorphisms of the IL-23 receptor gene are associated with CD[42]. IL-23 p19-/- mice are less susceptible to colitis in comparison with IL-12 p35-/- mice which do not express IL-12[43]. Mice deficient in receptor-related organ receptor (ROR)γ t, the lineage commitment transcription factor of Th17 cells, are resistant to inflammation in different models of colitis[44]. Most of the proinflammatory effect of Th17 has been attributed to the expression of IL-17. IL-17 is known to induce the expression of proinflammatory factors such as TNF-α, IL-6, IL1, iNOS, metalloproteinases and chemokines, which also play a role in CAC[45]. Finally, IL-23 expression is increased in several types of human cancer including CRC[46], and IL-23 p19-/- mice are demonstrated to be more resistant to tumor development[47]. Overall, these data suggest that tumor-promoting Th17 cells rather than Th1 cells might sustain inflammation in CD, thus explaining the increased risk of CAC in CD patients (Figure 1).

Cytotoxic T cells

CD8+ T cells, NK and natural killer T (NKT) cells have been shown to play a role in cancer immunity. Their role, initially limited to the capacity to kill dysplastic target cells, has been recently extended demonstrating a more complex contribution to the antitumor immune response.

A central role in cancer immunosurveillance is attrib-

uted to CD8+ cytotoxic T cells. After presentation of tumor-related antigens by antigen-presenting cells, CD8+ T cells become activated and release different cytotoxic molecules responsible for target cell killing. Activated CD8+ T cells express high levels of IFN-γ and FasL[48]. While FasL, a membrane bound molecule, induces apoptosis by interacting with Fas expressed on the surface of dysplastic cells[49], IFN-γ has been shown to enhance the expression of Fas in colorectal cancer cell lines thus enhancing the killing process[50]. Perforin, granzyme A and granzyme B are also expressed by CD8+ T cells and their effect on target cells is to induce a permeabilization of the cell membrane and cell death[51,52].

NK cells are large granular lymphocytes with both cytotoxicity against tumor and cytokine-producing effector function. NK cells are involved in the rejection of *in vivo* implanted tumors in a manner dependent on the presence or absence of signals on the target cells. The lack of MHC class I expression on the surface of target cells or the upregulation of NKG2D ligands can determine NK cells activation[53-55]. NKG2D ligands are expressed at various levels in CRC cell lines and the expression of one of them, MICA, was associated with a better prognosis in CRC patients[56]. Once activated, NK cells express high levels of IFN-γ, perforin and granzymes which induce apoptosis in target cells. Interestingly, IL-21, a cytokine highly expressed in both UC and CD, has been shown to activate NK cells[57]. However, whether IL-21-induced activation of NK cells plays a role in anti-tumor immunity is still unclear[58-60].

In contrast to NK cells, which lack the T cell receptor (TCR), NKT cells express a limited variety of TCRs. Although NKT cells have NK-like cytolytic activity, they are considered regulators of the immune response, being able to express both Th1- and Th2-related cytokines. In tumor immunity, NKT cells have been considered as both enhancers and suppressors of the anti-tumor activity. A subset of NKT cells (type I NKT), characterized by the expression of V-α-14-Jα-18 TCR-α chain, has been shown to enhance tumor immunity by IFN-γ expression and NK cell activation[61,62]. Moreover, IFN-γ indirectly promotes the activation of CD8+ T cells by inducing the expression of IL-12 in antigen-presenting cells[63]. Tumor infiltration by type I NKT cells in CRC patients has been positively correlated with the disease-free survival[64]. In contrast to type I NKT cells, type Ⅱ NKT cells, which do not express the V-α-14-Jα-18 TCR-α chain, have been associated with suppression of antitumor immunity. Type Ⅱ NKT cells express IL-13, which has been shown to induce the expression of the immunosuppressive cytokine TGF-β in myeloid cells[56,65]. Interestingly, the selective activation of type Ⅱ NKT cells enhanced CT26 cell growth in a mouse model of CRC metastasis[66]. In UC, activation of type Ⅱ NK cells and expression of IL-13 characterize the "atypical" Th2 immune response observed in these patients[11]. It is tempting to speculate that activation of type Ⅱ NKT cells in UC might contribute to CAC development by selectively dampening the antitumor immune response while sustaining mucosal inflammation and cancer development.

Innate immune cells

The role of innate immune cells in sporadic CRC progression has started to be unveiled (For review, see Mantovani *et al*[67]). However, whether these cells are also important in the development of CAC is still unclear.

The role of innate immunity in the development of CAC is suggested by recent findings that Toll-like receptors (TLRs) are important in inflammation and CAC. TLRs form a family of membrane-bound receptors expressed by cells of different lineages such as epithelial cells, macrophages and dendritic cells. TLRs "sense" the presence of bacterial compound present in the extracellular space. The interaction between the microbiota and the intestinal mucosa through TLRs is required to maintain intestinal homeostasis. Recent genetic studies suggest that polymorphisms in the genes encoding TLRs are associated with increased risk of IBD and disease extension[68,69]. Moreover, TLR4 is demonstrated as being upregulated in intestinal epithelial cells of patients with active IBD[70]. With regard to CAC, it was shown that intestinal bacteria are required for tumor development in models of CAC. Furthermore, deficiency of MyD88, a molecule involved in TLR intracellular signaling, significantly exacerbated chemically-induced colitis[71] and reduced tumor number and size in sporadic and colitis-associated CRC models[72,73].

CAC: the role of cytokines and chemokines

As mentioned above, immune cells actively contribute to CAC by expressing soluble factors (e.g. cytokines and chemokines) and the role of some of these has been extensively investigated.

IL-6

IL-6 is a multifunctional cytokine important for immune responses, cell survival, apoptosis, and proliferation. IL-6 has been linked to IBD pathogenesis. Atreya *et al*[74] have demonstrated that IL-6 expression in the mucosa of IBD-affected patients induces T cell resistance to apoptosis, thus contributing to chronic inflammation. Accordingly, a correlation between IL-6 levels and the clinical activity of IBD has been demonstrated[75,76]. With regard to CAC, IL-6 expressed during colitis was shown to promote tumor growth in mice[26]. In this model, selective inhibition of the TGF-β signaling in T cells was associated with an enhanced expression of IL-6. In turn, IL-6 trans-signaling, mediated by the interaction of IL-6 and the soluble form of the IL-6 receptor α (sIL-6α) with the gp130 receptor expressed on the surface of dysplastic cells, enhanced tumor cell proliferation. Moreover, in a similar model, Grivennikov *et al*[77] demonstrated that IL-6 expressed by lamina propria myeloid cells protects normal and transformed epithelial cells from apoptosis in a STAT-3-dependent manner, demonstrating the critical oncogenic function of this cytokine-activated transcription factor. Recently, higher expression of IL-6 and STAT3 was observed in both patients with active UC and those who had progressed to CAC, compared with patients with inactive disease or control patients[78]. In the same study, patients with either inactive or active UC, compared with

control individuals, showed increased expression of suppressor of cytokine signaling 3 (SOCS3), which limits the ability of IL-6 to activate STAT3. On the other hand, the expression of SOCS3 was decreased in patients with UC who had progressed to CRC. In the AOM/DSS mouse model of CAC, IEC-specific SOCS3 gene disruption led to increased size, number and load of colonic tumors and this was associated with increased STAT3 and nuclear factor-κB (NF-κB) activation in colon[79].

TNF-α

Tumor necrosis factor (TNF)-α is a pivotal cytokine in the pathogenesis of IBD and anti-TNF-α monoclonal antibody (MAb) therapy is routinely used in UC and CD patients[80]. Although TNF-α has been classically considered as an anticancer agent, it is currently recognized that chronically elevated TNF-α in tissues may promote tumor growth, invasion and metastasis[81]. Indeed, mice deficient for the p55 TNF-α receptor subunit were protected from tumor development in the AOM/DSS model of CAC[82]. Moreover, in the same study, repetitive anti-TNF-α treatment not only suppressed colitis in mice but also prevented CAC.

The effect of TNF-α signaling in CAC is mostly due to the intracellular activation of NF-κB. NF-κB is a pleiotropic transcription factor with a key role in innate and adaptive immunity and is required for the expression of various proinflammatory factors[83]. In addition to its critical function in inflammation, NF-κB activation can support carcinogenesis by increasing cell proliferation and angiogenesis, inhibiting cell death, and promoting cell invasion and metastasis[84]. Greten *et al*[85] have shown that blocking NF-κB activation in the intestinal epithelium dramatically reduced the incidence of CAC, and this was associated with enhanced epithelial cell apoptosis during early tumor development. Interestingly, no reduction of intestinal inflammation was observed in these mice, thus indicating that prosurvival signals provided by NF-κB in epithelial cells play a role in CAC initiation independent of the inflammation severity.

IL-10

IL-10 is an immunomodulatory cytokine and its main biological function is to limit and terminate inflammatory responses. Experimental data indicate that IL-10 might play a role in the pathogenesis of IBD and CAC. Indeed, patients carrying mutations of IL-10 receptor that abrogate IL-10 signaling develop more aggressive disease. Moreover, IL-10-deficient mice spontaneously develop colitis[86] and CAC[87] when infected with certain enteric bacteria such as *H. hepaticus*. In this model, colitis and CAC could be prevented by administering exogenous IL-10, thus indicating that IL-10 is pivotal in the control of inflammation and inflammation-related cancer in the gut. Analogous to IL-6, IL-10 activates STAT3 in target cells. However, the final effect is inhibition of NF-κB activation[88,89] and reduction of the expression of proinflammatory cytokines such as TNF-α, IL-6 and IL-12[90]. IL-10 has also been shown to act as an antiangiogenic factor[91].

A source of IL-10 is represented by regulatory T cells (Tregs), a class of immunosuppressive T cells. Interestingly, in a model of sporadic CRC, Erdman *et al*[92] showed that the transfer of wild type Tregs, but not IL-10-/- Tregs, in CRC-susceptible $Apc^{min-/+}$ mice prevented the development of adenomas and induced rapid tumor regression. These data suggest that, besides its role as a negative controller of the immune system, IL-10 might directly suppress the growth of transformed epithelial cells. Accordingly, Tregs transfer was associated with induction of epithelial cell apoptosis and downregulation of Cox-2, a molecule involved in dysplastic cell survival and proliferation.

TGF-β

Another important immunosuppressive cytokine is transforming growth factor (TGF)-β. TGF-β tightly controls the activation of the immune system and the inhibition of TGF-β signaling causes autoimmune diseases involving several organs including the gut. Moreover, the inhibition of TGF-β signaling operated by the intracellular inhibitory molecule Smad7 in gut lamina propria cells has been shown to contribute to chronic gut inflammation observed in IBD[90].

TGF-β plays an important role in epithelial cell differentiation and growth arrest. Accordingly, TGF-β signaling is found to be altered in sporadic CRC[93]. In contrast, the role of TGF-β in CAC is still unclear. Using a T cell-specific dominant negative TGF-β receptor II transgenic mouse, Becker *et al*[26] demonstrated that TGF-β signaling-mediated negative control of IL-6 expression in T cells is required to inhibit dysplastic epithelial cell proliferation. Conversely, IL-6 has been shown to inhibit TGF-β signaling by inducing Smad7 expression[94]. Smad3 is a key intracellular mediator of the anti-inflammatory and immunosuppressive activity of TGF-β in the colon. Accordingly, Smad3-deficient mice develop CAC that is dependent on the presence of enteric bacteria[95]. Despite the role of TGF-β as immunosuppressant and inhibitor of dysplastic cell growth, TGF-β signaling acts, under certain conditions, as a tumor promoter. Indeed, TGF-β-induced suppression of tumor-specific CD8+ T cells might favor tumor growth and progression[96].

Chemokines

Chemokines and their receptors play an integral role in IBD by regulating the accumulation of immune cells at the site of intestinal inflammation[97].

Monocyte chemoattractant protein 1 (MCP-1, CCL2), a member of the CCβ family of chemokines, is a known chemotactic factor regulating the recruitment of monocytes/macrophages and other inflammatory cells to sites of inflammation *via* activation of the CCR2 receptor[98]. The expression of MCP-1 is increased in the mucosa of patients with IBD[99]. Popivanova *et al*[100], using a model of CAC, showed that CCL2 blockade reduces the infiltration of COX-2-expressing F4/80-positive cells and suppresses COX-2 expression by infiltrating macrophages, resulting in retardation of cancer progression.

The role of chemokines in CAC is further supported by recent studies on chemokine decoy receptor D6. D6, like all decoy receptors, does not induce a conventional intracellular signal but mediates high-affinity ligand binding and efficient ligand degradation. D6 expression is increased in patients with IBD and with CAC compared with healthy subjects. In the AOM/DSS model of CAC, D6-deficient mice showed increased expression of chemokines and higher accumulation of inflammatory cells in comparison to the wild type, resulting in the development of more severe colitis and higher incidence of CAC[101].

CONCLUSION

Clinical and experimental data indicate that chronic inflammation increases the risk of developing CAC, acting at different stages of the carcinogenesis process. The constant release of free radicals is known to be genotoxic leading to the dysregulation of important oncogenes and onco-suppressors. Moreover, it is also known that the release of cytokines such as IL-6 and TNF-α during chronic colitis can promote tumor growth and that low expression of immunosuppressive cytokines such as TGF-β and IL-10 can exacerbate this process. However, it is also clear that what we call macroscopically chronic inflammation may be the result of very different kinds of immune responses and their impact on CAC development is still unclear.

Many lines of evidence indicate that IFN-γ expressed by Th1 cells protects from tumorigenesis in different experimental models. Indeed, IFN-γ is critical in the activation of cytotoxic cells and antitumor activity. Moreover, IFN-γ renders dysplastic cells more susceptible to cell-mediated cytotoxicity. In contrast, Th2- and Th17-mediated immune responses in the gut seem to promote CAC development. Therefore, it is tempting to speculate that different Th-driven "chronic inflammations" of the gut could be associated with different risk of CAC (Figure 2). If this concept will turn out to be true, not only generic immunosuppressive therapy but also modulation of the ongoing intestinal immune response could be considered as an approach in the prevention of CAC in IBD patients.

In clinical practice, many anti-inflammatory drugs and immune-modulators are routinely used in the therapy of IBD. Their efficacy is based on the capacity to reduce clinical manifestations related to disease and to reduce the *in situ* macroscopic/microscopic inflammation. However, in most of the cases little is known about their impact on the immune response at a molecular level and the consequent effect on colon carcinogenesis. An exception is represented by 5-ASA. Clinical data indicate that the long term use of 5-ASA might prevent CAC in UC patients, acting as an anti-inflammatory agent and interfering with cancer cell growth[102]. However, whether 5-ASA might act in part by modulating the activity of the immune system, sustaining immunosurveillance, has not yet been investigated. The impact of other anti-inflammatory drugs and immune-modulators on UC-related colon carcinogenesis, and their capacity to improve or dampen the immunosurveillance against dysplastic cells, are still unknown. The long term evaluation of patients undergoing different therapeutic regimens will help address this issue.

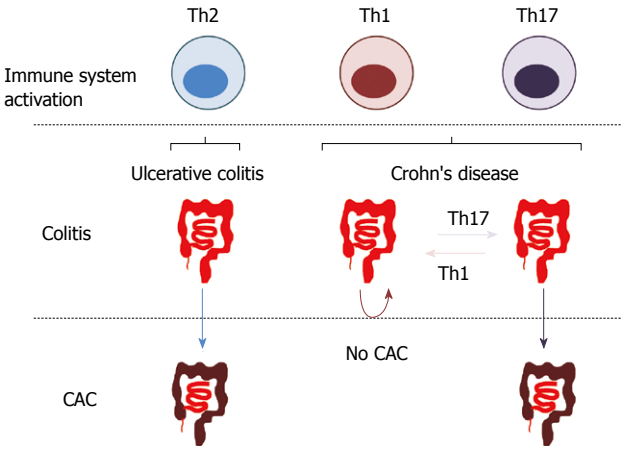

Figure 2 Hypothetical relationship between inflammatory bowel disease and colitis associated colorectal cancer. The T helper (Th)2 immune response characterizing ulcerative colitis determines an elevated risk of developing colitis associated colorectal cancer (CAC). In Crohn's disease, while a Th17-mediated immune response could cause inflammation and enhance CAC risk, the shift towards a Th1-mediated colitis could lower the incidence of CAC.

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REVIEW

Dual protective role of HO-1 in transplanted liver grafts: A review of experimental and clinical studies

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Abstract

Liver transplantation is considered as the most effective treatment for end-stage liver disease. However, serious complications still exist, particularly in two aspects: ischemia and subsequent reperfusion of the liver, causing postoperative hepatic dysfunction and even failure; and acute and chronic graft rejections, affecting the allograft survival. Heme oxygenase (HO), a stress-response protein, is believed to exert a protective function on both the development of ischemia-reperfusion injury (IRI) and graft rejection. In this review of current researches on allograft protection, we focused on the HO-1. We conjecture that HO-1 may link these two main factors affecting the prognosis of liver transplantations. In this review, the following aspects were emphasized: the basic biological functions of HO-1, its roles in IRI and allograft rejection, as well as methods to induce HO-1 and the prospects of a therapeutic application of HO-1 in liver transplantation.

Key words: Liver transplantation; Heme oxygenase-1; Allograft rejection; Ischemia/reperfusion injury

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INTRODUCTION

Transplantation remains the main therapeutic option for patients with end-stage liver disease. Thanks to the clinical use of immunosuppressants, acute rejections have been brought under substantial control. However, the adverse effects of these drugs, such as the development of blood hypertension, hyperlipidemia, diabetes, renal failure, and *de novo* tumors in transplanted patients, are significant, increasing the postoperative mortality. The severe side effects of the immunosuppressants limit their success in attenuating acute rejection. In addition, surgery and preservation of the allografts result in a cascade of ischemia-reperfusion injury (IRI) in the transplantation, for which there are still no effective therapeutic interventions. Consequently, the strategies to simultaneously attenuate IRI and induce donor-specific tolerance would considerably improve the quality of life and survival of the transplant recipients.

The liver, an immunologically privileged organ, bears inherent tolerogenic properties in the event of orthotopic

liver transplantation (OLT). Liver allografts could be established and maintained even without immunosuppressants[1]. In humans, liver transplants can also confer protection on other organ grafts stemming from the same donor[2]. Based on the aforementioned characteristics of the liver, it seems more feasible to induce a donor-specific tolerance in liver transplantations than in the case of transplantations of other solid organs.

More attentions have been paid to heme oxygenase (HO)-1 because of its cytoprotective, antioxidant, maintaining microcirculation, modulating the cell cycle and anti-inflammatory functions[3]. In the process of a liver transplantation, many cell types, including Kupffer cells, endothelial cells, and dendritic cells (DCs), can induce an HO-1 overexpression to prevent IRI and rejections[4-6]. Since HO-1 seems to be involved in both processes, it may act as a linkage between IRI and rejection in liver transplantation in order to induce donor-specific tolerance.

BASIC BIOLOGICAL FUNCTIONS OF HO-1 AND ITS BYPRODUCTS

HOs are rate-limiting enzymes in the heme catabolism. The heme catabolism by HO-1 produces carbon monoxide (CO), free iron, and biliverdin that is subsequently converted to bilirubin by biliverdin reductase[7]. Three HO isozymes have been identified: HO-1, HO-2 and HO-3. HO-1 is an inducible enzyme, while the other two are expressed constitutively[8].

HO-1 is a bona fide 32-kDa stress protein (Hsp32), variously manifested in endothelial, epithelial, smooth muscle and other cell types. HO-1 plays a protective role in many disease models *via* its anti-inflammatory, anti-apoptotic, and anti-proliferative actions[3]. Three products of the heme metabolism are considered to be beneficial due to their immunomodulatory, anti-apoptotic, and vasoactive properties.

CO, despite its potential toxicity, has recently caused a great interest because of its function as a signaling molecule with vasodilatory effects mediated by cGMP, and its antiapoptotic and anti-inflammatory effects[9,10]. CO can travel freely throughout intracellular and extracellular compartments and exert a wide spectrum of modulating physiological effects on multi-systems[11].

Bilirubin, a byproduct, is found to exert a beneficial influence on many diseases, including atherosclerosis, inflammatory, autoimmune, degenerative diseases, and cancer, in which it serves as a highly lipophilic antioxidant[12]. It can slightly reduce ethanol-induced lipid peroxidative injury by decreasing MDA content[9]. In addition, Takamiya *et al*[13] have demonstrated that HO-1 stabilizes mast cells (MCs) in order to exercise an anti-inflammatory action through bilirubin.

Furthermore, Fe, the third product, despite its cytotoxic pro-oxidant effects, induces an over-expression of ferritin, which in turn has strong antioxidant effects through the depletion of free iron and also by other less characterized effects that result in the induction of tolerogenic dentritic cells[14].

HO-1 ATTENUATES LIVER IRI IN LIVER TRANSPLANTATION

IRI is a continual process that culminates in hepatocellular injury. Clinically, it remains a major obstacle to liver transplantation and can lead to hepatic dysfunction and even post-transplantation failure. As a result, the mechanisms and prevention of cellular injury during hepatic ischemia and subsequent reperfusion needs to be elucidated[15].

Kupffer cells, the resident macrophage population within the liver, play key roles in IRI. They are activated after reperfusion by various stimuli in an autocrine fashion by Toll-like receptor 4 signaling[16], or by complement activation[17,18]. After being activated, they release inflammatory cytokines and free radicals, such as reactive oxygen species (ROS), tumor necrosis factor α (TNFα), interleukin 1 (IL-1), nitric oxide (NO), thromboxanes, and leukotrienes[19], and recruit neutrophils to the liver. TNF and IL-1 can also recruit and activate CD4+T-lymphocytes which maintain Kupffer cell activation by secretion of the granulocyte stimulating factors or interferon (IFN)-γ[20,21]. Of all the hepatic cells, the nonparenchymal sinusoidal endothelial cells (SECs) are most susceptible to IRI[22]. SECs are activated by tissue anoxia that disturbs the intracellular energy metabolism and enzyme function, leading to their apoptosis. These cause marked microcirculatory disturbances, leukocyte and platelet adhesion, diminished blood flow and continuation of the ischemic process, resulting in massive hepatic necrosis[23]. Thus, the inhibition of SEC apoptosis may be a useful therapeutic strategy to reduce the risk of ischemia injury in liver preservation. Yue *et al*[5] have found that the apoptosis of SECs was attenuated after the TAT-HO-1 was transducted into the liver, which may be associated with an increased expression of Bcl-2 and a reduced expression of Bax.

It is well known that it is of critical importance to attenuate IRI in liver transplantation. Both HO-1 and its products of degradation play a role in attenuating IRI (Figure 1). The findings that $Hmox^{-}/^{-}$ animals are more susceptible to IRI injury than the $Hmox^{-}/^{+}$ and $Hmox^{+}/^{+}$ animals indicate that HO-1 may play a potent protective role in IRI[24]. A further study has shown that donor livers with an enhanced HO-1 expression lowered the serum ALT/AST levels of the recipient, alleviated allograft injury, and suppressed cytokine release[4]. Luke Devey described a mechanism that HO-1 could drive macrophage differentiation down an "anti-inflammatory" pathway[24]. Therefore, preconditioning the donor liver, especially its Kupffer cells with a strong induction of HO-1, plays a potential protective role. Kupffer cells are not only the main factor associated with liver IRI, but also a major site of expression of the hepatic HO-1. Based on these findings, we can assume that HO-1 in Kupffer cells is

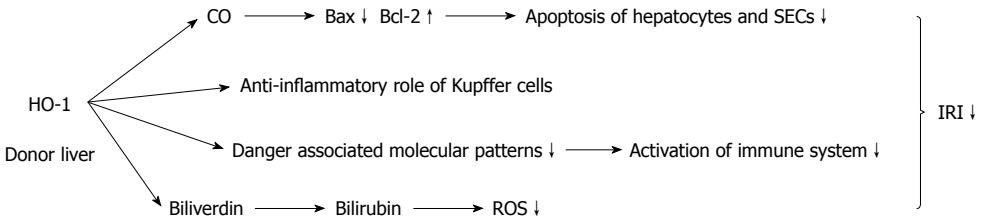

Figure 1 Function of heme oxygenase 1 and its degradation product in ischemia and reperfusion injury during liver transplantation. HO-1: Heme oxygenase 1; CO: Carbon monoxide; SEC: Sinusoidal endothelial cell; ROS: Reactive oxygen species; IRI: Ischemia-reperfusion injury.

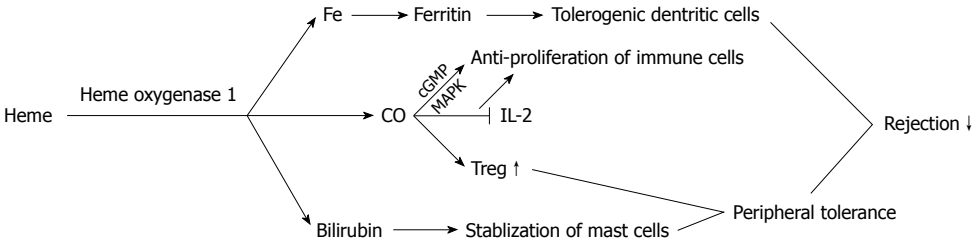

Figure 2 Function of heme oxygenase 1 degradation product to reduce rejection. CO: Carbon monoxide; Treg: Regulatory T cells; IL: Interleukin.

induced exclusively to exert a protective function in the event of IRI. Additionally, HO-1 can modulate each stage of the immune activation pathway such as limiting the production of damage-associated molecular patterns, modulating T cell activation, and enhancing immunological tolerance[25].

As previously described, CO mediates a cytoprotective and anti-inflammatory effect in I/R related oxidative injury. It significantly reduces the messenger RNA (mRNA) levels of the proapoptotic Bax, while it upregulates the anti-apoptotic Bcl-2. Bax and Bcl-2 are both found to be expressed in hepatocytes and SECs at the sinusoidal space. Therefore, CO reduces the IRI-mediated apoptosis through an overexpression of Bcl-2 and diminished Bax expression. This protective role of CO is mediated by an activation of the soluble guanylyl cyclase, as demonstrated by the fact that 1H-(1,2,4)oxadiazole (4,3-α) quinoxaline-1-one (ODQ; a soluble guanylyl cyclase inhibitor), completely reversed its beneficial effect[26].

The oxidation of bilirubin by ROS results in the conversion of bilirubin to biliverdin, the latter being a precursor of bilirubin in the heme degradation that is recycled to bilirubin in mammals by biliverdin reductase. This recycling process between bilirubin and biliverdin is believed to be behind one of the explanations for bilirubin's powerful antioxidant effects in the redox cycle[27]. However, the exact mechanism of the protective role of HO-1 remains to be fully explained.

Contrary to the aforementioned protective role of HO-1 against oxidant-induced injury and the induction of HO-1 as an adaptive response against oxidative damage, Froh *et al*[28] reported that a cobalt protoporphyrin (CoPP)-induced HO-1 over-expression increases liver injury, as demonstrated by an over-expression of hepatic ALT, aggravation of cell necrosis, and fibrosis. They suggested that high levels of HO-1 may sensitize cells to oxidative stress due to an accumulation of free divalent iron, thereby increasing oxidative injury. Since Kupffer cells are the main source of HO-1 in the liver, an increased expression of HO-1 may also aggravate the activation of Kupffer cells, thus increasing the formation of inflammatory and fibrogenic mediators. The controversial role of the HO-1 expression in human liver allografts of either cytoprotection or increased cytotoxicity ought to be investigated in more detail in the future.

HO-1 REDUCES REJECTIONS IN LIVER TRANSPLANTATION

The spontaneous graft tolerance of the liver is an active process which depends upon the transfer of donor leukocytes in the liver. Migration within the recipient's lymphoid system results in an early immune activation of recipient lymphocytes, with their subsequent deletion from exhaustion. Regulatory T cells and antigen presenting cells (APCs) are both involved in inducing donor-specific tolerance.

One highly significant tolerance inducing mechanism is the suppression of allogeneic responsiveness by regulatory T cells. Regulatory T cells (Tregs) are a subset of T lymphocytes that play a core role in immunological suppression and the termination of immune responses. Deficiency or dysfunction of these cells may lead to autoimmunity or an aggravated pathogen-induced inflammation[29]. The end-products of HO-1 degradation do not have much organ specificity to reduce rejection (Figure 2). CO could provide one mechanism by which the regulatory capacity of Tregs is generated. CO has been shown to have broad anti-proliferative effects in human CD4+T cells[30]. Generally, CO is thought to perform those anti-proliferative actions by modulating the activity of guanylate cyclase to increase the cellular cGMP

levels, and through the MAPK (mitogen activated protein kinase) pathway. More specifically, CO was also shown to block the production of IL-2, a principal cytokine responsible for T cell proliferation[31]. CO, functioning as a nonspecific suppressor, also fits with the observation that Treg cells are capable of suppressing the proliferation of various immune cell types without major histocompatibility complex restriction and in a non-Ag-specific manner.

Recently, the existence of interactions between Tregs and MCs has been demonstrated[32]. The secretion of immunosuppressive mediators, such as transforming growth factor β, forms the basis of the pivotal role of MCs in inducing allograft tolerance. IL-9 is the functional link by which activated Tregs recruit and activate MCs to mediate regional immune suppression. This is demonstrated by the fact that by neutralizing IL-9, allograft rejection in tolerant mice is greatly accelerated[33]. However, the degranulation of intragraft or systemic MCs causes the loss of Tregs and MCs from the graft, and impairs Treg function. As a result, rejection occurs in the established tolerant allograft[34]. Therefore, it seems important to stabilize the MCs in order to sustain allograft tolerance. As described above, the liver more easily induces tolerance than other organs. Although MCs are not abundant in the liver, an inhibition of MC degranulation seems important, because of the hepatic immunological privilege. Takamiya *et al*[13] illustrated that an overexpression of HO-1 suppresses compound 48/80-, IgE-induced MC degranulation through bilirubin. Yasui *et al*[35] demonstrated that the upregulation of HO-1 within the MCs also inhibits their cytokine production associated with a selective suppression of the DNA-binding activity of the AP-1 transcription factors. As a result, to induce HO-1 within MCs may be another target to affect Tregs. On the other hand, because MCs are involved in the fibrosis of chronic rejection, inhibiting MC degranulation and cytokine production may control the progression of chronic rejection. Whether the HO-1 overexpression of the donor's or the recipient's MCs is more important, needs to be ascertained.

It is well known that the Treg functions depend on the activity of APCs, and the HO-1 of APCs may also influence the Tregs. George *et al*[36] have demonstrated that lack of HO-1 in the APCs significantly impairs the suppressive function of Treg cells on effector T cells, which indicated the importance of HO-1 within APCs. APCs are involved in initiating rejection and can also be mediated to induce tolerance. Perfusion of donor liver with HO-1 inducers will up-regulate HO-1 expression of many cells in the liver, including hepatic dentritic cells. This pathway contains some mechanism for HO-1 to reduce both acute and chronic rejections (Figure 3). There are two recognition patterns associated with organ transplantation rejection: direct and indirect recognition induced by donor and recipient APCs, DCs are the main APCs in the liver, the only cell type that can activate naïve CD4+T cells. Hepatic DCs differ from those in other organs, because they are less immunostimulatory in response to diverse antigens. Some data have also shown that compared with splenic and blood DCs, freshly isolated mouse liver DCs express lower levels of Toll-like receptor 4 mRNA and are less able to activate allogeneic T cells, or polarize naïve T cells toward Th1 responses to LPS[37]. Immature DCs (imDCs) lack the capability of presenting alloantigen to alloreactive T cells because of a low expression of costimulatory molecules[38]. As it is shown, imDCs express HO-1, but apparently reduce or lose the ability of expression as they become mature[39]. There is expanding evidence that donor hepatic imDCs can downregulate immune responses, thus inducing and maintaining peripheral T-cell tolerance[39]. Furthermore, it has been hypothesized that the presence of large numbers of imDCs within the donor liver that circulate and repopulate the recipient contribute to microchimerism, another mechanism associated with donor-specific tolerance[40]. However, in the event of an acute liver transplant rejection, when the imDCs undergo maturation upon alloantigen stimulation, they induce an acute rejection through direct recognition. Consequently, keeping DCs in their immature state is crucial in order to induce an antigen-specific tolerance in liver transplantation.

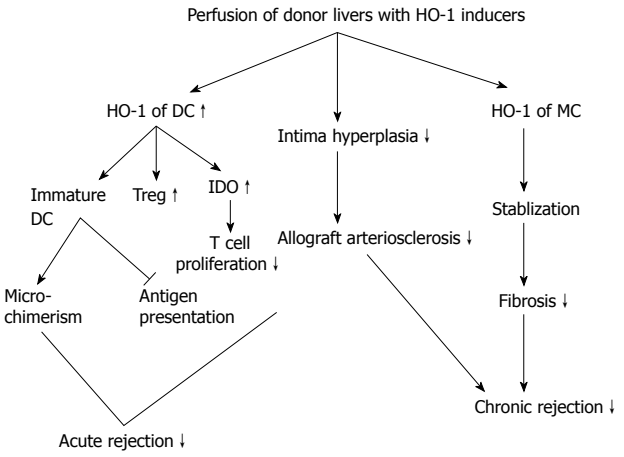

Figure 3 Perfusion of donor liver with heme oxygenase 1 inducers could reduce rejections through many pathways. HO-1: Heme oxygenase 1; DC: Dendritic cell; Treg: Regulatory T cells; IDO: Indoleamine 2,3-dioxygenase; MC: Mast cell.

Many studies have supported the idea that an induction of HO-1 or its products can inhibit DC maturation. Chauveau *et al*[39] have proven that the induction of HO-1 directs DC refractory to an LPS-induced maturation. Recent studies have also demonstrated that an HO-1 overexpression inhibits the secretion of cytokines critical for DC maturation, such as IL-12[41]. Indoleamine 2,3-dioxygenase (IDO) is a further mechanism associated with the immunosuppressive activity of imDCs, whereby IDO can inhibit T cell proliferation through tryptophan degradation, and induce Tregs as well[42,43]. An upregulation of HO-1 resulted in an IDO overexpression through CO[6]. The above-mentioned information all demonstrates that by inducing HO-1, the development of DCs could be directed selectively toward a tolerogenic DC type. In chron-

Table 1 Upregulation of heme oxygenase-1 in donor liver to alleviate ischemia-reperfusion injury and rejection

Effective product	Targets	Results	Ref.
HO-1	KC	Preventing IRI	[4]
HO-1	Attenuating apoptosis of SEC	Alleviating IRI	[5]
HO-1	Inhibiting DC maturation	Reducing rejection	[6]
HO-1	Anti-inflammatory differentiation of KC	Preventing IRI	[24]
HO-1	Modulating oxidative stress and proinflammatory mediators	Alleviating IRI	[30]
CO	Suppressing T cell proliferation	Reducing rejection	[31]
HO-1	Microchimerism	Inducing allograft tolerance	[41]
CO	Inhibiting TLR-induced DC maturation	Reducing rejection	[42]
HO-1	Suppressing intragraft infiltration of KC and neutrophils, preventing proinflammatory cytokine and chemokine expression	Alleviating IRI	[50]
HO-1	Inducing Treg	Inducing allograft tolerance	[56]
Biliverdin	Decreasing P-selectin, ICAM-1, iNOS and IL-6	Alleviating IRI	[60]

HO-1: Heme oxygenase 1; CO: Carbon monoxide; KC: Kupffer cell; SEC: Sinusoidal endothelial cell; DC: Dendritic cell; TLR: Toll-like receptor; Treg: Regulatory T cells; ICAM-1: Intercellular adhesion molecule-1; iNOS: Inducible nitric oxide synthase; IL: Interleukin; IRI: Ischemia-reperfusion injury.

ic rejection, graft arterial vasculature remodels after the transplantation. Chronic graft dysfunction is characterized by the development of intimal hyperplasia and narrowing of the vessel lumen[44]. Cheng *et al*[45] have found that a loss of HO-1 in DCs or *HO-1* gene silencing by small interfering RNA upregulated the MHCII expression through CIITA-driven transcriptional regulation and transcription 1 (STAT1) phosphorylation. They have also illustrated that an inhibition of HO-1 in DCs aggravated the development of transplant arteriosclerosis by increasing intimal hyperplasia, and by activating a CD4(+) T cell allograft response mediated by an MHCII upregulation. Therefore, we conclude that the activity of HO-1 is an important regulatory mechanism affecting multiple levels of the immune response to induce tolerance. Elucidating its effects on specific immune cells will aid the development of therapeutic strategies for a variety of inflammatory disorders, including autoimmune diseases and transplant rejection.

UP-REGULATION OF HO-1 AS POTENTIAL THERAPY ON GRAFT PROTECTION IN LIVER TRANSPLANTATION

Based on the aforementioned information, we can conclude that HO-1 plays a potential protective role in both IRI and graft rejection, whereby HO-1 may act as a link between these two events (Table 1). Thus, by upregulating HO-1 within the donor liver allograft, a preconditioned pre-transplantation hepatic status can be provided that may uphold allograft survival and normal function for a long time.

HO-1 is highly inducible by a variety of stimuli including heme, NO, cadmium, growth factors, and hyperoxia. These diverse stimuli act *via* a similarly broad range of signaling pathways. The nuclear factor (NF)-κB and activator proteins-1 and -2 lie in the promoter region of HO-1.The transcription factor NF-E2-related factor-2 is recognized as the key mediator of HO-1 induction and the protective functions of HO-1, as seen in experimental models both *in vivo* and *in vitro*[46]. Due to the potential toxicity of the conventional HO-1 inducers, such as hemin and CoPP, a number of new strategies, including protein transduction, traditional Chinese medicine, adenoviral transduction and others, have attracted substantial interest as potential HO-1 inducers[5,47,48].

Protoporphyrines are prototypic HO-1 inducers *in vitro*. Depending on the metal atom of the porphyrines, the enzymatic HO-1 function is activated (e.g. iron or cobalt atom). However, since porphyrines are heavy metals, their clinical usage has many limitations.

Simvastatin, clinically used as a lipid-lowering drug, is another inducer of HO-1. Lee *et al*[49] have shown that the protective effect of statins on vessels is produced by HO-1. Uchiyama *et al*[50] further demonstrated that simvastatin increases the HO-1 expression by inducing a nuclear translocation of the heat shock factor 1 in vascular endothelial cells. However, there are still several unresolved problems. Simvastatin is administered orally in clinical applications. Uchiyama *et al*[50], however, induced HO-1 by an intraperitoneal injection of a high-dose of simvastatin in their experiments. In view of its potential for a clinical application, a pilot study is necessary to evaluate whether simvastatin administered orally also induces HO-1.

In recent years, traditional Chinese herbal medicine has become popular in inducing HO-1. Sinomenine, a pure alkaloid extracted from the Chinese medical plant *Sinomenium acutum*, has been investigated for its protective effect on hepatic cells affected by an overexpression of HO-1 to attenuate IRI[5]. *Isodon Serra* (*I. Serra*) is another Chinese medicinal herb that has been found to possess the capacity to induce HO-1. It is a perennial herb that has been used widely for the treatment of arthritis, enteritis, jaundice, hepatitis, lepromatous leprosy, ascariasis and acute cholecystitis[51]. Moreover, it has been used in China to treat esophageal cancer. It has an anti-proliferative effect on melanoma cells and many other kinds of malignant cells[52,53]. However, our studies focused on other functions, besides its anti-tumorigenic activities[54,55]. Matsushima *et al*[56] have proven that crassin acetate, a

coral-derived cembrane diterpenoid, can effectively induce HO-1 mRNA/protein expression and HO-1 enzymatic activity in DCs. Nodosin and Oridonin, extracts obtained from *I. Serra*, also belong to a type of diterpenoid. Our previous studies elucidated that Oridonin has an immunosuppressive effect by regulating the cell mitosis cycle and modulating the signal mechanisms of four cytokines (IL-2, IFN-γ, IL-12 and TNFα)[54]. Oridonin, a potent HO-1 inducer, is a promising immunosuppressive drug. Hu *et al*[55] have shown that Oridonin upregulated the HO-1 expression at both the transcriptional and translational levels, and accordingly promoted HO-1 activity *in vitro* in their experiments. However, the exact mechanisms of our findings remain to be further investigated. Nodosin, another *I. serra* extract, also induces HO-1. We used a Nodosin solution *in vitro* to perfuse the isolated liver, while lactic Ringer's solution was used as control. The results showed that the expression of HO-1 in both the mRNA and the protein is higher in the perfused group than in the control group[57]. The potential role of *I. Serra* extract in the upregulation of HO-1 suggested that it is a novel nontoxic drug candidate for liver allograft protection. More models and methods used for the study of *I. Serra* extracts need to be defined in the future investigations. We believe that in the near future, the full pharmacological activity and detailed mechanisms of *I. Serra* will be further described.

FUTURE PROSPECTS

In a clinical setting, however, the inducible HO-1 system still has several limitations. The different effects of HO-1, which are neither exclusively cytoprotective nor exclusively cytotoxic, should be further investigated. The HO-1 induced cytoprotection might be restricted to a narrow threshold of overexpression. Besides, there are no available reagents that can specifically induce HO-1. Therefore, the unintended effects of treatment with non-specific HO-1 inducers would likely present a disadvantage[10]. Although an adenoviral-based HO-1 gene transfer has been attempted *in vivo*, the efficiency of viral transfection is organ dependent. CO may represent a candidate for the treatment of transplanted patients against IRI. However, its therapeutic window must be carefully considered, because the inhalation of high levels can be toxic or even be lethal. Biliverdin and reduced bilirubin may also represent possible candidates for clinical application. We have recently demonstrated that biliverdin had a protective effect in stringent rat liver models of IRI, as evidenced by an improved portal blood flow/bile production and a reduction in hepatocellular damage. It also improved the survival rate in a syngeneic rat OLT model after prolonged cold ischemia[58]. However, because bilirubin in excess can cause neurotoxicity and can act as a lytic agent binding to erythrocyte membranes, the therapeutic window of biliverdin must be examined in detail prior to its clinical use.

From the above, the question arises if HO-1 or its products can be used clinically. Although CO is toxic, beneficial results can be obtained with relatively low doses for appropriate length of time. In rodents, the administration of biliverdin or bilirubin in the first few weeks of life did not reveal much toxicity. Recent evidence indicates that they are not only non-toxic at physiological concentrations in normal cells, they may also have important anti-oxidant, anti-inflammatory, or anti-apoptotic properties[59,60].

Based on this review, which reveals that HO-1 is associated with both processes of IRI and acute rejection, we can conclude that the preconditioning of the donor liver with an upregulation of HO-1 not only attenuates IRI, but also reduces rejection after liver transplantation. HO-1, therefore, seems to stand out as a potential key therapeutic target to maintain graft function and improve the recipients' prognosis in liver transplantation. However, due to its limitations, the therapeutic role of HO-1 must undergo further critical analysis.

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ORIGINAL ARTICLE

Effect of preoperative FOLFOX chemotherapy on CCL20/CCR6 expression in colorectal liver metastases

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Abstract

AIM: To evaluate the influence of preoperative FOLFOX chemotherapy on CCL20/CCR6 expression in liver metastases of stage Ⅳ colorectal cancer (CRC) patients.

METHODS: Using Real Time-PCR, enzyme-linked immunosorbent assay, Western Blots and immunohistochemistry, we have analyzed the expression of CCL20, CCR6 and proliferation marker Ki-67 in colorectal liver metastasis (CRLM) specimens from stage Ⅳ CRC patients who received preoperative FOLFOX chemotherapy (n = 53) and in patients who did not receive FOLFOX chemotherapy prior to liver surgery (n = 29).

RESULTS: Of the 53 patients who received FOLFOX, time to liver surgery was ⩽ 1 mo in 14 patients, ⩽ 1 year in 22 patients and > 1 year in 17 patients, respectively. In addition, we investigated the proliferation rate of CRC cells in liver metastases in the different patient groups. Both CCL20 and CCR6 mRNA and protein expression levels were significantly increased in patients who received preoperative FOLFOX chemotherapy ⩽ 12 mo before liver surgery (P < 0.001) in comparison to patients who did not undergo FOLFOX treatment. Further, proliferation of CRLM cells as measured by Ki-67 was increased in patients who underwent FOLFOX treatment. CCL20 and CCR6 expression levels were significantly increased in CRLM patients who had undergone preoperative FOLFOX chemotherapy.

CONCLUSION: This chemokine/receptor up-regulation could lead to increased proliferation/migration through an autocrine mechanism which might be used by surviving metastatic cells to escape cell death caused by FOLFOX.

Key words: FOLFOX chemotherapy; CCL20/CCR6 expression; Colorectal liver metastases; Proliferation

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INTRODUCTION

Colorectal cancer (CRC) constitutes one of the most common causes of cancer death in the western world. CRC is also a tumor with a high propensity for metastatic spread, mostly to liver and lungs. Liver metastases develop in approximately 50% of CRC patients at some point in the course of their disease and worsen the prognosis for patient survival dramatically[1,2]. To date, long-term survival of patients with colorectal liver metastases (CRLM) can only be achieved by surgical resection. However, in cases where metastases are considered unresectable, chemotherapy is the treatment of choice, although 5-year survival with chemotherapy alone is very poor. Thus, surgical treatment of resectable liver metastases has become the standard treatment[3].

However, surgical resection can only be successful when liver metastases can be thoroughly resected and provided there is no other non-resectable distant metastasis. Moreover, the primary tumor needs to be resectable. Since all these criteria are often not fulfilled, cancer recrudescence occurs in 45% to 75% of CRLM patients within the first five years after primary tumor resection[3,4]. For these reasons, adjuvant chemotherapy has been evaluated in patients with resectable CRLM. To date, the standard approach with regard to adjuvant chemotherapy for CRC patients is a combination of oxaliplatin, fluorouracil and leucovorin[5,6]; termed FOLFOX. Perioperative FOLFOX chemotherapy has been shown to reduce the risk of cancer relapse by a quarter[7]. Thus, the combination of FOLFOX chemotherapy and surgery is a promising tool to improve the prognosis of CRC patients.

It has been shown that both the chemokine CCL20 and its unique receptor CCR6 are expressed in CRC cells[8-11], providing a basis for efficient autocrine and paracrine loops[12]. CCL20 stimulation of CCR6-bearing CRC cells led to increased proliferation and migration *in vitro*[8,9]. Moreover, our recent data suggest that interactions between CCL20 and the corresponding receptor CCR6 are critical components in the regulation of CRC progression and organ selective CRC metastasis to the liver[10,11].

The purpose of this study was to retrospectively analyze the impact of FOLFOX chemotherapy administered to stage Ⅳ CRC patients on CCL20/CCR6 expression in liver metastases. Eighty-two patients underwent radical surgery of the primary CRC and of synchronous and metachronous liver metastases. Twenty-nine patients underwent liver surgery without preoperative FOLFOX and 53 patients received FOLFOX prior to liver surgery. We compared CCL20/CCR6 expression in liver resection specimens from both groups and correlated their expression with proliferation of CRLM cells.

MATERIALS AND METHODS

Materials

Surgical specimens and corresponding normal tissue from the same samples were collected from patients who underwent surgical resection at our department between 2002 and 2008.

Informed written consent for tissue procurement was obtained from all patients and the study was approved by the local ethics commission of the Ärztekammer des Saarlandes.

Eighty-two patients were included in the study, comprising CRC patients who had FOLFOX chemotherapy before liver surgery (n = 53) and CRC patients who did not have FOLFOX before liver surgery (n = 29). Of the 53 patients who received FOLFOX, time to liver surgery was ⩽ 1 mo in 14 patients, ⩽ 1 year in 22 patients and > 1 year in 17 patients, respectively. In every patient sample the corresponding non-affected normal liver tissue was also analyzed, thus a total of 164 samples were analyzed. In the 53 patients who received FOLFOX chemotherapy before liver surgery, two cancers were classified as pT1, six as pT2, forty as pT3 and five as pT4, with positive nodal involvement in 40 cases, according to the UICC TNM classification[13]. In the twenty-nine patients who received no FOLFOX chemotherapy before liver surgery, six cancers were classified as pT1, two as pT2, nineteen as pT3 and two as pT4, with positive nodal involvement in 10 cases. The clinical data and patient characteristics were obtained from a prospective database and are summarized in Table 1. In the group who did not receive FOLFOX, 8 patients underwent CRLM resection less than 6 mo and 21 patients underwent CRLM resection 6 mo and longer after resection of primary tumor. In the patient group who received FOLFOX chemotherapy, 7 underwent CRLM resection less than 6 mo after resection of primary tumor and 45 patients underwent CRLM resection 6 mo and longer after resection of primary tumor. One patient underwent resection of primary tumor and CRLM resection at the same time (Table 2).

Tissue preparation

Tissue specimens were collected immediately after surgical resection, snap frozen in liquid nitrogen and then stored at -80℃ until they were processed under nucleic acid sterile conditions for protein and RNA extraction. For corresponding normal tissue we used adjacent non-affected tissue to the same resected specimen. All tissues obtained were reviewed by an experienced pathologist and examined for the presence of tumor cells. As minimum criteria for usefulness for our study, we only used tumor tissues in which tumor cells constituted at least > 75% of the tumor biopsy.

Single-strand cDNA synthesis

Total RNA was isolated using RNeasy columns from Qiagen (Hilden, Germany) according to the manufacturer's instructions. RNA integrity was confirmed spectrophotometrically and by electrophoresis on 1% agarose gels. For cDNA synthesis, 5 μg of each patient total RNA sample were reverse-transcribed in a final reaction volume of 50 μL containing 1 × TaqMan RT buffer, 2.5 μmol/L random hexamers, 500 μmol/L each dNTP, 5.5 mmol/L $MgCl_2$, 0.4 U/μL RNase inhibitor, and 1.25 U/μL Multiscribe RT. All RT-PCR reagents were purchased from Applied

Table 1 Clinical characteristics of patients with colorectal liver metastasis

Characteristic	CRLM with FOLFOX[1] (*n* = 53)	CRLM without FOLFOX[2] (*n* = 29)
Localization of primary tumor		
Colon	25	13
Rectum	28	16
Gender		
Male	29	17
Female	24	12
Age at surgery (yr)		
Median	61.4	66.7
Range	35-79	43-77
Largest tumor diameter (cm)		
Median	4.7	4.9
Range	1.3-9.7	1.2-10.1
Tumor (T)-category of primary tumor		
pT1	2	6
pT2	6	2
pT3	40	19
pT4	5	2
Lymph node metastasis (N-category)[3]		
Positive	40	10
Negative	13	19
Grade		
G1	0	1
G2	32	17
G3	21	11

[1]Colorectal cancer patients with FOLFOX chemotherapy before colorectal cancer liver metastasis (CRLM) surgery; [2]Colorectal cancer patients without FOLFOX chemotherapy before CRLM surgery; [3]N-category significantly different between FOLFOX and non-FOLFOX patients ($P < 0.05$).

Biosystems (Foster City, CA). The reaction conditions were 10 min at 25℃, 30 min at 48℃, and 5 min at 95℃.

Real-time PCR

All Q-RT PCR assays containing the primer and probe mix were purchased from Applied Biosystems, (Applied Biosystems, Foster City, CA) and utilized according to the manufacturer's instructions. PCR reactions were carried out using 10 μL 2 × Taqman PCR Universal Master Mix No AmpErase® UNG and 1 μL gene assay (Applied Biosystems, Foster City, CA), 8 μL Rnase-free water and 1 μL cDNA template (50 mg/L). The theoretical basis of the qRT assays is described in detail elsewhere[14]. All reactions were run in triplicate along with no template controls and an additional reaction in which reverse transcriptase was omitted to assure absence of genomic DNA contamination in each RNA sample. For the signal detection, ABI Prism 7900 sequence detector was programmed to an initial step of 10 min at 95℃, followed by 40 thermal cycles of 15 s at 95℃ and 10 min at 60℃ and the log-linear phase of amplification was monitored to obtain C_T values for each RNA sample.

Gene expression of all target genes was analyzed in relation to the levels of the slope matched housekeeping genes phosphomannomutase (PMM1) and β2-microglobulin (β2M)[15]. Data analysis was performed according to the relative standard curve method. Data are presented in relation to the respective housekeeping genes.

Table 2 Period between resection of primary tumor and resection of colorectal liver metastasis

Interval	CRLM with FOLFOX[1] (*n* = 53)	CRLM without FOLFOX[2] (*n* = 29)
< 6 mo	7	8
⩾ 6 mo	45	21
0	1	0

[1]Colorectal cancer patients with FOLFOX chemotherapy before colorectal cancer liver metastasis (CRLM) surgery; [2]Colorectal cancer patients without FOLFOX chemotherapy before CRLM surgery.

Isolation of total protein

Protein lysates from frozen tissue were extracted with radioimmunoprecipitation (RIPA) buffer containing Complete, a protease inhibitor cocktail (Roche, Penzberg, Germany). Total protein quantification was performed using the Pierce BCA protein assay reagent kit (Pierce, Rockford, IL., USA).

Sandwich-type enzyme-linked immunosorbent assay

The chemokine protein levels in the different tissue lysates were determined by sandwich-type enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions. Samples were assayed in duplicate with all values calculated as the mean of the two measurements. CCL20 levels were assayed using a validated commercial ELISA (Duo Set R&D Systems, DY360, Minneapolis, MN, USA). The absorbance was read at 450 nm in a 96-well microtiter plate reader. The chemokine concentration from each tissue lysate was normalized to the total protein content of each sample.

Western blotting analysis

Total protein (25 μg/lane) was separated by SDS-PAGE using a 10% gel and blotted onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked by incubation in Tris-buffered saline (TBS) containing 5% nonfat dry milk and 0.1% Tween 20 for 2 h at room temperature and then incubated overnight at 4℃ with goat anti-human CCR6 antibody (diluted 1:500, C2099-70B, Biomol, Hamburg, Germany). Blots were then washed and incubated at room temperature for 1 h with donkey anti-goat HRP antibody (diluted 1:5000, sc-2056, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bands were visualized by ECL Western blotting analysis systems (Amersham Biosciences, Piscataway, NJ, USA). The human cell lysate HL-60 (sc-2209, Santa Cruz Biotechnology, Santa Cruz, CA, USA) served as positive control.

Immunohistochemistry

Resected specimens were routinely fixed with formalin in the immediate postoperative period and paraffin-

embedded within the first three hours after procurement. Before staining, 4 μm thick sections were mounted on Superfrost Plus slides, deparaffinized with xylene, and rehydrated in graded ethanol to deionized water. The sections were treated with an antigen retrieval solution (Target Retrieval, Dakocytomation, Carpinteria, CA) and microwaved. CCL20 and CCR6 staining was performed according to the avidin-biotin-peroxidase reaction (Vectastain ABC ELITE Kit, Vector Laboratories Inc., Burlingame, CA) and Ki-67 staining was performed according to the APAAP method (Dako REAL Detection System, Dako, Glostrup, Denmark, K5000). For CCL20 and CCR6 staining, but not for Ki-67 staining, slides were immersed in 3% hydrogen peroxide for 10 min and then treated with avidin and biotin (Avidin/Biotin blocking kit, Vector Laboratories Inc., Burlingame, CA). Sections for CCL20, CCR6 and Ki-67 staining were incubated with serum followed by an overnight incubation with goat anti-human CCR6 polyclonal antibody (1:125, Biomol, Hamburg, Germany, C2099-70B), goat anti-human CCL20 polyclonal antibody (1:150, R&D, Abingdon, UK AF360) or Ki-67 MIB-1 monoclonal antibody (1:75, Dako, Glostrup, Denmark, M7240). Consequently, immunostaining with the avidin-biotin-peroxidase reaction (Vectastain ABC ELITE Kit, Vector Laboratories Inc., Burlingame, CA) was performed on CCL20 and CCR6 slides and a chromogene aminoethyl-carbazide solution (Tissugnost, Darmstadt, Merck) was used. For detection of Ki-67, the alkaline phosphatase-antialkaline phosphatase complex (APAAP) was used with Fast Red Substrate as chromogen (Dako, Glostrup, Denmark, K0597). Consequently, for all sections counterstaining was performed in hematoxylin solution. Negative controls were performed in all cases omitting primary antibody.

Statistical analysis

All data are presented as mean and SE (standard of the mean). Statistical calculations were done with the MedCalc (MedCalc software, Mariakerke, Belgium) software package[16]. The parametric Student's *t*-test was applied, if normal distribution was given; otherwise, the Wilcoxon's rank sum test was used. Statistical significance was considered on a two-sided significance level (α) of 0.05.

RESULTS

Characteristics of patients

Prior to CRLM resection, 82 patients were enrolled in our study over a 6 year period. The median age of patients at surgery was 61.4 years (35-79) in the FOLFOX group and 66.7 years (43-77) in the patient group without FOLFOX. There were 29 males and 24 females in the FOLFOX group and 17 male and 12 female patients in the non-FOLFOX patient group. The demographic and clinical characteristics of patients are shown in Tables 1 and 2. FOLFOX and non-FOLFOX patients showed no statistically relevant differences with respect to T-stage, grading and timing between primary tumor resection and CRLM resection. However, with respect to N-stage our data revealed a significant difference between FOLFOX and non-FOLFOX patients ($P < 0.05$), as shown in Table 1. Thus, the non-FOLFOX group included a higher percentage of patients without lymph node metastasis (65.5%) compared to the FOLFOX group under investigation (24.5%).

Impact of preoperative FOLFOX chemotherapy on CCL20 expression

Significantly greater levels of CCL20 mRNA and CCL20 protein expression were observed in CRLM tissues compared to corresponding normal tissue in all patients ($P < 0.05$ and $P < 0.001$, respectively) (Figures 1 and 2). However, patients who were preoperatively treated with FOLFOX chemotherapy showed significantly higher levels of CCL20 mRNA and CCL20 protein expression as compared to patients without FOLFOX treatment ($P < 0.05$) (Figures 1A and 2A). When the interval between FOLFOX treatment and surgery was considered, only patients who received FOLFOX chemotherapy $\leqslant$ 12 mo before liver surgery expressed significantly higher amounts of CCL20 mRNA and CCL20 protein, as compared to patients without FOLFOX treatment, respectively ($P < 0.05$) (Figures 1B and 2B).

Immunostaining of the CRLM tissue revealed positive staining for CCL20 in 56% (16/29) of patients without and in 87% (46/53) of patients with preoperative FOLFOX chemotherapy (Figure 3A and B), respectively. CCL20 was immunolocalized with lesser intensity in the tumor tissue sections of CRLM patients without FOLFOX, as shown for a representative patient in Figure 3A, compared to patients who underwent preoperative FOLFOX chemotherapy, as shown in Figure 3B for a representative patient.

Impact of preoperative FOLFOX chemotherapy on CCR6 expression

Both CCR6 mRNA and CCR6 protein expression levels were significantly increased in CRLM tissues of all patients compared to the corresponding normal liver tissue ($P < 0.05$) (Figure 4). Further, CCR6 mRNA and CCR6 protein expression levels were significantly higher in CRLM tissues of patients who underwent preoperative FOLFOX chemotherapy compared to patients without FOLFOX ($P < 0.05$) (Figure 4A). CCR6 up-regulation was limited to those patients who received preoperative FOLFOX chemotherapy $\leqslant$ 12 mo before liver surgery ($P < 0.05$) (Figures 4B and 5).

Immunostaining revealed positive staining for CCR6 in 59% (17/29) of patients without and in 87% (46/53) of patients with preoperative FOLFOX chemotherapy (Figure 3C and D), respectively. CCR6 staining intensities were stronger in the FOLFOX group. However, intense laminar CCR6 immunostaining was found mainly in the benign-appearing tissue sections of CRLM patients. Thus, CCR6 staining localized to a streak of hepatocytes along the tumor invasion front. These hepatocytes appeared clearly distinct from normal hepatocytes (Figure 3C and D).

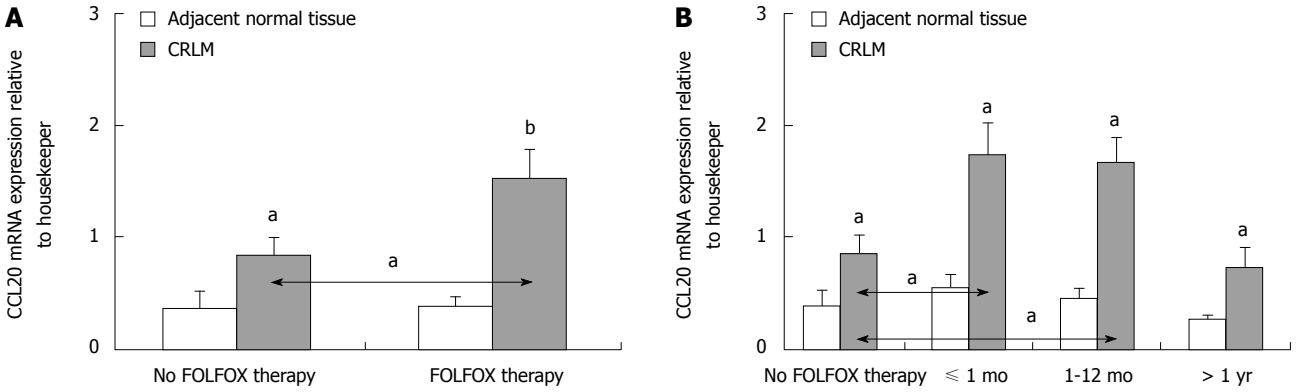

Figure 1 CCL20 mRNA expression relative to PMM1 and β2M in colorectal liver metastasis patients with (*n* = 53) and without (*n* = 29) FOLFOX chemotherapy before colorectal liver metastasis surgery (A) and itemized according to different time periods of FOLFOX treatment before colorectal liver metastasis surgery (no FOLFOX therapy, *n* = 29; FOLFOX treatment ≤ 1 mo, *n* = 14; FOLFOX treatment ≤ 1 year, *n* = 22; FOLFOX treatment > 1 year, *n* = 17) (B). Q-RT-PCR data are expressed as mean ± SE, $^{a}P < 0.05$ and $^{b}P < 0.001$, respectively. CRLM: Colorectal liver metastasis.

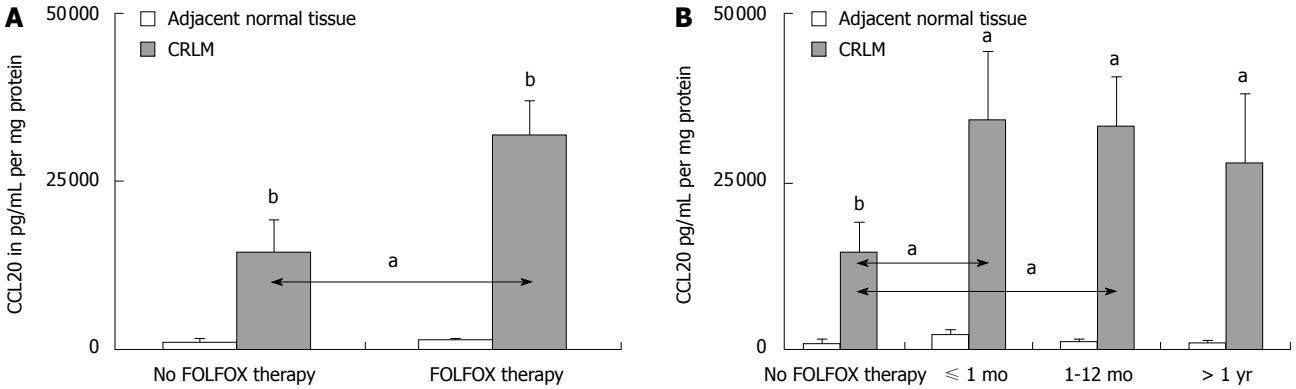

Figure 2 CCL20 protein concentrations (pg/mL pro mg total protein) in colorectal liver metastasis patients with (*n* = 53) and without (*n* = 29) FOLFOX chemotherapy before colorectal liver metastasis surgery (A) and itemized according to different time points of FOLFOX treatment before colorectal liver metastasis surgery (no FOLFOX therapy, *n* = 29; FOLFOX treatment ≤ 1 mo, *n* = 14; FOLFOX treatment ≤ 1 year, *n* = 22; FOLFOX treatment > 1 year, *n* = 17) (B). Protein data are expressed as mean ± SE, $^{a}P < 0.05$ and $^{b}P < 0.001$, respectively. CRLM: Colorectal liver metastasis.

Impact of FOLFOX on proliferation of CRLM cells

Proliferation of CRLM cells as demonstrated by Ki-67 staining revealed a more frequent immunostaining pattern in CRLM tissues of patients who underwent preoperative FOLFOX (Figure 3F) compared to patients without FOLFOX (Figure 3E). In CRLM patients without FOLFOX treatment (*n* = 29) (Figure 3E) we observed weak or no Ki-67 immunostaining in 11 patients (38%), moderate immunostaining intensities in 13 patients (45%) and strong immunostaining intensities in 5 patients (17%). In CRLM patients with preoperative FOLFOX treatment (*n* = 53) (Figure 3F) we observed weak or no Ki-67 immunostaining in 16 patients (30%), moderate immunostaining intensities in 11 patients (21%) and strong immunostaining intensities in 26 patients (49%).

DISCUSSION

At present, standard treatment of CRC patients without distant metastasis consists of surgical resection of the primary tumor followed by adjuvant FOLFOX chemotherapy in patients with lymph node metastasis[17]. For patients with synchronous liver metastasis there are three treatment options: colectomy with synchronous or heterochronous CRLM surgery; perioperative chemotherapy with FOLFOX, FOLFIRI or CapeOX followed by colectomy and CRLM resection; and colectomy followed by chemotherapy and staged CRLM resection[5,6]. As liver resection offers the chance of long-term survival only for patients with resectable CRLM, chemotherapy is often applied to render formerly unresectable CRLM patients resectable. Moreover, superior survival for patients who have undergone resection has been demonstrated by several studies[18,19]. This improved survival may be due to the lower tumor burden.

The impact of chemotherapy on metastatic lymph node lesions was addressed in another study, where patients with 4 or more lymph node metastases around the primary cancer were considered to benefit from perioperative chemotherapy[20]. Chemotherapy options for metastatic CRC have significantly changed in recent years. While the optimal adjuvant systemic chemotherapy has yet to be determined, FOLFOX treatment seems to be one of the most effective CRLM treatment options[21] and perioperative FOLFOX chemotherapy is most commonly used to reduce the risk of cancer relapse in CRC patients[7].

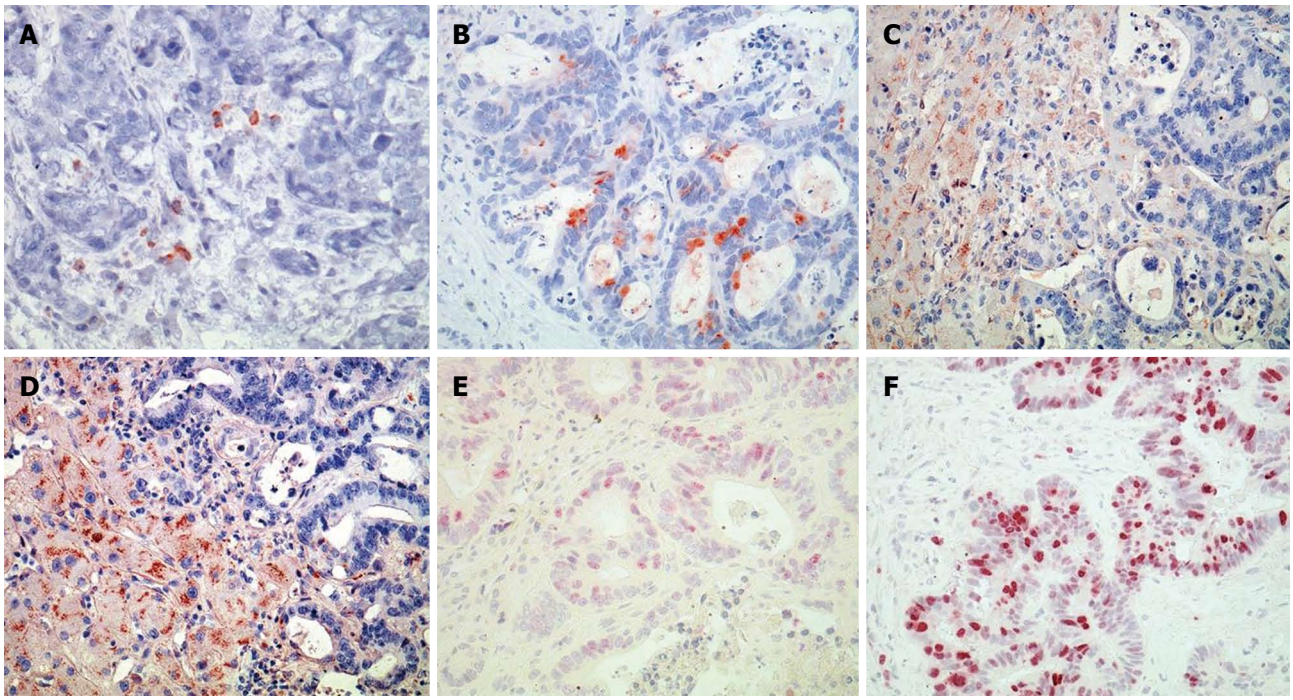

Figure 3 Immunohistochemical staining (original magnification × 200). Representative examples of CCL20 (A, B), CCR6 (C, D) and Ki-67 (E, F) protein expression in colorectal liver metastasis specimens from a colorectal liver metastasis patient without (A, C, E) and with preoperative FOLFOX treatment less than 1 mo before surgery (B, D, F), respectively.

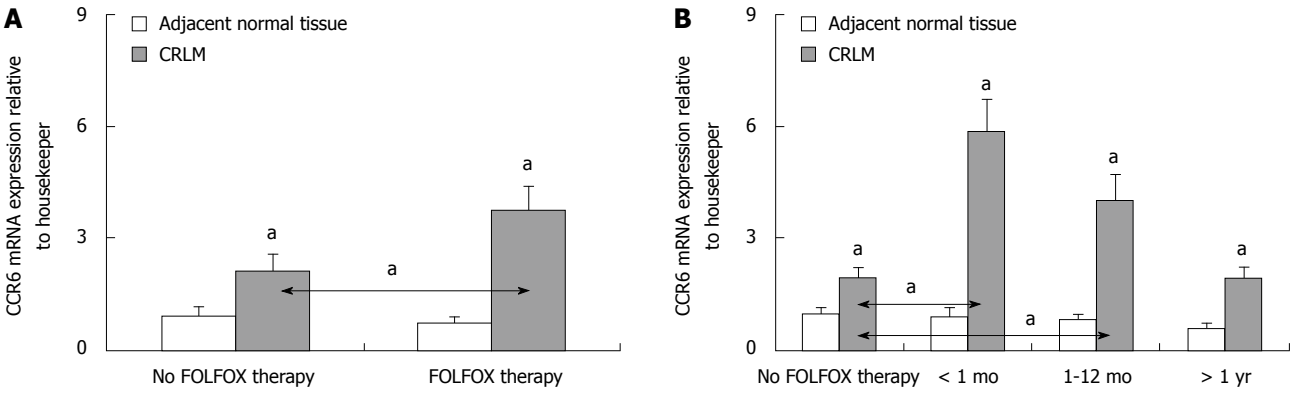

Figure 4 CCR6 mRNA expression relative to PMM1 and β2M in CRLM patients with (*n* = 53) and without (*n* = 29) FOLFOX chemotherapy before CRLM surgery (A) and itemized according to different time points of FOLFOX treatment before CRLM surgery (no FOLFOX therapy, *n* = 29; FOLFOX treatment ≤ 1 mo, *n* = 14; FOLFOX treatment ≤ 1 year, *n* = 22; FOLFOX treatment > 1 year, *n* = 17) (B). Q-RT-PCR data are expressed as mean ± SE, $^{a}P < 0.05$.

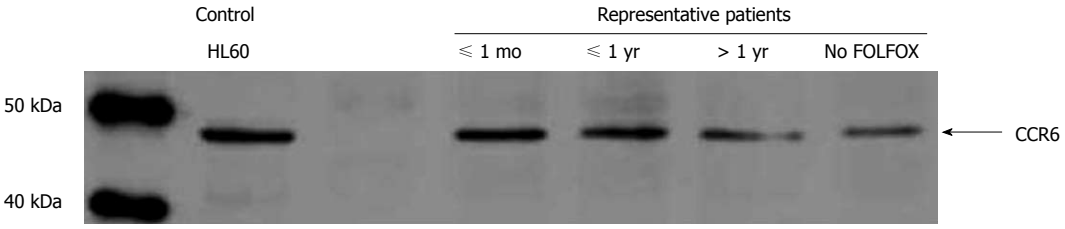

Figure 5 Expression of chemokine receptor CCR6 in colorectal liver metastasis patients itemized according to different time points of FOLFOX treatment before colorectal liver metastasis surgery as determined by Western blotting analysis. Total cell lysates of tumor (P) were immunoblotted with antibodies specifically recognizing chemokine receptor CCR6. Acute leukemia cell line HL60 served as a positive control for the detection of CCR6.

Although pathological effects of chemotherapy for CRLM have been discussed[21-24], the pathological response to chemokine expression in CRLM after chemotherapy has not been reported. Since CCL20 and CCR6 have both been

shown to be expressed on CRC cells[10,11] and CCL20 stimulation of CCR6-bearing CRC cells led to increased proliferation and migration *in vitro*[8,9], we investigated the impact of FOLFOX chemotherapy in stage Ⅳ CRC patients on CCL20/CCR6 expression in liver metastatic tissue.

Our results have shown that both CCL20 and CCR6 expression were significantly increased in patients who had received preoperative FOLFOX chemotherapy ≤ 12 mo before liver surgery as compared to patients who had not received FOLFOX chemotherapy prior to liver surgery. While CCL20 expression was significantly elevated in CRLM tissues, we detected CCR6 signals only sporadically in the tumor cells. Yet, CCR6 expression appeared rather cumulative in deformed hepatic cells along the tumor invasion front, which may represent a stimulative signal for the tumor to further expand into the neighboring hepatic tissue. Further, we demonstrated that CRLM cells of patients who had preoperative FOLFOX chemotherapy are characterized by an increased proliferation rate. This was measured by the expression of the human Ki-67 protein which is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes[25].

Investigating the proliferation of CRLM cells in CRLM resection specimens, we measured proliferation after the event of FOLFOX exposure. FOLFOX treatment promotes apoptosis of CRC cells, thus inhibiting their proliferation. However, after FOLFOX chemotherapy, not only cell proliferation as measured by KI-67 staining, but also CCL20 and CCR6 expression levels, were increased as compared to patients without FOLFOX chemotherapy. *In vitro* data have shown an increased proliferation and migration of CCR6-bearing tumor cells after CCL20 stimulation[8,9]. Thus, the observed up-regulation of CCL20 expression by surviving metastatic CRC cells after FOLFOX treatment might represent a CCL20/CCR6 dependent autocrine mechanism, potentially leading to increased proliferation and migration. Since FOLFOX chemotherapy induces non-specific cell death, this mechanism might be used by surviving metastatic cells within the liver to escape FOLFOX-induced cell death. Interestingly, if the interval between preoperative FOLFOX chemotherapy and liver surgery was > 1 year, the up-regulation of neither CCL20 nor CCR6 remained statistically significant. Generally, this suggests that the up-regulation of CCL20 and CCR6 by FOLFOX is a temporary event.

The disruption of chemokine/chemokine receptor interactions is a promising strategy in the treatment of cancer. A CCR5 inhibitor is already in the clinic for the treatment of human immunodeficiency virus (HIV)-infected patients. Other different chemokine antagonists are currently under investigation in phase Ⅰ-Ⅲ trials for infectious diseases, autoimmune diseases and cancer. Since CCR6 and CCL20 may play a role in CRC, leading to proliferation and migration *via* autocrine or paracrine mechanisms, progression of CRC might be advantaged by CCR6/CCL20 interactions. Thus, the effect of chemotherapy on the expression of cancer-related chemokines and their receptors might explain in part the frequent recurrence of metastasis in patients after this treatment. Therefore, CCL20 and CCR6 interactions may constitute a potential new target for specific treatment interventions in the treatment of CRC. Such a novel approach might be effective in combination with FOLFOX as preoperative treatment prior to resection of CRLM.

COMMENTS

Background

Although long-term survival of patients with colorectal liver metastases (CRLM) can only be achieved by surgical resection, cancer recrudescence can only be avoided if CRLM are thoroughly resected and if no other non-resectable distant metastases are present. As liver resection offers the chance of long-term survival only for patients with resectable CRLM, chemotherapy is often applied to render formerly unresectable CRLM patients resectable. Thus, a combination of perioperative chemotherapy and surgery is frequently applied to improve prognosis in colorectal cancer (CRC) patients.

Research frontiers

As a standard approach of adjuvant chemotherapy, a combination of oxaliplatin, fluorouracil and leucovorin (termed FOLFOX) is most commonly used to reduce the risk of cancer relapse in CRC patients. Although pathological effects of chemotherapy for CRLM have been discussed, the pathological response regarding chemokine expression in CRLM after chemotherapy has not been reported. Thus, application of FOLFOX may enhance the expression of chemokines which are known to be up-regulated with CRC.

Innovations and breakthroughs

Recently, interactions of the chemokine/chemokine receptor pair CCL20/CCR6 became known as critical components in the regulation of CRC progression and organ selective CRC metastasis to the liver. Thus, the authors retrospectively analyzed the impact of FOLFOX chemotherapy in stage Ⅳ CRC patients on CCL20/CCR6 expression in liver metastases. The results have shown that both CCL20 and CCR6 expression levels were significantly increased in patients who had received preoperative FOLFOX chemotherapy ≤ 12 mo before liver surgery as compared to patients who had not received FOLFOX chemotherapy prior to liver surgery.

Applications

As the disruption of chemokine/chemokine receptor interactions is a promising strategy in the treatment of cancer, various chemokine antagonists are currently under investigation in phase I-III trials for infectious diseases, autoimmune diseases and cancer. Since CCL20/CCR6 interactions may play a role in the progression of CRC, the effect of chemotherapy on the expression of cancer-related chemokines and their receptors might explain in part the frequent recurrence of metastasis in patients after this treatment. Thus, CCL20/CCR6 interactions may constitute a potential target for specific CRC treatment interventions, especially in combination with FOLFOX as preoperative treatment prior to CRLM resection.

Peer review

The authors have investigated the expression of CCL20/CCR6 in resected liver metastases of colorectal cancer with regard to preoperative FOLFOX chemotherapy. In their retrospective data analysis, they found a correlation of chemotherapy with an upregulation of CCL20/CCR6 after FOLFOX treatment within 12 mo before surgery. This result is a descriptive observation without further elucidation of the underlying mechanisms. However, it may be a basis for further research in this field to clarify tumor cell resistance against specific chemotherapeutic agents.

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ORIGINAL ARTICLE

Interferon-γ inhibits ghrelin expression and secretion *via* a somatostatin-mediated mechanism

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Abstract

AIM: To investigate if and how the proinflammatory cytokine interferon γ (IFNγ) affects ghrelin expression in mice.

METHODS: The plasma concentration of ghrelin, and gastric ghrelin and somatostatin expression, were examined in wild-type mice and mice infected with *Helicobacter pylori* (*H. pylori*). Furthermore, ghrelin expression was examined in two achlorhydric mouse models with varying degrees of gastritis due to bacterial overgrowth. To study the effect of IFNγ alone, mice were given a subcutaneous infusion of IFNγ for 7 d. Finally, the influence of IFNγ and somatostatin on the ghrelin promoter was characterized.

RESULTS: *H. pylori* infection was associated with a 50% reduction in ghrelin expression and plasma concentration. Suppression of ghrelin expression was inversely correlated with gastric inflammation in achlorhydric mouse models. Subcutaneous infusion of IFNγ suppressed fundic ghrelin mRNA expression and plasma ghrelin concentrations. Finally, we showed that the ghrelin promoter operates under the control of somatostatin but not under that of IFNγ.

CONCLUSION: Gastric infection and inflammation is associated with increased IFNγ expression and reduced ghrelin expression. IFNγ does not directly control ghrelin expression but inhibits it indirectly *via* somatostatin.

Key words: Ghrelin; Interferon-γ; Somatostatin; Inflammatory diseases; *Helicobacter pylori*

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INTRODUCTION

The gastric peptide hormone ghrelin is, in adults, predominantly produced in P/D$_1$ endocrine cells in humans or in A-like endocrine cells in rats and mice, which are located in the oxyntic glands of the gastric corpus[1-5]. Within the oxyntic glands, ghrelin-containing cells are found from the neck to base in both rats and humans[2,6-8]. Ghrelin-producing cells are also found in the antrum of the stomach and proximal small intestine as well as in other organs[2,9-14], but these sites are of lesser importance as the plasma ghrelin concentrations are reduced by 65% after gastrectomy[13]. Plasma ghrelin consists of two forms; the active acylated ghrelin, which is the ligand for the GH secretagogue (GHS) receptor, and the non-acylated ghrelin, which constitutes greater amounts in the blood than the acylated form[11]. Ghrelin is involved in energy homeostasis and ghrelin plasma concentrations are decreased in obesity and increased in states of negative energy balance such as fasting, anorexia or cachexia[11] as well as being inversely correlated to body mass index (BMI) and insulin secretion[11,13]. Ghrelin plasma concentrations increase before meals and decrease after eating[15,16]. However, to what degree ghrelin is important as a meal initiator or cause of increased caloric ingestion in obesity has not yet been determined[17].

Recently, several studies have found that infection with the gram-negative bacteria *Helicobacter pylori* (*H. pylori*) reduces ghrelin concentrations in both humans[7,18,19] and rodents[20]. With regard to various upper gastrointestinal diseases, plasma concentrations of ghrelin were lowest in chronic gastritis and gastric ulcer and highest in acute gastritis[21]. Furthermore, children infected with *H. pylori* have faltering growth[22,23], which suggests that *H. pylori* could alter signals from the stomach related to the control of growth and body weight[24].

The inflammation that occurs in the *H. pylori*-infected host is a Th1-dominated immune reaction which is regulated by, among others, the lymphocyte-derived cytokine interferon-γ (IFNγ)[25]. In the gastrin knockout (KO) mouse, which is another model for chronic gastritis due to bacterial overgrowth, we and others have also found increased gastric production of IFNγ and expression of IFNγ regulated transcripts[26,27]. Furthermore, IFNγ is one of the major cytokines behind the inflammatory response to *H. pylori* as no inflammation occurs during *H. pylori* infection without the presence of IFNγ[25]. Finally, infusion of IFNγ triggers inflammation *in vivo* without *H. pylori*[26]. Since approximately 50% of the world population is infected with this bacteria[28], knowledge of the factors modulating body weight during *H. pylori* infection could have great impact on health in general. Since little is known about the factors that regulate ghrelin expression during *H. pylori* infection and gastric inflammation[29], we examined the effect of IFNγ on ghrelin expression in mice.

MATERIALS AND METHODS

Mice

Groups of wild-type (wt) C57BL/6J mice (aged 12-16 wk), KO mice which are gastrin deficient (aged 12-16 wk or 48-56 wk)[30], histidine decarboxylase (HDC) KO mice (aged 48-56 wk)[31] and matching control mice were used. All mice were male mice that had been backcrossed to the C57BL/6J mouse strain. The mice were kept under specific pathogen-free conditions and monitored according to the Federation of European Laboratory Animal Science Associations recommendations[32] with 12 h light, 12 h dark cycles. The study was approved by the Danish Animal Welfare Committee.

H. pylori infection

C57BL6/J mice (n = 10) were inoculated with a non-mouse-adapted clone of *H. pylori* strain 67:21, originally isolated from an antral biopsy obtained from a Swedish female with gastric ulcer. The strain is VacA$^+$ and contains the entire Cag pathogenicity island (PAI) with genetic stability in the Cag PAI[33]. The mice were inoculated every second day (three times) during a 5-d period. After the mice had been sacrificed, DNA was extracted and analyzed for the presence of *Helicobacter* species using a semi-nested polymerase chain reaction-denaturing gradient gel electrophoresis assay, specific for the genus *Helicobacter*, as described previously[34]. A matched group of uninfected C57BL6/J mice were used as controls. All animal experiments were approved by the Danish Animal Welfare Committee (2005/562-40) and the Danish Forest and Nature Agency (20010077355/6).

IFNγ infusion

Wild-type mice were given a continuous subcutaneous IFNγ infusion (8 μg/kg per hour or 24 μg/kg per hour for 7 d) for each group (n = 6) using osmotic minipumps (Alzet no.2001; Alza Corp., Cupertino, CA). Control mice received a saline infusion instead. The lower dose of IFNγ equals the dose of IFNγ used by Kang *et al*[26].

Tissue and plasma collection

The mice were anesthetized with intraperitoneal 2,2,2 tribromoethanol (Sigma-Aldrich Corp., St. Louis, MO), blood was collected in EDTA-tubes and the stomachs removed. The stomachs of all mice were dissected into fundus and antrum and immediately placed in liquid nitrogen. Plasma and tissue was subsequently stored at -80℃ until further analysis.

Measurement of plasma ghrelin

Plasma ghrelin was measured in EDTA plasma without extraction using RIA no. RK-031-31 (Phoenix Peptides, Belmont, CA). This assay measures the sum of Ser3-octanoyl and Ser3-des-octanoyl ghrelin peptides. The assay has a detection limit of 20 pmol/L, an interassay variation of 13%, and an intra-assay variation of 5%[4].

mRNA extraction and analysis

The stomachs were dissected into fundus and antrum and immediately placed in liquid nitrogen. RNA was extracted using the method described by Chomcynski and

Sacchi[35], and quantitative changes in the specific mRNA were determined by real-time PCR using the Lightcycler (Roche, Mannheim, Germany) as described by Chen *et al*[36]. Quantitations were performed using one of the following primer sets for each analysis: Ghrelin forward primer (FP) 5'-TCTGCAGTTTGCTGCTACTCA-3' and ghrelin reverse primer (RP) 5'-CCTCTTTGACCTCTTCCCAGA-3'; IFNγ FP 5'-CCTTTGGACCCTCTGACTTG-3' and IFNγ RP 5'-CATCCTTTTGCCAGTTCCTC-3'; gastrin FP 5'-CACTTCATAGCAGACCTGTCCA-3' and gastrin RP 5'-CTGGCCTCTGGAAGAGTGTT-3'; somatostatin FP 5'-CCCAGACTCCGTCAGTTTCT-3' and somatostatin RP 5'-TCAGAGGTCTGGCTAGGACAA-3'; iNOS FP 5'-ACCCCTGTGTTCCACCAGGAGATGTTGAA-3' and iNOS RP 5'-TGAAGCCATGACCTTTCGCATTAGCATGG-3' and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) FP 5'-GGTGCTGAGTATGTCGTGGA-3' and GAPDH RP 5'-GTGGTTCACACCCATCACAA-3'. Each run consisted of one negative control, one sample in which the Moloney murine leukemia virus reverse transcriptase had been omitted in the reverse transcription (RT) step, a standard curve generated by 3-fold serial dilution of RT reactions, and seven to nine RT reactions from each of the three strains. Expression of a given transcript was normalized to a GAPDH quantification performed on the same RT reaction as previously described[36].

Immunohistochemical analysis

Stomachs were rinsed in ice-cold PBS, fixed in 4% paraformaldehyde in PBS for 4-6 h and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin. Immunohistochemistry was performed using the rabbit ghrelin antibody H-031-31 diluted 1:1 000 (Phoenix Peptides) detected with Envision-DAB+ (Dako, Glostrup, Denmark) as previously described[37]. The specificity of the immunostaining was tested by absorbing the primary antibodies with antigen before applying them to the slides or omitting the primary antibody when purified antigen was not available. The morphometrical analysis was performed by cell counting in transversely cut sections as described[38].

Cell lines

NCI-H727 cells were grown in RPMI 1640 media (Invitrogen, Carlsbad, CA), 10% FBS (Biowest, Nuaillé, France), *penicillin* (100 U/mL) and *strep*tomycin (100 μg/mL) (Invitrogen) and cultured at 37℃ in 5% CO_2.

Plasmids and transient transfections

A 2.5 Kb fragment containing the mouse ghrelin promoter and exon one was amplified from C57BL6/J genomic DNA using the Expand kit (Roche, Mannheim, Germany) using mGhrMluI primer 5'-ATATACGCGTGTAGAACACTCACCCTAAATCTG-3' and mGhrXhoI primer 5'-ATATCTCGACTGCCTGGGGATGTGGTGCCTG-3'. The fragment was ligated into the pGL3 Bacic reporter vector (Promega, Madison, WI). The promoter sequence was confirmed by sequencing. One day before transfection, 500 000 NCI-H727 cells were seeded in 6-well dishes coated with 0.01% poly-L-lysine. The NCI-H727 cells were transfected using 6 μL TurboFect™ *in vitro* Transfection Reagent (Fermentas, Burlington, Canada); 2 μg ghrelin promoter plasmid and 1 μg pRL-0 (Promega, Madison, WI) were mixed with 200 μL GIBCO™ Opti-MEM I (Invitrogen, Carlsbad, CA) and incubated for 20 min before application to the cells. Twenty-four hours later, the cells were FBS starved in RPMI1640 media containing 0.5% FBS (Biowest, Nuaillé, France) for 24 h before treatment with forskolin (10 mg, Sigma-Aldrich, St. Louis, MO), IBMX (≥ 99.9%, Sigma-Aldrich, St. Louis, MO), octreotide (200 μg/mL, Mayne Pharma, Melbourne, Australia), or IFNγ (0.2 mg/mL, Immukine, Boehringer Ingelheim, Ingelheim, Germany), for 24 h alone or in combination. All treatments were performed in triplicate. IBMX and forskolin were dissolved in 99.9% DMSO (Merck, Darmstadt, Germany). Cells were then harvested and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System according to the instructions by the manufacturer (Promega, Madison, WI) and normalized to Renilla luciferase activity.

Statistical analysis

Student's unpaired *t*-test statistics were used and differences with a $P \leqslant 0.05$ were considered significant. Unless otherwise stated, results are given as mean ± SD.

RESULTS

H. pylori infection is associated with an IFNγ inflammatory response and with suppression of ghrelin expression

The mice were sacrificed 2 mo after inoculation, as earlier studies had shown that a *cag*-dependent inflammation of the corpus mucosa develops at this time and results in a severe active and chronic gastritis[39,40]. At 2 mo, seven out of ten mice tested positive for *H. pylori* using semi-nested PCR with primers for *H. pylori* CagA and urease genes. The non-infected mice were subsequently excluded. All control mice tested negative.

The infection of the mouse stomach by *H. pylori* caused a 2- to 3-fold increase in the fundic expression of IFNγ and of inducible nitric oxide synthase (iNOS) (Table 1). Furthermore, during the 2 mo infection, the *H. pylori*-infected mice did not gain weight in contrast to wild-type mice (Figure 1A). Ghrelin mRNA expression was reduced to 55% in the *H. pylori*-infected mice (Figure 1B). This was associated with a 49% decrease in the plasma ghrelin concentration (Figure 1C). The reduced ghrelin mRNA expression presumably reflects a reduced expression in each cell as opposed to cell atrophy, as the density of fundic ghrelin cells was unaffected (Table 1). In the infected mice the expression of antral somatostatin was suppressed, whereas the fundic somatostatin expression was increased (Table 1).

Ghrelin expression was reduced in old gastrin knockout mice, but not in young gastrin and old histidine decarboxylase knockout mice

To test whether the altered ghrelin expression could also be

Table 1 Fundic somatostatin mRNA increases after 2 mo of *Helicobacter pylori* infection in wild-type mice (mean ± SE)

	Fundus			Antrum		
	Control	*H. pylori*	*P*	Control	*H. pylori*	*P*
IFNγ mRNA	1.0 ± 0.2	1.8 ± 0.3	< 0.05	1.0 ± 0.1	4.1 ± 0.9	< 0.05
iNOS mRNA	1.0 ± 0.1	1.5 ± 0.1	< 0.05	1.0 ± 0.2	2.2 ± 0.5	< 0.05
Somatostatin mRNA	1.0 ± 0.2	1.4 ± 0.2	< 0.05	1.0 ± 0.2	0.4 ± 0.1	< 0.05
Ghrelin cells (#/mm mucosa)	25 ± 3	27 ± 5	NS	8 ± 2	7 ± 3	NS

The expression of interferon γ (IFNγ), iNOS and somatostatin mRNA in arbitrary units in *H. pylori*-infected mice (n = 7) 2 mo after inoculation or in uninfected control mice (n = 7). Ghrelin cell density was unchanged. *H. pylori*: *Helicobacter pylori*; NS: Non-significant.

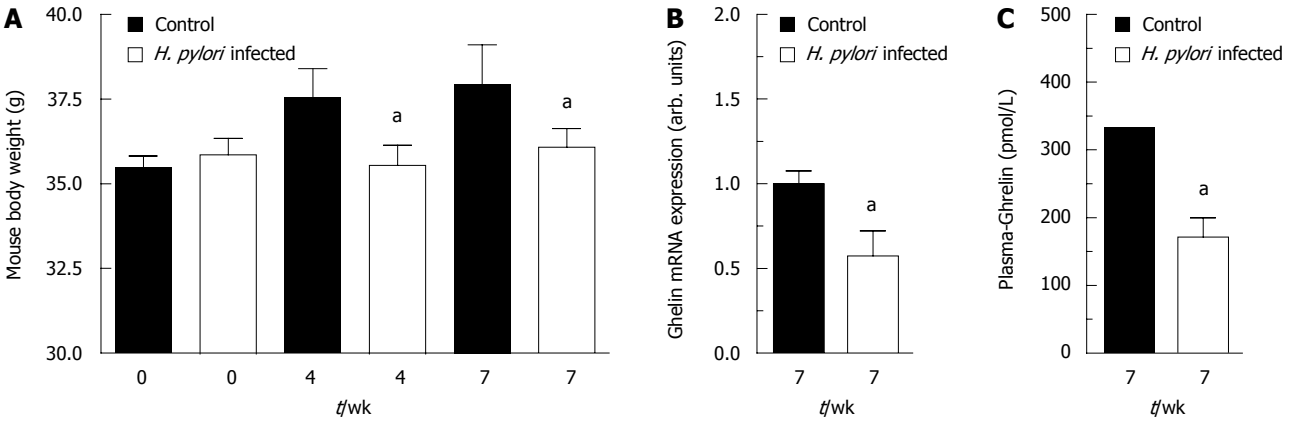

Figure 1 Mice infected with *Helicobacter pylori* have reduced ghrelin expression and do not gain weight. C57BL6/J mice were infected with *Helicobacter pylori* (*H. pylori*) strain 67:21 [this strain is VacA+ and contains a complete genetically stable Cag pathogenicity island (PAI)]. While the control mice gained weight the infected mice did not (A). Mice infected with *H. pylori* had reduced ghrelin expression in the stomach (B) and reduced ghrelin plasma concentrations (C). $^{a}P < 0.05$ *vs* control.

found in other mouse models with gastric inflammation, we examined the expression of IFNγ and ghrelin in two other mouse models; the achlorhydric gastrin KO mice and the hypochlorhydric histidine decarboxylase (HDC) KO mice[41,42].

IFNγ expression was not induced in the old HDC KO mice (Figure 2A), and the ghrelin expression was unchanged in these mice (Figure 2B). Young gastrin KO mice only had moderate inflammation, while the old mice had more inflammation when evaluated by higher IFNγ expression (Figure 2C). Ghrelin expression was unaffected in young gastrin KO mice but reduced in old gastrin KO mice (Figure 2D).

IFNγ suppresses fundic ghrelin mRNA expression and plasma ghrelin concentrations

Since both the *H. pylori* infection and the bacterial overgrowth in the gastrin KO mice were associated with increased expression of IFNγ and reduced ghrelin expression, we examined the effect of IFNγ on ghrelin expression. The fundic ghrelin expression was approximately 30 times higher than the antral (Figure 3A and B). The fundic expression of ghrelin mRNA was halved at both infusion rates of IFNγ (8 μg/kg per hour and 24 μg/kg per hour) examined compared to expression levels in wt mice (Figure 3A and B). In contrast, antral ghrelin expression did not alter significantly under IFNγ infusion at either dose. The reduction in ghrelin expression presumably reflects a reduced expression in each cell as opposed to cell atrophy, as infusion of IFNγ did not change the density of fundic ghrelin cells (Figure 3D). The reduced ghrelin expression was correlated with a 40% reduction in plasma ghrelin concentrations at 7 d of IFNγ infusion (Figure 3C). Furthermore, IFNγ infusion induced the expression of fundic somatostatin, whereas the antral somatostatin expression did not change under the influence of IFNγ (Table 2).

The ghrelin promoter operates under the control of somatostatin but not under that of IFNγ

We next examined the effect of IFNγ and somatostatin on the transcriptional regulation of ghrelin using a 2 kb ghrelin promoter construct. The experiments were carried out in NCI-H727 cells since these are carcinoid cells expressing both somatostatin receptor 2 (SSTR2) and SSTR5. This indicates that they could be a good model for A-like cells in the stomach (Døssing, unpublished data). Treatment with IFNγ did not affect the activity of the ghrelin promoter construct. In contrast, forskolin and IBMX both independently and together activated the 2 kb promoter (Figure 4). Moreover, treatment with octreotide (somatostatin analog) reduced the basal ghrelin promoter activity (Figure 4) as well as forskolin/IBMX-induced ghrelin promoter activation in a dose-dependent manner.

DISCUSSION

Our results show that the gastric expression of ghrelin

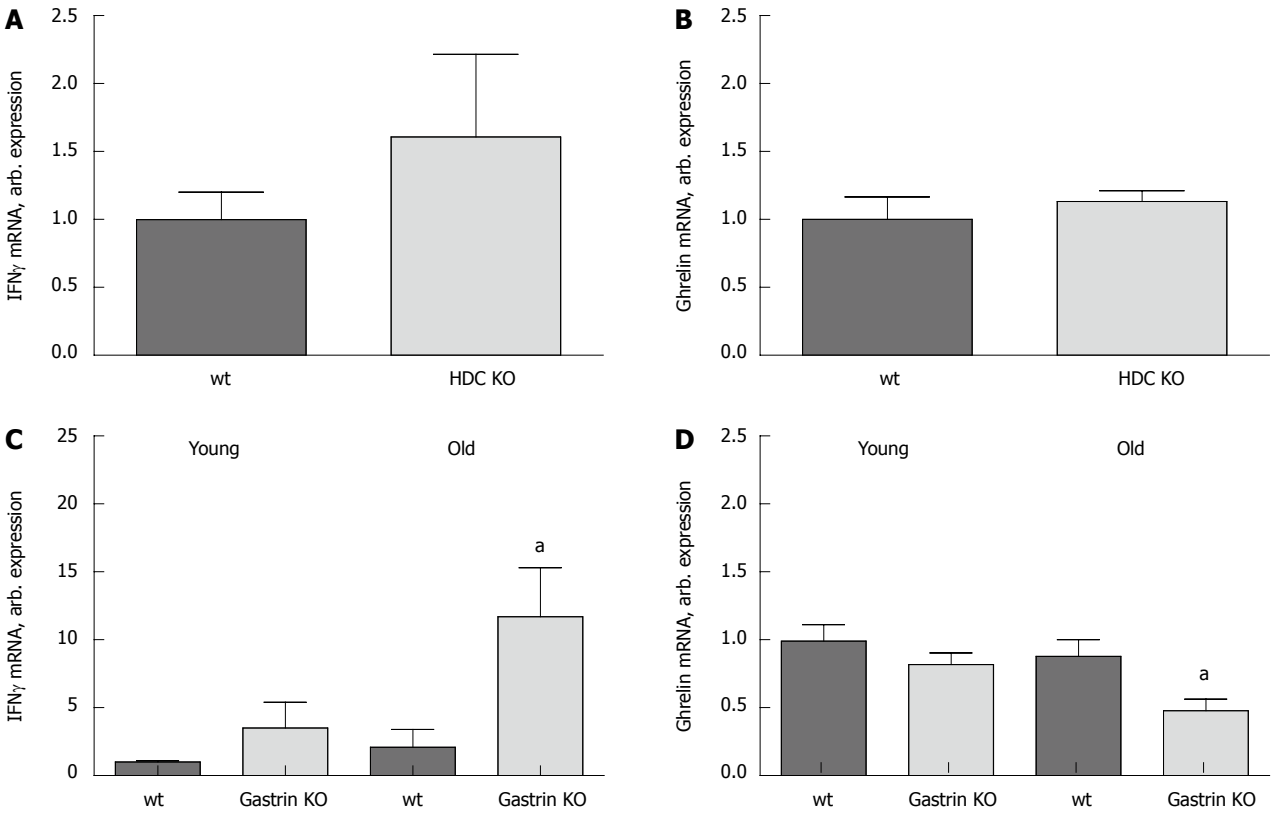

Figure 2 Interferon γ expression is increased and ghrelin expression is reduced in old but not young gastrin knockout mice and histidine decarboxylase knockout mice. The expression of interferon γ (IFNγ) and ghrelin mRNA in young (12-16 wk) and old (48-56 wk) achlorhydric gastrin KO mice and old (48-56 wk) hypochlorhydric histidine decarboxylase (HDC) KO mice (n = 6 in each group) is shown. There is no change in either IFNγ (A) or ghrelin (B) expression in HDC KO mice as compared to wt mice. While the gastric inflammation evaluated by expression of IFNγ increases when the gastrin KO mice get older (C), the expression of ghrelin decreases (D). $^{a}P < 0.05$.

Table 2 Fundic expression of somatostatin mRNA increases during subcutaneous interferon γ infusion (mean ± SE)

	Fundus			Antrum		
	Saline	+ IFNγ	*P*	Saline	+ IFNγ	*P*
Low Dose IFNγ						
Somatostatin mRNA	1.0 ± 0.2	1.9 ± 0.2	< 0.05	1.0 ± 0.2	0.9 ± 0.1	NS
IFNγ mRNA	1.0 ± 0.1	1.1 ± 0.2	NS	1.0 ± 0.1	1.2 ± 0.2	NS
High Dose IFNγ						
Somatostatin mRNA	1.0 ± 0.1	1.6 ± 0.1	< 0.05	1.0 ± 0.1	1.1 ± 0.1	NS
IFNγ mRNA	1.0 ± 0.2	1.3 ± 0.1	NS	1.0 ± 0.1	1.2 ± 0.1	NS

The fundic and antral expression of somatostatin mRNA and endogenous interferon γ (IFNγ) mRNA in arbitrary units in mice infused with either IFNγ or saline (n = 6 in each group). Low dose IFNγ = 8 μg/kg per hour for 7 d and high dose IFNγ = 24 μg/kg per hour for 7 d. NS: Non-significant.

mRNA and the plasma concentration of ghrelin are reduced during gastric infection, either due to bacterial overgrowth in general or to *H. pylori* infection specifically. Both types of infection are associated with an IFNγ inflammatory response. Furthermore, infusion of IFNγ alone could mimic the changes in ghrelin expression and plasma concentration seen during *H. pylori* infection and bacterial overgrowth.

The observation of reduced ghrelin expression in *H. pylori*-infected mice is in agreement with several studies that found reduced ghrelin concentrations in both humans[7,18,19] and rodents[20] infected with *H. pylori*. However, others have reported ghrelin plasma concentration to be unaffected[43,44] or even to increase during *H. pylori* infection[45]. These discrepancies could be due to differences in the severity of the infection. We found gastric ghrelin expression unaffected in young gastrin KO mice and HDC KO mice, both with only mild inflammation as evaluated by the IFNγ response. However, as the gastrin KO mice got older and developed a more severe gastric inflammation, the ghrelin expression decreased. Similar observations have also been observed in humans, where a correlation between increasing degree of chronic inflam-

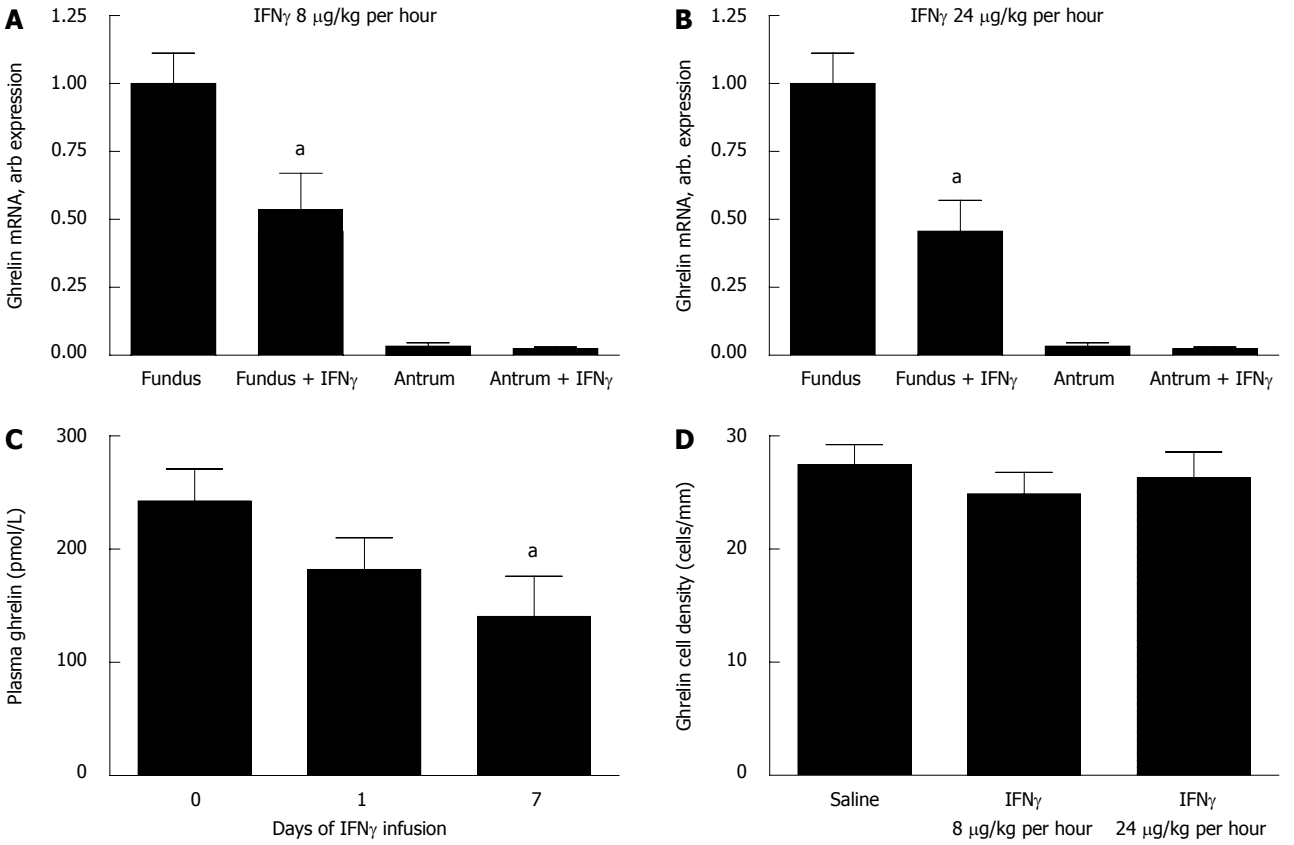

Figure 3 Interferon γ suppresses ghrelin expression in the gastric fundus and ghrelin concentrations in plasma independently of loss of ghrelin-producing cells. Mice infused with interferon γ (IFNγ) as compared to saline infused mice (n = 6 in each group). IFNγ was infused at low dose (LD) = 8 μg/kg per hour for 7 d or high dose (HD) = 24 μg/kg per hour for 7 d. Low (A) and high (B) dose IFNγ infusion decreases the expression of ghrelin mRNA to a similar degree. HD IFNγ represses ghrelin concentrations in plasma gradually over time (C). The density of fundic ghrelin cells is not altered by IFNγ infusion (D). $^aP < 0.05$.

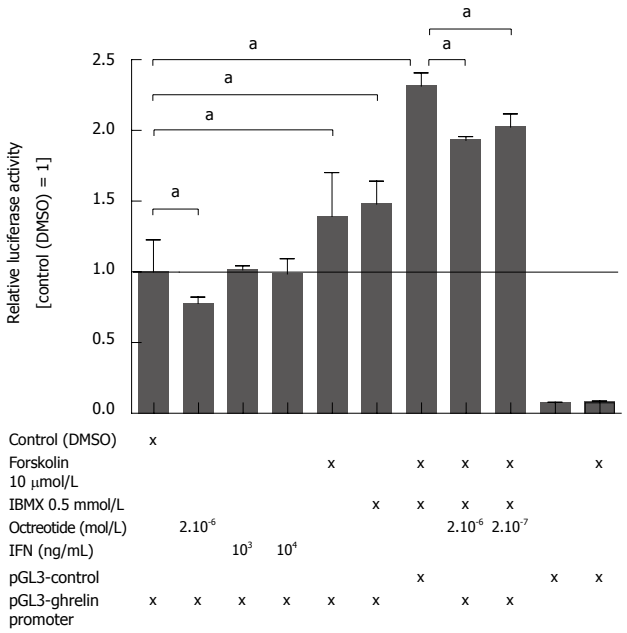

Figure 4 The somatostatin receptor agonist octreotide inhibits activation of the ghrelin promoter. Pulmonary carcinoid NCI-H727 cells were transfected with a 2 kb mouse ghrelin promoter construct and treated for 24 h with forskolin, IBMX, octreotide, and interferon γ (IFNγ) alone or in combination, after which the ghrelin promoter response was measured by the dual-luciferase reporter assay system. Octreotide inhibited both basal and IBMX and/or forskolin-induced ghrelin expression. Forskolin and IBMX were dissolved in dimethyl sulfoxide (DMSO). $^aP < 0.05$.

mation, severity of glandular atrophy and metaplasia and decreasing ghrelin expression was found[8,19].

Reduced gastric ghrelin expression could either be due to a reduced number of ghrelin cells or reduced expression within each cell. Ghrelin-producing cells are primarily found in the oxyntic glands in the stomach[2,3]. The expression of ghrelin mRNA was more than 30 times higher in the fundus than antrum in control mice. Reduced numbers of ghrelin cells in fundic mucosa has indeed been seen in *H. pylori*-infected humans[7] and this correlates with a reduction in plasma ghrelin concentrations.

In our study, the density of ghrelin cells did not change indicating that our observations are due to reduced expression within the cells. The mechanism leading to reduced ghrelin expression during *H. pylori* infection is poorly understood. We found that IFNγ had no direct effect on the ghrelin promoter, suggesting that the IFNγ inhibition of ghrelin expression is mediated *via* other transmitters. In our study, fundic somatostatin was increased both during gastric inflammation and during infusion of IFNγ. Somatostatin is the universal inhibitor of secretion from endocrine cells[46] and also inhibits ghrelin secretion[47]. We show that octreotide (a somatostatin analog) inhibits the activity of the ghrelin promoter. Increased corpus somatostatin has also been found during *H. pylori* infection[48]. However, reduced fundic somatostatin expression has been found in other studies[49,50]. One explanation for

these differences is the degree of fundic atrophy, which affects both ghrelin and somatostatin expression[7].

Immunoregulation of somatostatin has also been demonstrated in *in vitro* studies[51]. These showed that TNFα and IL-1β stimulated somatostatin secretion. IL-4 also stimulated somatostatin secretion and together these changes could explain the hypochlorhydria seen in mice infected with *H. felis*[52]. However, in that study, infusion of IFNγ resulted in a reduction of fundic somatostatin. We have no explanation for the difference in response to IFNγ. The proinflammatory cytokine IL-1β also influences ghrelin levels and seems to suppress excess ghrelin secretion in *H. pylori*-infected mice[53]. Thus, not only IFNγ but other cytokines as well are associated with reduced ghrelin expression.

We have shown that gastric infections either due to *H. pylori* or bacterial overgrowth are associated with reduced fundic ghrelin expression and increased IFNγ production. Infusion of IFNγ in mice alone mimics the changes seen in the mice with gastric infections. Stimulation with IFNγ does not directly inhibit the ghrelin promoter; instead the inhibition is mediated through somatostatin.

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COMMENTS

Background

Ghrelin is involved in energy homeostasis and ghrelin plasma concentrations are decreased in obesity and increased during fasting, anorexia or cachexia. Recently, several studies have found that infection with *Helicobacter pylori* (*H. pylori*) reduces ghrelin concentrations in both humans and rodents. Furthermore, children infected with *H. pylori* have faltering growth, suggesting that *H. pylori* may alter signals from the stomach related to the control of growth and body weight. The mechanism(s) through which inflammation modulates ghrelin expression are, however, poorly understood.

Research frontiers

Chronic gastritis induced by *H. pylori* is a Th1-dominated immune reaction which is regulated by, among others, the lymphocyte-derived cytokine interferon-γ (IFNγ). In the gastrin knockout (KO) mouse which is a model for chronic gastritis due to bacterial overgrowth, increased gastric production of IFNγ has been found. Since little is known about the factors that regulate ghrelin expression during *H. pylori* infections and gastric inflammation, the authors examined if, and through which mechanisms, IFNγ modulates ghrelin expression in mice.

Innovations and breakthroughs

H. pylori-infected mice and old gastrin KO mice with inflammation due to bacterial overgrowth of the stomach display an increased expression of IFNγ and a decreased expression of ghrelin. The changes in ghrelin and somatostatin expression can be duplicated by infusion of IFNγ alone. IFNγ does not directly suppress ghrelin expression but inhibits it indirectly by increasing somatostatin secretion.

Applications

A better understanding of the mechanisms that control ghrelin expression during inflammation by either *H. pylori* alone or by gastric bacterial infections in general aids in the understanding of factors modulating growth and body weight during infection. This could have great impact on general health in the population.

Terminology

IFNγ is a cytokine that is important for innate and adaptive immunity against bacterial infections and for tumor control. The most important functions of IFNγ come from its immunostimulatory and immunomodulatory effects. Ghrelin is a hormone produced in the oxyntic glands of the gastric corpus. It is a growth hormone promoting intestinal cell proliferation, and is involved in energy homeostasis. Ghrelin expression was, in this study, found to be inhibited by octreotide, which is an analog of somatostatin. Somatostatin acts as a general inhibitor of secretion from, and growth of, endocrine cells. Somatostatin is widely distributed throughout the body including several locations in the digestive system such as the stomach, intestine and delta cells of the pancreas.

Peer review

This is the first experimental study on the effect of IFNγ on ghrelin expression. It is a very well designed study, using a careful combination of several animal models and different techniques, and the discussion is well structured. The study is valuable in the context of providing evidence on the indirect regulation of ghrelin expression and secretion by IFNγ mediated through somatostatin.

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BRIEF ARTICLE

Ethanol injection is highly effective for hepatocellular carcinoma smaller than 2 cm

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Abstract

AIM: To analyze the long-term prognosis in a cohort of western cirrhotic patients with single hepatocellular carcinoma treated with ethanol injection.

METHODS: One-hundred forty-eight patients with solitary hepatocellular carcinoma were enrolled. The tumor diameter was lower than 2 cm in 47 patients but larger in the remaining 101 patients. The impact of some pre-treatment clinical and laboratory parameters and of tumor recurrence on patients' survival was assessed.

RESULTS: Among the pre-treatment parameters, only a tumor diameter of less than 2 cm was an independent prognostic factor of survival. The occurrence of new nodules in other liver segments and the neoplastic portal invasion were linked to a poorer prognosis at univariate analysis. Patients with a single hepatocellular carcinoma smaller than 2 cm showed a better 5-year cumulative survival (73.0% *vs* 47.9%) (P = 0.009), 3-year local recurrence rate (29.1% *vs* 51.5%) (P = 0.011), and 5-year distant intrahepatic recurrence rate (52.9% *vs* 62.8%) (P = 0.054) compared to patients with a larger tumor.

CONCLUSION: The 5-year survival rate of patients with single hepatocellular carcinoma < 2 cm undergoing ethanol injection is excellent and comparable to that achieved using radiofrequency ablation.

Key words: Hepatocellular carcinoma; Cirrhosis; Percutaneous ethanol injection; Prognosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth more common cancer worldwide, and is the leading cause of death among patients with cirrhosis in Europe and the USA[1-3]. Since the rate of tumors diagnosed early in a subclinical stage is still low, screening and surveillance strategies in cirrhotic patients based on an ultrasound (US) study of the liver and serum assay of α-fetoprotein (AFP) have been developed and applied in both the Eastern and

Western world. Accordingly, a trend towards a progressive decrease of the mean diameter of HCC diagnosed by screening during the last few years has been demonstrated, and this seems to improve patients' prognosis by increasing their access to curative treatments[4].

Surgical resection and liver transplantation provide the best effective cure in HCC. However, resection is usually offered to cirrhotics with a single lesion and preserved liver function without severe portal hypertension, while liver transplantation is an effective option for patients with tumors within the Milan criteria (one nodule < 5 cm or no more than 3 nodules < 3 cm), but can only be considered in a limited number of patients due to the problem of organ shortage[5]. US-guided percutaneous ablation is currently regarded as the first line approach in the treatment of early-stage HCC deemed unsuitable for surgery or liver transplantation. Percutaneous ethanol injection (PEI) was the first percutaneous treatment introduced in clinical practice; it has been widely used during the last 20 years with excellent results[6], and was recommended as the standard ablation therapy of HCC according to the European guidelines for the management of HCC published in 2001[7]. At the end of the nineties, radiofrequency ablation (RFA) became available and progressively replaced PEI[8,9]. Accordingly, a question about the usefulness of PEI in the treatment of HCC has been recently raised. In agreement with Forner *et al*[10], we think that PEI is still useful for the treatment of lesions located at risky sites for RFA, for residual areas of viable tumors after RFA, and as a bridge treatment in HCC patients listed for liver transplantation. Furthermore, according to the present guidelines for the management of HCC[11], the efficacy of PEI is probably similar to that of RFA in patients with compensated cirrhosis and single tumors smaller than 2 cm in which 5-year survival has been shown to be higher than 70% in eastern series[9,12,13].

The aim of this study was to assess the factors affecting long term prognosis in a single-centre cohort of western cirrhotic patients with single HCC treated with PEI, focusing on the subgroup of patients with small tumors smaller than 2 cm.

MATERIALS AND METHODS

Two hundred-eighteen cirrhotic patients with single HCC treated with PEI in our centre during the period 1991-2008 were evaluated. In all patients cirrhosis was confirmed by histological and/or clinical findings (blood chemistry, US, and/or endoscopic signs of liver cirrhosis and/or portal hypertension). Most patients were diagnosed during a 6 mo-interval screening program for early diagnosis of HCC based upon ultrasound study of the liver and serum assay of AFP. After detection of the suspicious HCC nodule, all patients underwent characterization of the lesion using computed tomography (CT), magnetic resonance imaging (MRI), contrast enhanced ultrasound (CEUS), and/or fine-needle biopsy, in accordance to the current diagnostic guidelines; for this reason, most of the lesions diagnosed before 2001 were further evaluated using cyto-histological assessment, while lesions larger than 2 cm detected after 2001 were mainly diagnosed using two imaging studies showing coincident typical dynamic findings after contrast enhancement; a cytological and/or histological evaluation using fine-needle biopsy was reserved for lesions showing non-typical features on dynamic imaging study[7]. After 2005 this policy was extended to lesions less than 2 cm in size[11].

Patients were usually staged before treatment using CT scan. MRI was reserved for patients with contraindications to the administration of iodinated contrast media.

Among the 218 patients included in the study group, 48 were excluded because PEI was associated with other locoregional therapies as initial treatment, 19 because complete necrosis was not achieved after treatment completion, and 3 because of insufficient follow-up data. Hence, the study refers to 148 patients with a minimum post-treatment follow-up period of 6 mo. One hundred-three patients were treated during the period 1991-2000 while the remaining 45 patients underwent PEI during the period 2001-2008. All patients gave informed consent to all diagnostic investigations and therapeutic procedures and the study protocol conforms to the ethical guidelines of the of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. For the whole population, the last follow-up data were recorded on February 2010.

The patients were 109 men and 39 women (mean age 67 ± 8 years, range 34-87 years). The cirrhosis etiology was post-hepatic C in 107 cases, post-hepatic B in 18 cases, post-hepatic C and B in 7 cases, alcoholic in 10 cases, and cryptogenic in 6 cases. According to the Child-Pugh scoring system[14], 130 patients were scored Class A, and 18 Class B. Fifteen patients had mild ascites, while 6 patients showed partial non-neoplastic thrombosis of the portal system according to dynamic imaging studies[15]. The mean HCC diameter was 2.8 cm (range 1.1-5.8 cm). Forty-seven patients (31.8%) had a single tumor < 2 cm, 57 (38.5%) between 2 and 3 cm, and 44 (27.0%) > 3 cm. In only 3 cases the tumor diameter exceeded 5 cm. Overall, a cytological and/or histological diagnosis of HCC was available in 63/148 HCCs (well differentiated in 52 cases, moderately differentiated in 10 cases, and poorly differentiated in 1 case).

All patients were excluded from resective surgery due to one or more of the following reasons: severe portal hypertension, impaired liver function, refusal of surgery, presence of severe comorbidities increasing the surgical risk, and severely impaired clotting parameters. Four patients were waiting for liver transplantation and PEI was used as a bridging treatment; all these patients were transplanted and the follow-up period ended at the moment of transplantation. For patients diagnosed after 2000, when RFA became available in our centre, the main reasons for choosing PEI for treatment were the following: impaired clotting parameters preventing the use of large bore needles, location of the tumor in a dangerous position for RFA (e.g. near the gall bladder, the glissonian capsule, or an intestinal loop adjacent to the liver edge), location of

Table 1 Clinical and biochemical risk factors influencing overall survival *n* (%)

Variables	Median (Interquartile range)	*P* value at Log-Rank[1]	Cox regression[2]
Pre-PEI			
Age (yr)	67 (61-72)	0.640	-
Male gender	109 (73.6)	0.640	-
HCV Pos	114 (77.0)	0.615	-
HBsAg Pos.	25 (16.9)	0.624	-
Child B	18	0.329	-
AST (IU/L)	66.5 (44.5-89.5)	0.794	-
ALT (IU/L)	64 (41.5-106.5)	0.877	-
TAP	79 (69-86)	0.248	-
Tot. Bilirubin	1.0 (0.80-1.45)	0.438	-
Albumin	3.6 (3.3-3.9)	0.116	-
PLT	100 (76.5-139.5)	0.342	-
AFP	11.1 (6-31)	0.330	-
Ascites	15 (10.1)	0.108	-
Portal thrombosis	6 (4.1)	0.133	-
Size < 2 cm	47 (31.8)	0.009	0.421 (0.216-0.821), *P* = 0.011
Post-PEI			
Portal invasion	14 (9.5)	0.003	-
Local recurrence	56 (37.8)	0.210	-
Distance recurrence	61 (41.2)	0.003	-
Global recurrence	86 (58.1)	0.180	-

Hepatocellular carcinoma size: 25, 5 (20-34). [1]*P* value at Log-Rank: Univariate: Results are expressed as *P* level at Log-Rank (Mentel-Cox); [2]Cox Regression: Results are expressed as HR (95% CI) and *P* level at Cox Regression analysis (backward wald). HCV: Hepatitis C virus; AFP: α-fetoprotein; PEI: Percutaneous ethanol injection; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PLT: Platelets.

the tumor near a large vessel lowering the level of heating needed to induce tumor necrosis, and patient choice.

Patients were usually treated by multisession US-guided PEI on an out-patient basis. After local antisepsis and intradermal local anaesthesia at the site of the needle insertion, sterile 95% ethanol (Salf, Bergamo, Italy) was injected into the lesion using either a 20-cm-long Chiba needle with an inner calibre of 20 Gauge (Ekoject, Hospital Service, Italy) or a PEI dedicated 20-cm long multi-hole 21-Gauge needle (Peit Needle, Hospital Service, Italy) at a dosage of 1-8 mL per session. The Chiba needles were preferentially employed to treat small tumors while the multi-hole needles were usually reserved for lesions larger than 2-3 cm. Treatment was performed once or twice per week and the total amount of ethanol injected was calculated according to the numerical expression $V=(4/3)\pi(r+0.5)^3$, where V (in mL) is the volume of ethanol and r (in cm) is the radius of the lesion increased by 0.5 cm based on the concept that the volume of ethanol injected must overcome the theoretical volume of the lesion[16]. The number of PEI procedures needed to achieve tumor ablation was 6 on average (range 1-11) in the whole population and 2 (range 1-4) in the subgroup of patients with HCC of up to 2 cm. The amount of ethanol injected per session ranged between 2 and 9 mL, with no more than 15 min needed for each PEI session. The effectiveness of PEI was assessed by CT scan with contrast enhancement performed within one month after treatment completion. Complete necrosis was considered achieved when the lesion appeared as a non perfused area during the arterial phase of the study. In the case of intolerance to iodinated contrast media, MRI with gadolinium contrast enhancement was used. During the last 7 years, we added CEUS in the post-PEI assessment of the treated lesion; our policy was to perform CEUS about one month after PEI; in case of presence of residual arterial enhancement, suggesting the presence of a viable tumor, the lesion was immediately re-treated without further imaging evaluation while in the case of absence of contrast enhancement in the arterial phase, suggesting complete necrosis, we used CT or MRI as a confirmatory test[17].

After completion of treatment, the patients were followed by serum assay of AFP and US liver study performed every 3 mo. Local recurrence of the treated lesion was suspected on the basis of size increase and/or US pattern change; in this case, CEUS, CT scan or MRI were performed to assess the presence of arterial enhancement areas suggesting recurrent disease. Furthermore, each new lesion visualized by US in a liver segment different from that of the first neoplasm was characterized using CEUS, CT scan, MRI, and/or US-guided liver biopsy, following the current guidelines for HCC diagnosis[7,11].

The following clinical and biochemical parameters assessed before PEI were analyzed to examine their value as predictive factors for survival (Table 1): age, gender, hepatitis B surface antigen (HBsAg), hepatitis C virus (HCV) antibody, Child-Pugh class, aspartate aminotransferase (AST) level, alanine aminotransferase (ALT) level, prothrombin time ratio, serum bilirubin, serum albumin, platelet count, presence of ascites, presence of portal thrombosis, serum AFP and HCC size ≤ 2 cm. Finally, the impact on survival of local recurrence (HCC recurrence in the same liver segment), distant recurrence (HCC recurrence in a liver segment different from that of the

Table 2 Cumulative survival and disease free survival rates

Months	Overall survival		Disease free survival	
	%	*n*	%	*n*
0		148		148
6	100	147	89.2	132
12	95.8	135	71.0	99
24	86.7	99	48.5	51
36	73.4	66	33.5	27
48	64.1	42	32.1	23
60	55.9	33	24.1	9
Median (95% CI)	78 (54.38-101.62)		24 (16.90-31.09)	

first neoplasm), portal invasion (imaging detection or cytological diagnosis of neoplastic thrombosis of the portal tree), and disease free survival (DFS, defined as the interval in months between last PEI session until local HCC recurrence and/or appearance of new HCC lesions within or outside the liver) were also evaluated.

Statistical evaluation

Overall survival was defined as the interval in months between the first PEI session until death or the last recorded follow-up. Cumulative survival and recurrence curves were obtained using the Kaplan-Meier curves. For each variable taken into account, the differences between curves were assessed using the *post hoc* log-rank test. For age, ALT level, AST level, prothrombin time ratio, bilirubin level, albumin level, platelet count, and AFP, the patients were separated into groups: those $\leqslant$ the median and those > the median. For the purpose of the study, patients were split into two subgroups according to a HCC size smaller or larger than 2 cm. The parameters significant at univariate analysis were tested using the Cox's proportional hazard model. The post treatment parameters of neoplastic portal invasion, local recurrence, distant recurrence, and DFS were only tested by univariate analysis. Correlation was analyzed with the Spearman rank test. A *P* value of $\leqslant$ 0.05 was considered significant. All statistical analyses were performed using the SPSS™ 13.0 software package.

RESULTS

The mean follow-up period was 42 mo (range 8-166 mo), and was longer than 60 mo in 34 patients. In no case was the PEI procedure associated with mortality or complications requiring emergency treatment. Seeding of HCC to the abdominal wall was not recorded.

The 1-, 3-, and 5-year survival rate of the entire cohort was 95.8%, 73.4%, and 55.9%, respectively, and the estimated median survival rate was 78 mo [CI 95% 54.38-101.62] (Table 2). During the follow-up, 4 patients underwent OLT and 51 (34.5%) died. The causes of death were: liver failure due to progression of the neoplasm involving more than 50% of the liver parenchyma with or without invasion of the main intrahepatic vessels in 35 cases (68.6%), liver failure unrelated to tumor progression in 7 cases (13.7%), bleeding from esophageal or gastric varices in 2 cases (2.9%), and other extrahepatic diseases in 3 cases (sepsis, myocardial infarction, pulmonary embolism, 5.9%). The cause of death was unknown in 4 cases (7.8%).

Fifty-six patients showed local HCC recurrences, 61 distant recurrences, and 31 both local and distant recurrences. The median time elapsed between the PEI completion and the first detection of local or distant recurrence were 12 mo (CI 95% 7.0-16.5), and 21 mo (CI 95% 14.0-32.0), respectively. No cases of local recurrence were recorded after 36 mo from PEI completion. The overall DFS rate was 71.0%, 33.5%, and 24.1% at 1, 3, and 5 years, respectively (Table 2). Local recurrences were treated with PEI, RFA or transarterial chemoembolization (TACE) in 41 cases (73.2%). The recurrent lesions in segments different from that of the first neoplasm were treated with PEI, RFA, TACE or systemic therapy including tamoxifen, octreotide, or sorafenib in 34 cases (55.7%). In 15 cases of local recurrence and in 27 cases of distant recurrence, no treatment was applied due to one or more of the following reasons: end stage liver failure, multifocal or infiltrative pattern of recurrence with or without vascular invasion, and concomitant diagnosis of extrahepatic spreading.

Of the whole population, HCC size < 2 cm was the only pre-PEI parameter significantly linked to survival ($P = 0.009$). This parameter resulted to be an independent predictor of survival after multivariate analysis using the Cox regression model [HR 0.421 (0.216-0.821); $P = 0.011$]. Among the post-treatment parameters, only distant recurrence and portal invasion were significantly linked to survival ($P = 0.003$ for both parameters) (Table 1).

For further analysis, the 47 patients with a single HCC smaller than 2 cm were compared to the 108 patients with tumors larger than 2 cm. The clinical (age, sex, Child-Pugh class, ascites, portal thrombosis) and laboratory (HBsAg positive, anti-HCV positive, ALT, AST, prothrombin time ratio, bilirubin, albumin, platelet count, AFP) features were not significantly different between groups. As expected, the 1-, 3-, and 5- year survival rate of the patients with HCC < 2 cm (97.8%, 86.3%, and 73.0%) was significantly better than that of patients with larger tumors (94.8%, 67.5%, and 47.9%) ($P = 0.009$) (Figure 1). The estimated median survival of patients with HCC < 2 cm was longer than that of patients with HCC > 2 cm [93 mo (CI 95% 43.9-142.1) *vs* 60 mo (CI 95% 41.9-79.4)]. Furthermore, the cumulative 1-, 2- and 3-year local recurrence (13.2%, 21.6%, and 29.1% *vs* 28.2%, 43.7%, and 51.5%) of the patients with HCC < 2 cm was significantly lower than that of patients with larger tumors (log rank χ^2 0.825, $P = 0.011$). Likewise, the cumulative distant recurrence at 1-, 3-, and 5-years in patients with HCC < 2 cm was lower (2.3%, 27.2%, and 52.9% *vs* 11.0%, 49.6%, 62.2%); this difference demonstrated a clear trend towards statistical significance (log rank χ^2 5.338, $P = 0.054$). In order to analyze the possible influence of local on distant recurrence, we investigated the correlation between these 2 events. Interestingly, 62/92 (67.4%) of patients without local did not experience distant recurrence, while 31/56 (55.4%) of subjects with local developed distant recurrence of HCC.

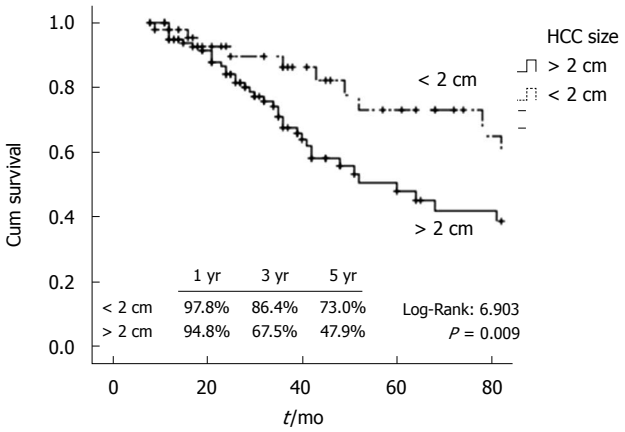

Figure 1 Comparison between cumulative survival rates in 47 patients with solitary hepatocellular carcinoma < 2 cm (dotted line) and 101 patients with solitary hepatocellular carcinoma > 2 cm (continuous line). HCC: Hepatocellular carcinoma.

On this ground, we found a positive correlation at Spearman-rho test (Coefficient: 0.224; $P = 0.006$) and calculated that subjects with local recurrence had an increased risk to develop distant recurrence with an OR of 1.698 (95% CI 1.165-2.473).

DISCUSSION

PEI has been the first ablation technique extensively used for the treatment of HCC and is usually indicated for lesions smaller than 3 cm in diameter since complete tumor necrosis may be achieved in 90%-100% of tumors smaller than 2 cm, 70%-80% of lesions between 2 and 3 cm, and about 50% of lesions between 3 and 5 cm[18]. Furthermore, PEI has been recently shown to achieve complete necrosis of the treated tumors at explant analysis in 38/59 patients (64.3%) undergoing liver transplantation[19]. However, PEI has been largely replaced by RFA during the last years, and this is due to the best predictability of the necrotic effect in all tumor sizes and to the best effectiveness in tumors larger than 2 cm[11]. According to some recent randomized trials involving tumors with a maximal size of 3 or 4 cm, RFA is more efficacious than PEI in terms of initial complete tumor necrosis rate (93%-100% *vs* 66%-100%), 3-year survival rate (63%-81% *vs* 48%-67%) and local tumor recurrence (8%-14% *vs* 22%-34%)[20-25]. Furthermore, a few recent meta-analytic studies support the superiority of RFA versus PEI in terms of patient survival and local disease recurrence[26-28]. However, when the analysis is restricted to lesions smaller than 2 cm, the superiority of RFA is questionable: a recent meta-analysis about the clinical outcomes of RFA, PEI and percutaneous acetic acid injection for HCC shows that for lesions smaller than 2 cm there is no significant difference between RFA and PEI for the proportion of patient mortality and for local recurrence[28].

Our long term cohort study confirms that PEI is still an effective treatment for compensated cirrhotics with a single HCC < 2 cm since the 5-year survival rate was as high as 73.0% and a tumor diameter up to this size was the only pre-treatment parameter independently linked to survival. This datum is comparable to that shown in eastern single center series. In a group of 270 cirrhotics with HCC treated by PEI, Ebara *et al*[13] described a subgroup of 96 Child-Pugh Class A patients with solitary HCC smaller than 2 cm with a 5-year survival of 78.3%. Omata *et al*[9] reported a survival rate of 70% in a series of 144 patients with a single HCC < 2 cm undergoing PEI. Less favourable results were shown by Arii *et al*[29] in a retrospective multicenter Japanese survey, showing a 54% survival rate in 767 patients with Stage I HCC < 2 cm submitted to PEI.

Available data from the literature do not provide unequivocally a survival benefit of RFA over PEI in these patients. A 5-year survival of 83.8% was reported by Tateishi *et al*[30] in a cohort of 87 patients with HCC lower than 2 cm treated by RFA. A similar rate of 5-year survival (83.8%) has been recently reported for Child-Pugh Class A patients with a single lesion of up to 2 cm treated with RFA and registered by the Liver Cancer Study Group of Japan[31]. However, in the recent western series by Livraghi *et al*[32] involving 218 patients with a single HCC < 2 cm undergoing ablation with RFA, the 5-year survival rate was 55% for the whole population and 68% for the subgroup of potentially operable patients.

In our cohort of patients, intrahepatic HCC progression was the cause of death in more than two thirds of the cases, and the 5-year survival of patients with a single nodule < 2 cm was significantly better than that of patients with HCC > 2 cm (73.0% *vs* 47.9%). This is not surprising, considering that in tumors > 2 cm the success of ethanol in achieving necrosis of the entire mass is limited by intratumoral septa, and that in most of these tumors PEI does not induce a peritumoral necrosis, preventing the persistence of peripheral minute neoplastic foci or extra-tumoral satellites[10]. Accordingly, in our group of patients, the cumulative local recurrence rate of tumors smaller than 2 cm was significantly lower than that of patients with larger HCC. The reappearance of a viable tumor in a lesion assessed as completely necrotic shortly after ablation implies, in most cases, the need for additional locoregional therapy and is negatively linked to survival. In a large study by Sala *et al*[33], among Child-Pugh Class A patients, the 5-year survival rate of patients achieving a sustained complete response at the end of follow-up was significantly higher than that of patients without a sustained complete response due to initial treatment failure, late local recurrence, or appearance of new HCC nodules outside the segment of the first neoplasm. Similarly, in our study, distant intrahepatic recurrence was linked to survival and occurred significantly more frequently in patients with tumors larger than 2 cm. This may be in part related to the insufficient local disease control obtained by PEI in tumors more than 2 cm large. Indeed, the reappearance of viable tumor tissue may promote the HCC spreading within the liver through microvessel invasion and satellitosis. The observation of a significantly increased risk of new HCC lesions within the liver in patients with local recurrence in this series reinforces this hypothesis.

In conclusion, our study shows that a tumor diameter of up to 2 cm is an independent predictor of survival in cirrhotics with single HCC treated by PEI. In this subgroup of patients, we observed a 5-year survival rate as high as 73.0%, significantly better than that observed in patients with solitary larger tumors. This datum supports the hypothesis that in compensated cirrhotic patients with small HCC up to 2 cm submitted to ablation the long term prognosis is excellent independently from the application of PEI or RFA as therapeutic procedure[10]. Although PEI could be less effective than RFA in providing complete necrosis of the tumor even in this subset of patients, this is counterbalanced by the universal feasibility of PEI in tumors located at risky sites for RFA, or in which a thermal ablation may be less efficient due to the proximity of large vessels. For these reasons, a large prospective randomized study, designed to assess the overall survival as primary end point according to the principles of the intention-to-treat analysis, taking into account the site of the tumor in the liver, and including a rigorous cost-effectiveness evaluation , is still needed.

COMMENTS

Background

Small hepatocellular carcinomas of up to 2 cm are increasingly being recognized due to the diffusion of the screening program for early diagnosis of this tumor in liver cirrhosis. The ideal treatment of such small lesions is a controversial issue.

Research frontiers

Prospective randomized studies comparing the available treatments for small hepatocellular carcinoma are lacking and should be planned.

Innovations and breakthroughs

This study shows that the survival rate of compensated cirrhotic patients with solitary hepatocellular carcinoma of up to 2 cm treated with percutaneous ethanol injection is higher than 70%. Furthermore, in the series of 148 patients with a single tumor up to 5.8 cm in size treated with this technique, a tumor diameter equal or lower than 2 cm was the only factor significantly linked to survival.

Applications

In authors' opinion, percutaneous ethanol injection is still a valuable treatment of small hepatocellular carcinoma of up to 2 cm in size emerging in liver cirrhosis. In this subset of patients, the demonstration of a better therapeutic performance of radiofrequency ablation in terms of overall survival and cost-effectiveness is still lacking.

Terminology

Percutaneous ethanol injection is a well standardized technique of chemical ablation of hepatocellular carcinoma. When introduced within the neoplasm through a fine-needle, ethanol shows a direct cytotoxic effect and produces coagulative necrosis, followed by fibrosis. In addition, due to the lesive effect of ethanol on the endothelial cells, tumor ischemic necrosis is elicited by thrombosis of the small tumor feeding vessels. The effectiveness of this technique has been questioned after the introduction in clinical practice of the thermal ablation techniques of liver tumors.

Peer review

This is an interesting series showing a continued role for PEI for single small HCC. However, my guess is that RFA has replaced PEI in most centers related to the number of procedures to achieve benefit. There are not many reports from western countries, making this report of interest and worthy of consideration for publication.

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BRIEF ARTICLE

Colorectal cancer screening behavior and willingness: An outpatient survey in China

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Abstract

AIM: To identity the factors influencing colorectal cancer (CRC) screening behavior and willingness among Chinese outpatients.

METHODS: An outpatient-based face-to-face survey was conducted from August 18 to September 7, 2010 in Changhai Hospital. A total of 1200 consecutive patients aged ⩾ 18 years were recruited for interview. The patient's knowledge about CRC and screening was pre-measured as a predictor variable, and other predictors included age, gender, educational level, monthly household income and health insurance status. The relationship between these predictors and screening behavior, screening willingness and screening approach were examined using Pearson's χ^2 test and logistic regression analyses.

RESULTS: Of these outpatients, 22.5% had undergone CRC screening prior to this study. Patients who had participated in the screening were more likely to have good knowledge about CRC and screening (OR: 5.299, 95% CI: 3.415-8.223), have health insurance (OR: 1.996, 95% CI: 1.426-2.794) and older in age. Higher income, however, was found to be a barrier to the screening (OR: 0.633, 95% CI: 0.467-0.858). An analysis of screening willingness showed that 37.5% of the patients would voluntarily participated in a screen at the recommended age, but 41.3% would do so under doctor's advice. Screening willingness was positively correlated with the patient's knowledge status. Patients with higher knowledge levels would like to participate in the screening (OR: 4.352, 95% CI: 3.008-6.298), and they would select colonoscopy as a screening approach (OR: 3.513, 95% CI: 2.290-5.389). However, higher income level was, again, a barrier to colonoscopic screening (OR: 0.667, 95% CI: 0.505-0.908).

CONCLUSION: Patient's level of knowledge and income should be taken into consideration when conducting a feasible CRC screening.

Key words: Colorectal cancer; Screening; Behavior; Willingness; Survey

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies worldwide. The Asian Pacific Working Group on CRC has suggested that some Asian ethnic groups (e.g. Japanese, Korean and Chinese) are more susceptible to CRC than others, with an incidence similar to that of the West[1]. The incidence of CRC in China has increased rapidly since the 1980s[2,3]. CRC now ranks as the fifth leading cause of cancer-related deaths[4]. Screening is an effective tool for early diagnosis[5], but the compliance rates have been low in many countries[6-10]. A study of community-based CRC screening in Hangzhou, China, which involved a population of 34 726 individuals, revealed that the compliance rates for fecal occult blood test (FOBT) and colonoscopy were only 17.5% and 2.8%, respectively[11]. These figures are extremely low compared with those of the United States (overall screening rates were nearly 55% in 2008[12]).

Reasons for low compliance rates may vary among countries. A community-based screening among residents of Beijing revealed that busy work schedules and the complexity of screening procedures were the main barriers to CRC screening[13]. Lack of financial support, fear of pain and the necessity of bowel preparation were barriers to colonoscopic screening in a Hangzhou-based study[14]. Other researchers have doubted the feasibility of population screening in China, due to the requirements for a high awareness of the disease, sufficient medical resources and strong financial support[15]. Opportunistic screening (also called individual screening), which is performed on request from a physician or healthcare provider when a patient presents for consultation for other health reasons[16], has been widely used in most cancer screening protocols throughout the world[17] and may be also suitable for Chinese outpatients. Individuals with more personal experience with illness are more likely compliant with the CRC screening[18]. China has a large population but with an uneven distribution of health resources. Additionally, the general population has a low awareness of CRC and inadequate knowledge regarding CRC and screening[19,20].

Although CRC screening of outpatients might be effective, there have been few studies exploring its availability in China. The 2010 National Institutes of Health (NIH) State-of-the-Science Conference, which aimed to enhance the use and quality of CRC screening, recommended that studies should be carried out about patient screening preferences and other factors influencing informed, shared decision making regarding the choice of CRC screening modalities[12]. Therefore, our study was intended to explore outpatients' screening behavior and willingness as well as to identify influencing factors in Shanghai, China.

MATERIALS AND METHODS

Study population

From August 18 to September 7, 2010, 1200 consecutive outpatients were recruited for our survey from the Outpatient Department of Changhai Hospital, a tertiary care hospital in Shanghai, China. Both sporadic and hereditary cases of CRC were the target of early detection. All these outpatients were over 18 years of age, able to communicate properly and free of mental disorders. Patients with a medical emergency or incurable tumor were excluded. Health care workers including doctors, nurses, medical educators and medical students were not included as subjects of this study.

Study design

A self-designed questionnaire was developed after a literature review, and revised by epidemiologists and clinicians. The following contents were included: (1) Patient general information; (2) evaluation of CRC and screening knowledge; (3) previous screening behavior; (4) screening willingness; and (5) preferred approach. Available screening approaches in our hospital could be classified as fecal test (e.g. FOBT, stool DNA test), blood test (biomarkers in clinical research) and colonoscopy.

A pilot test was conducted in 50 outpatients by trained interviewers on August 18 to verify the feasibility of the survey. The questionnaire was distributed to the patients upon their arrival at the clinic, who were asked to answer the questions under the guidance of interviewers while waiting to see a doctor. To ensure the quality of the survey, additional information about screening was offered to guide the patient's choice of screening approach. This study was approved by the Ethics Committee of Changhai Hospital, and all patients gave written informed consent.

Comparing with other scoring system that evaluated the knowledge about CRC and screening[20,21], several simple factors were taken into account in the evaluation: (1) is the patient familiar with CRC; (2) does the patient understand at least one of the clinical manifestations of CRC; (3) has the patient ever heard of cancer screening; and (4) is the patient familiar with colonoscopy as an early detection method for CRC? Patients were classified as having a high level of knowledge (answered all the questions above), a low level (answered no more than 2 questions) and a moderate level (between high and low).

Statistical analysis

Data were managed using Microsoft Excel software, and duplicate questionnaires were excluded. The results were tabulated and analyzed with the PASW Statistics for Windows release 18.0 (SPSS, Inc., Chicago, Illinois). The primary outcomes were the patient's previous screening behavior, screening willingness and preferred screening approach. Pearson's χ^2 test was used to quantify the association between the outcomes and the predictor variables, which included gender, age, possession of health insurance and monthly household income. A bivariate logistic regression model was used to examine the association between the outcomes and levels of education and knowledge about CRC. Statistical significance was considered at $P < 0.05$, and odds ratios (OR) were given with 95% confidence intervals (CI).

Table 1 Characteristics of respondents

Patient characteristics	Number (n = 1001)	Percent (%)
Gender		
Female	510	50.9
Male	491	49.1
Age (yr)		
< 40	397	39.7
⩾ 40	604	60.3
Educational level		
Primary or no schooling	69	6.9
Secondary education	445	44.5
Higher education	487	48.7
Monthly household income		
< 4000 RMB (yuan)[1]	538	53.7
⩾ 4000 RMB (yuan)	463	46.3
Health insurance		
No[2]	358	35.8
Yes	643	64.2
Previous CRC screening		
No	775	77.4
Yes[3]	226	22.6
Screening willingness		
Voluntary attendance	375	37.5
Under recommendation	413	41.3
No attendance	213	21.3
Preferred screening approach		
Blood test	249	24.9
Fecal test	186	18.6
Colonoscopy	322	32.2
Not specified	244	24.4
Knowledge about CRC and CRC screening		
Low	288	28.8
Moderate	247	24.7
High	466	46.6

[1]Renminbi is the official currency of the People's Republic of China; [2]Including the status of health insurance application; [3]Including colonoscopy, fecal occult blood test (FOBT) and double contrast barium enema (DCBE). CRC: Colorectal cancer.

RESULTS

A total of 1200 consecutive patients were recruited for the survey. Of these, 1029 (85.75%) were successfully surveyed, and 171 (14.25%) did not respond to this survey. Among the 1029 respondents, 28 (2.72%) were found to have unfilled sections, but no duplicate data were detected. Ultimately, 1001 patients were included in our analysis. A total of 604 patients were not less than 40 years of age, which is the recommended minimal screening age in China for sporadic CRC[22]. The mean age of the patients was 45.25 years (range, 18-86 years). Patients were classified as having a high (n = 466, 46.6%), moderate (n = 247, 24.7%) or low (n = 288, 28.8%) levels of knowledge according to our definitions. Other predictor variables, such as educational level and monthly household income, are listed in Table 1.

Previous CRC screening behavior

Among the 1001 included patients, 22.5% (n = 226) had previously undergone CRC screening. The most common examination method used was colonoscopy (91.6%); other methods (FOBT or double contrast barium enema, DCBE) accounted for a small proportion (8.4%). Factors influencing the participation in the screening were age, possession of health insurance, monthly household income and status of CRC knowledge (Table 2). Patients who had been screened tended to have a good knowledge of CRC and screening (OR: 5.299, P < 0.001), have health insurance (OR: 1.996, P < 0.001) and are older in age (OR: 3.834, P < 0.001). High income, however, was found to be a barrier to the screening (OR: 0.633, P < 0.003).

Screening willingness

The analysis of screening willingness revealed that 37.5% of patients (n = 375) would voluntarily agree to be screened at the recommended age; 41.3% (n = 413) would need a physician's recommendation before attending the screening; and 21.3% (n = 213) refused to be screened (Table 1). We categorized the screening willingness into "attendance" (n = 788) and "rejection" (n = 213) and found that knowledge regarding CRC was the only factor influencing the screening willingness (Table 3). Patients with a high level of knowledge about CRC were more willing to attend the screening than those with a poor knowledge of CRC (OR: 4.352, P < 0.001).

Screening approach

The analysis of patients' preference in screening approach revealed that colonoscopy was the most commonly preferred approach (32.2%, n = 322), while blood testing ranked second (24.9%, n = 249), and a fecal test was the least popular option (18.6%, n = 186). However, 24.4% of patients (n = 244) expressed an equivalent preference for all screening approaches (Table 1).

Colonoscopy is the most precise screening approach for CRC. Thus, the screening approaches were characterized into "precise modes (colonoscopy)" and "normal modes (blood and fecal tests)", and factors influencing the patient's selection of screening approach were investigated. Both CRC-associated level of knowledge and monthly household income influenced the choice of screening approach (Table 4). With an increase in knowledge, the proportion of patients selecting a precise screening approach was increased from 25.4% to 54.4% (P < 0.001). Patients with higher incomes, however, prefer not to adopt precise screening approaches on average (P = 0.010).

DISCUSSION

In this outpatient-based study, we found that a high level of knowledge regarding CRC and screening techniques, possession of health insurance or advanced age were stimulus factors for prior CRC screening. Most of the patients were willing to participate in the screening, but 41.3% were willing to do so under doctor's recommendations before attendance. Level of knowledge was the only factor that influenced screening willingness. Outpatients with a higher level of knowledge were willing to participate in the screening and select colonoscopy as the screening approach. Higher income level, however, was a barrier to both the previous screening and the preference of colonoscopy as a screening methodology. These

Table 2 Factors associated with outpatients' previous screening behavior *n* (%)

Variable	Previously screened		OR (95% CI)	*P* value
	No (*n* = 775)	Yes (*n* = 226)		
Gender				
Female	401 (78.6)	109 (21.4)	1.000	0.365
Male	374 (76.2)	117 (23.8)	1.151 (0.856-1.548)	
Age (yr)				
< 40	356 (89.7)	41 (10.3)	1.000	< 0.001
⩾ 40	419 (69.4)	185 (30.6)	3.834 (2.657-5.532)	
Health insurance				
No	303 (84.6)	55 (15.4)	1.000	< 0.001
Yes	472 (73.4)	171 (26.6)	1.996 (1.426-2.794)	
Educational level				
Primary or no schooling	52 (75.4)	17 (24.6)	1.000	-
Secondary education	319 (71.7)	126 (28.3)	1.208 (0.673-2.169)	0.526
High education	404 (83.0)	83 (17.0)	0.628 (0.346-1.141)	0.127
Monthly household income, RMB (yuan)				
< 4000	397 (73.8)	141 (26.2)	1.000	0.003
⩾ 4000	378 (81.6)	85 (18.4)	0.633 (0.467-0.858)	
Level of knowledge				
Low	261 (90.6)	27 (9.4)	1.000	-
Moderate	213 (86.2)	34 (13.8)	1.543 (0.902-2.639)	0.113
High	301 (64.6)	165 (35.4)	5.299 (3.415-8.223)	< 0.001

OR: Odds ratio; CI: Confidence interval.

Table 3 Factors associated with outpatients' screening willingness *n* (%)

Variable	Screening willingness		OR (95% CI)	*P* value
	Rejection[1] (*n* = 213)	Attendance[2] (*n* = 788)		
Gender				
Female	120 (23.5)	390 (76.5)	1.000	0.089
Male	93 (18.9)	398 (81.1)	1.317 (0.971-1.786)	
Age (yr)				
< 40	76 (19.1)	321 (80.9)	1.000	0.207
⩾ 40	137 (22.7)	467 (77.3)	0.807 (0.589-1.105)	
Health insurance				
No	73 (20.4)	285 (79.6)	1.000	0.630
Yes	140 (21.8)	503 (78.2)	0.920 (0.670-1.265)	
Educational level				
Primary or no schooling	16 (23.2)	53 (76.8)	1.000	-
Secondary education	109 (24.5)	336 (75.5)	0.931 (0.511-1.695)	0.814
High education	88 (18.1)	399 (81.9)	1.369 (0.748-2.506)	0.309
Monthly household income, RMB (yuan)				
< 4000	123 (22.9)	415 (77.1)	1.000	0.189
⩾ 4000	90 (19.4)	373 (80.6)	1.228 (0.905-1.668)	
Level of knowledge				
Low	106 (36.8)	182 (63.2)	1.000	-
Moderate	52 (21.1)	195 (78.9)	2.184 (1.481-3.221)	< 0.001
High	55 (11.8)	411 (88.2)	4.352 (3.008-6.298)	< 0.001

[1]Patients rejected to attend screening; [2]Patients would attend screening voluntarily or under recommendation. OR: Odds ratio; CI: Confidence interval.

results indicated that patients' knowledge and income status should be considered when launching a screening program among outpatients in Shanghai.

To our knowledge, this is the first study to investigate outpatients' CRC screening behavior and to identity their screening preferences in China. The advantages of this study are the use of a prospective face-to-face survey of consecutive outpatients and a relatively large sample size. We attempted to establish a simple method to rapidly evaluate patients' levels of knowledge regarding CRC and screening techniques. This method differs from other scoring systems. Our method allows the physician to evaluate the patient's level of knowledge through asking several simple questions, and an appropriate screening approach can be offered immediately following the evaluation.

Our results have several similarities to those of previous population-based studies that explored factors influencing CRC screening[20,23-26] and analyzed CRC screening willingness in Malaysia[21] and Taiwan[27]; however, there

Table 4 Factors associated with outpatients' choice of screening approach *n* (%)

Variable	Screening approach		OR (95% CI)	*P* value
	Normal[1] (*n* = 435)	Precise[2] (*n* = 322)		
Gender				
Female	233 (59.1)	161 (40.9)	1.000	0.340
Male	202 (55.6)	161 (44.4)	1.153 (0.864-1.539)	
Age (yr)				
< 40	163 (58.8)	114 (41.2)	1.000	0.593
⩾ 40	272 (56.7)	208 (43.3)	1.093 (0.810-1.476)	
Health insurance				
No	149 (59.1)	103 (40.9)	1.000	0.533
Yes	286 (56.6)	219 (43.4)	1.108 (0.815-1.505)	
Educational level				
Primary or no schooling	27 (54.0)	23 (46.0)	1.000	-
Secondary education	197 (57.9)	143 (42.1)	0.852 (0.469-1.547)	0.599
High education	211 (57.5)	156 (42.5)	0.868 (0.479-1.571)	0.640
Monthly household income, RMB (yuan)				
< 4000	226 (53.3)	198 (46.7)	1.000	0.010
⩾ 4000	209 (62.8)	124 (37.2)	0.677 (0.505-0.908)	
Level of knowledge				
Low	103 (74.6)	35 (25.4)	1.000	-
Moderate	136 (72.0)	53 (28.0)	1.147 (0.697-1.887)	0.590
High	196 (45.6)	234 (54.4)	3.513 (2.290-5.389)	< 0.001

[1]Blood and feces test; [2]Colonoscopy. OR: Odds ratio; CI: Confidence interval.

have also been some inconsistent results.

As shown in the previous studies, a better knowledge of CRC and screening is related to a higher participation rate in population-based screening[20,23-26]. Among our patients, better knowledge was associated with the previous screening. This association is consistent with qualitative evidence in which lack of knowledge about CRC and screening has been cited as a barrier to screening participation in the United States, Canada and China[28].

Lack of health insurance is an important barrier to the screening participation among ethnic groups with all levels of education[29,30]. The US-based 2005 National Health Interview Survey (NHIS) showed that 19% of respondents with no insurance reported having CRC screening (FOBT or endoscopy), compared with over 39% of those who had insurance[29]. In our study, health insurance status was positively associated with the screening behavior. This is an important finding for outpatient screening because more than half of the patients (64.2%) were covered by health insurance. Their compliance with CRC screening may be relatively easy to promote if appropriate screening advice is offered.

Factors that could enhance the screening willingness in previous studies included the followings: being a close relative of a CRC patient[31], perceived susceptibility, perceived less barriers to screening, doctor's recommendation and personal contact with friends or relatives having CRC[21]. In Taiwan, factors related to intentions to have FOBT were influenced by the inconvenience and the unpleasantness of the screening procedure. Participants' gastrointestinal symptoms or family histories and physicians' recommendation or patients' health conditions were relevant to the intentions for a flexible sigmoidoscopic or colonoscopic screening[27]. Additionally, a knowledge of CRC symptoms was associated with willingness to be screened in Malaysia on univariate analysis but not on multivariate analysis[21]. Among the patients in our survey, the knowledge regarding CRC and screening was an important factor that influenced screening willingness, meanwhile 41.3% patients expressed that they would need doctor's recommendation before attending the screening. So interventional studies which intend to increase the patients' knowledge regarding CRC and screening would help enhance the screening willingness.

Income level is another important factor affecting an individual's decision to be screened. Patients with more affluent socioeconomic status have been shown to have a higher average rate of screening than the less affluent[8,32,33]. However, in our study, the high-income patients were found to have a lower rate of screening and the reluctance of colonoscopic screening. This opposite phenomenon might be related to some cultural reasons. High-income patients live in better conditions and tend to get good treatment, so they are less concerned about using the preventive screening because they are more "healthy". Similar trend was detected in a Hong Kong population who perceived their health status to be good and had a less concern about contracting CRC than those who perceived a fair or poor health status[19]. The reluctance of high-income patients to take colonoscopic screening may also be influenced by the complexity of bowel preparation and the uncomfortable feeling caused by colonoscopy.

There are several limitations in this study. First, it was based in a single center. Our preliminary results on outpatient behavior and willingness cannot represent all the outpatients in Shanghai. Second, some patients (14.25%) did not respond to our survey, although great efforts were made to publicize the significance of the survey.

This may cause some patient selection bias, and a multicenter survey may be needed to confirm our results. However, our hospital, which is the largest endoscopy center in Shanghai, attracts many patients for this procedure. Therefore, our results are fairly representative of urban outpatient clinics.

In conclusion, most of the outpatients are willing to participate in CRC screening. A better knowledge about CRC and screening techniques is positively correlated with previous screenings, higher willingness to participate in the screening and a preference for colonoscopy as a screening methodology. However, a higher income level is a barrier to the screening behavior and the selection of colonoscopy. These results may have some implications for outpatient CRC screening and may help guide the further interventional studies.

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COMMENTS

Background

The incidence of colorectal cancer (CRC) in China has increased since the 1980s. Screening is an effective method for early detection of CRC.

Research frontiers

Opportunistic screening which screened CRC among outpatients might be more effective in China, but has not been well illustrated. Studies exploring patient's screening preferences and factors influencing the choice of colorectal cancer screening modalities are needed before the screening is started. In this study, the authors demonstrated the factors influencing outpatients' screening behavior and willingness in Shanghai, China.

Innovations and breakthroughs

This is the first study to report outpatients' screening behavior and willingness as well as to identify influencing factors in Shanghai, China. The results indicate that patients' levels of knowledge and income should be considered when launching a screening program among outpatients.

Applications

By understanding what factors will influence colorectal cancer screening behavior and willingness among Chinese outpatients, this study has provided some implications for screening practice and may help guide further interventional studies.

Peer review

It is a very interesting research for the readers, the conclusions are very valuable and it should be accepted for publication in the journal.

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BRIEF ARTICLE

High incidence of biliary complications in rat liver transplantation: Can we avoid it?

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Abstract

AIM: To investigate how to reduce the incidence of biliary complications in rat orthotopic liver transplantation.

METHODS: A total of 165 male Wistar rats were randomly divided into three groups: Group A, orthotropic liver transplantation with modified "two-cuff" technique; Group B, bile duct was cut and reconstructed without transplantation; and Group C, only laparotomy was performed. Based on the approaches used for biliary reconstruction, Group A was divided into two sub-groups: A1 (n = 30), duct-duct reconstruction, and A2 (n = 30), duct-duodenum reconstruction. To study the influence of artery reconstruction on bile duct complication, Group B was divided into four sub-groups: B1 (n = 10), duct-duct reconstruction with hepatic artery ligation, B2 (n = 10), duct-duct reconstruction without hepatic artery ligation, B3 (n = 10), duct-duodenum reconstruction with hepatic artery ligation, and B4 (n = 10), duct-duodenum reconstruction without hepatic artery ligation. The samples were harvested 14 d after operation or at the time when significant biliary complication was found.

RESULTS: In Group A, the anhepatic phase was 13.7 ± 1.06 min, and cold ischemia time was 50.5 ± 8.6 min. There was no significant difference between A1 and A2 in the operation duration. The time for biliary reconstruction was almost the same among all groups. The success rate for transplantation was 98.3% (59/60). Significant differences were found in the incidence of biliary complications in Groups A (41.7%), B (27.5%) and C (0%). A2 was more likely to have biliary complications than A1 (50% *vs* 33.3%). B3 had the highest incidence of biliary complications in Group B.

CONCLUSION: Biliary complications are almost inevitable using the classical "two cuff" techniques, and duct-duodenum reconstruction is not an ideal option in rat orthotopic liver transplantation.

Key words: Rat; Liver transplantation; Biliary complication; Animal model

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INTRODUCTION

The occurrence of biliary complications (BC) remains one of the most critical challenges in clinical liver transplantation. According to the literature, the incidence of biliary complication for living-donor liver transplantations (LDLT) is as high as 64%[1-9]. Biliary complications have made biliary reconstruction the "Achilles heel" of liver transplantation. In rat liver transplantation, the occurrence of biliary complications has become a confounding factor in the judgment of experimental results and an obstacle to the practice of transplantion. Unfortunately, few researches could be found to report the incidence of biliary complications in rat liver transplantation. In this study, we investigated the biliary complications after rat orthotopic liver transplantation (ROLT), trying to find a better approach to biliary reconstruction and reduce the incidence of biliary complications after transplantation.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200-250 g, were purchased from the Experimental Animal Center of Sun-Yat Sen University and fed in the specific pathogen free (SPF) animal lab. The weight of the recipient was similar to that of the donor. All animals had free access to food and water except the recipients, which had been fasted for 12 h before operation.

Technique

ROLTs were performed using the "two-cuff" technique established by Kamada[10,11]. All surgical procedures were performed by a single operator under naked eye. Napental was used for the anesthesia (40 mg/kg). All experiments were performed in compliance with the standards for animal use and care set by institutional animal care and use Committee.

Donor operation

After laparotomy, the left subphrenic vein was ligated and all the perihepatic ligaments were divided. The bile duct was incised and a stent (0.9-mm inner diameter, 4-mm length) was introduced and tied firmly. The right renal vein was dissociated and the right adrenal venous plexus was ligated. After 150 units of heparin was injected to form systemic heparinization, the liver was irrigated with physiological saline containing heparin (20 U/mL) through the aorta distal to the celiac artery. At the same time, infrahepatic inferior vena cava (IHIVC) and suprahepatic inferior vena cava (SHIVC) were dissected to allow outflow of the perfusate. When the liver turned khaki color, SHIVC was divided along the diaphragm (without the phrenic ring) , and the right renal vein, the portal vein (PV) and IHIVC were skeletonized and divided. The liver was then harvested and the graft was preserved at 4℃ in physiological saline with 20 U/mL heparin.

The PV was induced through the cuff (2-mm inner diameter, 3.5-mm length) and the distal end of the vein was completely reversed and fixed onto the cuff with a 5-0 silk ligation. The same method was used to prepare the cuff (3-mm inner diameter, 4-mm length) for IHIVC. The SHIVC was treated with two 8-0 silk sutures pierced via the two corners of the vein.

Recipient operation

After laparotomy, the self-made retractor was used to expose the operative area, and the left subphrenic vein. The transport vessels between the left liver and esophagus, hepatic artery and the right adrenal venous plexus were ligated orderly. One necessary step was to put a rubber under the SHIVC for the purpose of traction when removing the liver. Then IHIVC and PV were clamped to the anhepatic phase. SHIVC was blocked after exsanguination and the liver removed quickly. SHIVC was sutured by an end to end anastomosis (8-0, nylon suture), PV was reconstructed by means of cuff technique and the anhepatic phase was ended. The same method was used to reconstruct IHIVC. Based on the experimental design, the bile duct was reconstructed differently.

The recipient rats were fasted for at least 12 h after operation but water was permitted.

Biliary reconstruction and hepatic artery ligation

There were two ways to rebuild the biliary tract in this experiment: (1) end-to-end anastomosis with the stent; and (2) end-to-side anastomosis between bile duct and duodenum (1-2 cm away from the pylorus) with the stent.

Experimental design

One hundred and sixty-five male Wistar rats were randomly divided into three groups: Group A, orthotropic liver transplantation by modified two-cuff method; Group B, bile duct was cut and reconstructed without orthotopic liver transplantation; and Group C, sham-operation group. Based on the approaches of biliary reconstruction, Group A was divided into two sub-groups: A1 (n = 30), duct-duct reconstruction, and A2 (n = 30), duct-duodenum reconstruction. To study the influence of hepatic artery on bile duct complication, Group B was divided into four subgroups: B1 (n = 10), duct-duct reconstruction with hepatic artery ligation; B2 (n = 10), duct-duct reconstruction without hepatic artery ligation; B3 (n = 10), duct-duodenum reconstruction with hepatic artery ligation, and B4 (n = 10), duct-duodenum reconstruction without hepatic artery ligation. In Group C (n = 5), only laparotomy was performed.

Samples were harvested 14 d after operation or at the time when any significant biliary complication was found. Serologic samples were collected to test the levels of aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin (TBIL), direct bilirubin (DBIL), gummaglutamyl transpeptidase (GGT) and alkaline phosphatase (ALP). Biliary complications were determined by pathologic examination and serologic analysis.

Statistical analysis

Data were expressed as mean ± SD. Statistical differences

Table 1 Incidence rates of biliary complications in different groups

Groups	*n*	Abscess	Sludge	EBD	IBD	Total	
Group A							41.7%[e] (25/60)
A1	30	26.70% (8/30)	13.30% (4/30)	26.70% (8/30)	13.30% (4/30)	33.30% (10/30)	
A2	30	43.30% (13/30)	20% (6/30)	26.70% (8/30)	10% (3/30)	50%[a] (15/30)	
Group B							32.50% (13/40)
B1	10	10% (1/10)	0	30% (3/10)	0	30% (3/10)	
B2	10	10% (1/10)	0	20% (2/10)	10% (1/10)	20% (2/10)	
B3	10	30% (3/10)	20% (2/10)	40% (4/10)	10% (1/10)	50%[c] (5/10)	
B4	10	20% (2/10)	10% (1/10)	30% (3/10)	10%*t* (1/10)	30% (3/10)	
Group C	5	0	0	0	0	0	0
Total	105	26.70% (28/105)	12.40% (13/105)	26.70% (28/105)	9.52% (10/105)	36.20% (38/105)	

Some rats had two (or more) kinds of biliary complications at the same time. [a]$P < 0.05$ *vs* A1; [c]$P < 0.05$ *vs* B1, B2 and B4; [e]$P < 0.05$ *vs* groups B and C EBD: Extrahepatic biliary dilatation; IBD: Intrahepatic biliary dilatation.

between the control and the experimental groups were analyzed using analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

Operation time and success rate

In transplantation groups, the anhepatic phase was 13.7 ± 1.06 min, and cold ischemia time was 50.5 ± 8.6 min. There was no significant difference between A1 and A2 in operating time ($P > 0.05$). And the time for biliary reconstruction was almost the same among all groups ($P > 0.05$).

Recipients surviving at least 24 h were considered as success. The success rate of ROLT was 98.3% (59/60). Only one case in A1 died from bleeding at SHIVC 8 h after operation.

Biliary complications

The incidence rate of biliary complications was 41.7% in Group A, which was much higher than that in Group B (32.5%) and Group C (0%), with significant differences among the three groups ($P < 0.05$). After transplantation, A2 had a higher incidence of biliary complications than A1 (50% *vs* 33.3%, $P < 0.05$). B3 had the highest incidence of biliary complications among the groups without orthotopic liver transplantation ($P < 0.05$). No biliary complication was found in the sham-operation group.

General observation

The color of urine turned yellow in the rats with biliary complications. Other complications were dried hair, reaction retardation, reduced appetite and activities, and eye bleeding in some rats.

Gross anatomy

Biliary complications consisted of abscess, intrahepatic and extrahepatic biliary dilatation and biliary sludge. Abscess (35%) was most frequently seen in Group A, compared with dilatation of extrahepatic bile duct (25%) in Group B. B3 had the highest incidence of biliary complications in non-transplantation groups (50% *vs* 30%, 20% and 30%) and extrahepatic biliary dilatation and abscess were two of the most important complications in this group (Table 1).

Histopathology

In the samples with biliary complications, infiltration of a large number of mononuclear cells in the portal area was the most common change, followed by dilatation of bile ducts. Cellular infiltration of biliary wall and degeneration of epithelial cells, dilatation of the central vein could also be seen. Some samples even showed vacuolar degeneration, necrosis and fibrous tissue hyperplasia. In samples with severe biliary dilatation, the normal bile duct structure had completely disappeared, with a large number of infiltrated inflammatory cells.

Serology

The serum levels of AST, ALT, TBIL, DBIL, GGT and ALP in Group A and Group B were significantly higher than in Group C, particularly AST and TBIL, with significant difference among the three groups ($P < 0.05$). Significant difference could be easily observed between duct-duodenum reconstruction groups and duct-duct reconstruction groups (A2 *vs* A1, B3 *vs* B1, and B4 *vs* B2, $P < 0.05$). Among non-transplantation groups, the serological changes, especially the bilirubin level, were more remarkable in groups with hepatic artery ligation than in those without (B1 *vs* B2 and B3 *vs* B4, $P < 0.05$) (Table 2).

DISCUSSION

Biliary complication is the second most common cause of graft dysfunction in liver transplantation with an incidence rate of as high as 64%[1-9]. Biliary complications may be related to various factors, including hepatic artery thrombosis or stenosis, technical reasons, as well as ischemia-reperfusion injury and immunological injury. High incidence of biliary complication has made biliary reconstruction the "Achilles heel" of liver transplantation.

It has been proved that hepatic artery plays an important role in blood supply of bile duct, the artery must be reconstructed when injured or cut during operation. In the early 90s, Engermann *et al*[12] showed that reconstruction of the hepatic artery can significantly reduce the incidence of biliary complications such as biliary fistula and biliary obstruction after ROLT. But with the cuff method introduced in 1973, ROLT without hepatic artery (HA) recon-

Table 2 Serological values in different groups

	n	AST (U/L)	ALT (U/L)	TBIL (μ mol/L)	DBIL (μ mol/L)	GGT (U/L)	ALP (U/L)
A1	30	452.2 ± 296.9	223.7 ± 194.7	17.1 ± 26.0	14.8 ± 24.1	10.5 ± 6.2	366.5 ± 173.0
A2	30	534.2 ± 373.3	272.8 ± 274.7	11.6 ± 21.8	10.2 ± 20.2	8.0 ± 7.3	282.8 ± 154.9
B1	10	146.6 ± 43.6	75 ± 17.7	0.79 ± 1.59	0.54 ± 1.26	4.6 ± 3.8	208.6 ± 76.4
B2	10	108.7 ± 26.4	70.0 ± 6.6	0.33 ± 0.25	0.09 ± 0.12	2.5 ± 0.97	190.2 ± 44.2
B3	10	359.3 ± 452.4	126.6 ± 131.9	15.1 ± 31.3	11.5 ± 24.1	7.3 ± 5.1	240 ± 152.0
B4	10	271.2 ± 275.2	119.3 ± 138.2	9.7 ± 27.3	7.42 ± 21.5	5.7 ± 2.8	236.3 ± 158.3
Group C	5	81.7 ± 13.2	60.7 ± 4.5	0.13 ± 0.15	0 ± 0	2.7 ± 0.58	281.7 ± 139.3

ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; DBIL: Direct bilirubin; GGT: Gumma-glutamyl transpeptidase.

struction became globally accepted [13-18]. It is reasonable to speculate that biliary complications after liver transplantation might be more common in rats than that in human beings. Unfortunately, few studies can be found to investigate and report the incidence of biliary complications in ROLT. In our study, we simulated the biliary processes of ROLT in Group B, and found that the incidence of complications were significantly higher in groups with hepatic artery ligation than those without, which meant that hepatic artery might play an important role in the occurrence and development of biliary complications, but further investigations are needed to verify the definite mechanism.

Bile duct reconstruction by stent has been well accepted since Kamada introduced the "two-cuff" technique in ROLT[12-18]. But in view of our experience in over 600 cases of rat orthotopic liver transplantation, it seems that biliary complications after orthotopic liver transplantation are almost inevitable in the classical model. Several reasons might be contributed to this: Firstly, the wall of bile duct will become thicker as the rat grows up, constant inner diameter of the stent will definitely make the bile duct relatively narrow. Secondly, the stent would become a foreign body in bile, which will make the eddy come into being at the proximal stent and induce the sludge. So it is a great challenge to seek a new approach to modify the ROLT model. This new approach should reduce the incidence of biliary complications, and be easier to achieve.

Choledochojejunostomy has been proved to be an effective surgery to reconstruct the bile duct in clinical liver transplantation, which does not increase the incidence of biliary complications compared with the end-to-end anastomosis[19-22]. In our study, we simulated the method and designed the duct-duodenum reconstruction model. Unfortunately, this method did not show ideal results, the incidence of biliary complications was significantly higher in A2 (duct-duodenum reconstruction) than that in A2 (duct-duct reconstruction) due to the following reasons: in ROLT, the stent is directly driven into the upper part of the duodenum, which makes the stent easily obstructed by chime and then more likely to have biliary complications. Based on our study, although choledochojejunostomy has been widely used in liver transplantation, duct-duodenum reconstruction is obviously not an ideal choice in ROLT.

In conclusion, biliary complications are almost inevitable using the classical "two-cuff" technique. More attention should be paid to the occurrence of biliary complications in ROLT model. The established mode of hepatic artery and biliary reconstruction should be modified.

COMMENTS

Background

Biliary complication is the second most common cause of graft dysfunction in liver transplantation with an incidence rate of as high as 64%. Unfortunately, few researches could be found to report the incidence of biliary complications in rat liver transplantation. In this study, the authors investigated the biliary complications after rat orthotopic liver transplantation, trying to find a better way to perform biliary reconstruction and reduce the incidence of biliary complications after transplantation

Research frontiers

Classical rat liver transplantation model using the "two-cuff" technique introduced by Kamada has been well accepted since 1983, but the incidence of biliary complications remains extremely high, there is an urgent need to improve the technology for biliary reconstruction.

Innovations and breakthroughs

Most previous studies used the "two-cuff" technique to establish rat liver transplant models, but few focused on the high incidence of biliary complications. In view of the experience in over 600 cases of rat orthotopic liver transplantation in this study, biliary complications are almost inevitable by using the classical "two-cuff" technique.

Applications

More attention should be paid to the occurrence of biliary complications in ROLT model. The established mode of hepatic artery and biliary reconstruction should be modified.

Terminology

Biliary complications: often include hilar abscess, intrahepatic and extrahepatic biliary dilatation, biliary sludge. "Two-cuff" technique: A classical method used in rat liver transplantation, which was first introduced by Kamada *et al*. The key steps are: Portal vein and infrahepatic inferior vena cava are induced through the cuffs and the distal end of the vein is completely reversed and fixed onto the cuff with a 5-0 silk ligation.

Peer review

The paper is well designed and the experience is valuable to be published.

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BRIEF ARTICLE

Evaluation of transarterial chemoembolization combined with percutaneous ethanol ablation for large hepatocellular carcinoma

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Abstract

AIM: To assess the effects of combined transcatheter arterial chemoembolization (TACE) and percutaneous ethanol ablation (PEA) in patients with large hepatocellular carcinoma (HCC).

METHODS: A total of 63 patients with unresectable large HCC were treated with TACE followed by PEA. The largest dimension of the tumors ranged from 5.3 cm to 17.8 cm. The survival rates, acute effects, toxicity and prognostic factors were analyzed.

RESULTS: The cumulative survival rates at 1, 3 and 5 years were 59.4%, 28.4% and 15.8%, respectively (a median survival of 27.7 mo). Tumor area was reduced by more than 50% in 30 (47.6%) cases. In 56 cases with increased α-fetoprotein (AFP) values, AFP level was declined by more than 75%. The combined therapy was generally well tolerated. Only two patients died from variceal bleeding associated with the therapy. The Cox proportional hazards model showed that the number of tumors, the tumor margin and the ethanol dose were independent prognostic factors.

CONCLUSION: The combined TACE and PEA therapy is a promising approach for unresectable large HCC.

Key words: Hepatocellular carcinoma; Chemoembolization; Ethanol ablation; Combination therapy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, responsible for an estimated one million deaths annually. It has a poor prognosis due to its rapid infiltrating growth and complicating liver cirrhosis. Surgery is the only potential cure, but the resection rate for HCC is only 10%-30%. The remaining patients are subjected to various modes of non-surgical therapy. Transcatheter arterial chemoembolization (TACE) has become one of the most popular approaches of non-surgical treatment, being effective in reducing tumor size in HCC and improving survival[1-4]. However, tumor cells remain viable in and

around the capsule, which is supplied by both arterial and portal blood, and these cells are often responsible for later recurrence and spread[5-10]. Further treatment is needed to eradicate residual tumor cells. We used TACE combined with percutaneous ethanol ablation (PEA) to treat 63 patients with large HCC and retrospectively evaluated the effects of this combined therapy and the prognostic factors.

MATERIALS AND METHODS

Ethics

This study was approved ethically by the Sun Yat-Sen University Cancer Center. All patients provided informed written consent. This work was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association.

Patients

From November 2001 to January 2009, 63 consecutive patients with large unresectable HCC were enrolled to this study. In all the patients, the diagnosis of HCC was made based on the histologic or angiographic findings combined with serum α-fetoprotein (AFP) levels. In 41 (65.1%) patients, the diagnosis of HCC was confirmed by histologic examination. The remaining 22 patients were diagnosed according to the findings on ultrasound, CT and angiography, and serum AFP levels. The enrolling criteria were as follows: (1) lesions detectable on ultrasound and CT; (2) tumor/liver volume ratio not above 0.7:1; (3) serum transaminase level under 80 IU/L; and (4) no evidence of extrahepatic metastasis or ascites. Patients who had ascites, extrahepatic metastasis, severe cirrhosis (class C according to Child's classification), or Karnofsky performance score < 70 were excluded. The baseline characteristics of patients are shown in Table 1.

Table 1 Baseline data of the patients

Variables	Values
Mean age (yr)	57.2
Cases of HBV-related liver disease	61.0
Cases of HCV-related liver disease	0.0
Mean AST (U/L)	43.4
Mean ALT (U/L)	49.5
Mean total bilirubin (μmol/L)	26.2
Mean AFP (ng/mL)	963.9
Mean tumor size (cm)	8.3

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: α-fetoprotein.

Methods

TACE was performed in the following processes: a 5.0 French catheter (Terumo, Tokyo, Japan) was inserted into the femoral artery by the Seldinger's method. Celiac angiography and selective hepatic arterial angiography were routinely performed to observe the tumorous blood supply, distribution of hepatic arteries and collateral circulation routes. The tip of the catheter was placed at the feeding artery of the tumor, and embolization was performed using emulsionized mixture of lipiodol ultra-fluid (Guerbet, France), Perarubicin (50 mg/m^2) and DDP (80 mg/m^2). The maximum dose for the embolization depended on the size of the tumor, blood supply and hepatic function of the patient. When the tumor was filled well with emulsifier, the embolization ended.

After 1-2 times of TACE, PEA was performed using an ethanol solution (99% concentration, mixed with lipiodol, 9:1 volume ratio) slowly injected into the tumor through a 15-cm 21 gauge Chiba needle (Cook, Bloomington, IN) guided by CT scan. The size of needle, the amount of ethanol injected per procedure and the number of procedures for the entire treatment, were planned depending on the volume of the tumor and the extent of the transient high-density zones induced by ethanol diffusion on CT scans. The procedure was completed when the entire targeted tumor appeared with a high density. PEA was performed 2-5 times for each tumor. The amount of ethanol injected per procedure and per tumor was 3-20 mL (mean ± SD, 8.2 ± 3.4 mL) and per patient 5-40 mL (mean ± SD, 30.5 ± 6.6 mL).

The follow-up protocol after the initial combined therapy was planned according to the volume of the tumor, tumor blood supply and the extent of the high-density zones on CT scans. The standard TACE for a 8.0-10.0 cm HCC needs two steps (3 wk for each step) when a good tumor blood supply was displayed on enhanced CT scan, and the standard PEA protocol for a 5.0-6.0 cm HCC needs three steps (1.5 wk for each step) when tumor blood supply was obviously decreased on enhanced CT scan. The ethanol treatment was ended when the entire targeted tumor appeared with a high density.

The therapeutic efficacy was evaluated by CT scan two mo after the combined treatment.

Prognostic factors

Factors thought to influence survival were selected and classified to obtain survival rates using the Kaplan-Meier method. The significance of the differences was evaluated by the log-rank test with univariate analysis. The following prognostic factors were investigated: sex, age, number of lesions, tumor size, tumor extension, tumor margin (the tumor margin is the edge of the tumor, and the boundary between the tumor tissues and normal tissues was determined based on hepatobiliary phase images), AFP, portal thrombosis, ascites, Child grade, Okuda stage, times of TACE and PEA and the total ethanol dose. Variables with possible prognostic significance were selected, and each variable was divided into 2-4 classes (Table 2). Factors related to the survival rate were used as variables, and stepwise multivariate analysis was performed. Multiple regression analysis was performed using the Cox proportional-hazard model to calculate the relative-risk ratio between each factor and the survival rate.

RESULTS

Recent results, survival and prognostic factors

Tumor area was reduced by more than 50% in 30 (47.6%) cases. In 56 cases with increased AFP, AFP level was declined by more than 75%.

At the end of this study, 11 patients remained alive,

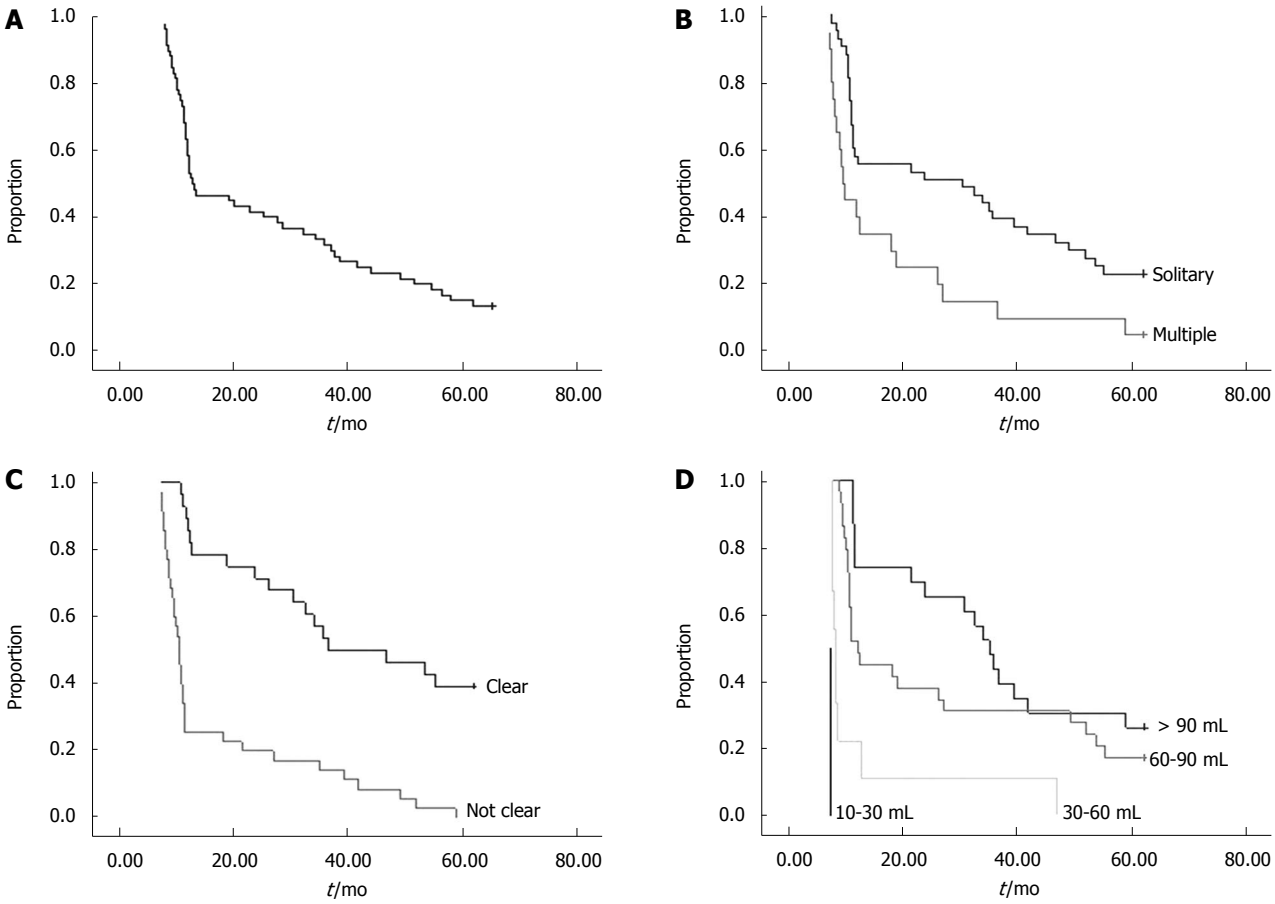

Figure 1 Overall cumulative survival curve and cumulative survival curves in patients based on the number of lesions, tumor margin and percutaneous ethanol ablation dose. A: Overall cumulative survival curve in 63 hepatocellular carcinoma (HCC) patients receiving combined therapy of transcatheter arterial chemoembolization (TACE) and percutaneous ethanol ablation (PEA); B: Cumulative survival curves in patients based on the number of lesions; C: Cumulative survival curves in patients based on the tumor margin; D: Cumulative survival curves in patients based on PEA dose.

Table 2 Variables and classes by univariate and multivariate analyses

Variables	Classes			
	A	B	C	D
Sex	M (52)	F (11)		
Age (yr)	< 55 (36)	> 55 (27)		
No. of lesions	Solitary (43)	Multiple (20)		
Tumor size (cm)	5-10 (41)	> 10 (22)		
Tumor extension	1 lobe (46)	2 lobe (17)		
Tumor margin	Clear (28)	Not clear (35)		
AFP	< 400 (22)	> 400 (41)		
Portal thrombosis	Absent (51)	Present (12)		
Ascites	Absent (56)	Present (7)		
Child grade	A (38)	B (25)		
Okuda stage	Ⅰ (29)	Ⅱ (34)		
TACE (number of times)	1 (11)	2 (26)	3 (20)	4 (6)
PEA (number of times)	1 (3)	2 (6)	3 (31)	> 4 (23)
Total ethanol dose	10-30 (2)	- 60 (9)	- 90 (29)	> 90 (23)

In the parenthesis are numbers of patients. AFP: α-fetoprotein; TACE: Transcatheter arterial chemoembolization; PEA: Percutaneous ethanol ablation.

and 52 patients had succumbed. The survival curve is shown in Figure 1A. Overall survival rates at one, three, and five years were 54.0%, 31.7% and 17.5%, respectively (median survival 27.7 mo).

Univariate analysis indicated that 11 factors significantly influence the survival. Sex, age and TACE times were not significant ($P > 0.05$), (Table 3).

The Cox proportional hazards model showed that only the number of tumors, tumor margin and the total ethanol dose were independent factors predicting survival (Table 4).

The overall survival rates at one, three and five years in the 43 patients with a solitary lesion were 58.1%, 39.5% and 23.3%, respectively, and were 45.0%, 15.0% and 5.0%, respectively in the 20 patients with multiple lesions. The mean survival of patients with a solitary lesion was significantly longer ($P = 0.0145$) than that of patients with multiple lesions (Figure 1B). In the patients with clear tumor margin ($n = 28$), the 1, 3, and 5-year survival rates were 89.3%, 53.6% and 39.3%, respectively, and these figures were significantly higher ($P = 0.0052$) than in the patients without clear tumor margin ($n = 35$), who had survival rates of 25.7% at one year, 14.3% at three years, and 0 at five years (Figure 1C). The mean 1-, 3- and 5-year survival rates were estimated to be 0%, 0% and 0%, respectively, in the 2 patients who received 10-30 mL total ethanol dose; 33.3%, 16.7% and 0% in the 9 patients who received 30-60 mL total ethanol dose; 51.7%, 31.0% and 16.1% in the 29 patients who received 60-90 mL total ethanol dose; and 73.9%, 43.5%, and 26.1% in the 23 patients who received

Table 3 Factors affecting survival by univariate analysis *n* (%)

Variables	Class	Survival			*P* value
		1 yr	3 yr	5 yr	
Sex	A	27 (51.9)	16 (30.8)	9 (17.3)	
	B	7 (63.6)	4 (36.4)	2 (18.2)	0.924[1]
Age (yr)	A	21 (58.3)	11 (30.6)	5 (13.9)	
	B	13 (48.1)	9 (33.3)	6 (22.2)	0.223[1]
No. of lesions	A	25 (58.1)	17 (39.5)	10 (23.3)	
	B	9 (45.0)	3 (15.0)	1 (5.0)	0.0145[1]
Tumor size	A	28 (68.3)	18 (43.9)	11 (26.8)	
	B	6 (27.3)	2 (9.1)	0 (0)	0.0041[1]
Tumor extension	A	29 (63.0)	18 (39.1)	11 (23.9)	
	B	5 (29.4)	2 (11.8)	0 (0)	0.0054[1]
Tumor margin	A	25 (89.3)	15 (53.6)	11 (39.3)	
	B	9 (25.7)	5 (14.3)	0 (0)	0.0052[1]
AFP	A	16 (72.7)	10 (45.5)	9 (40.9)	
	B	18 (43.9)	10 (24.4)	2 (4.9)	0.0030[1]
Portal thrombosis	A	31 (60.8)	18 (35.3)	11 (21.6)	
	B	3 (25.0)	2 (16.7)	0 (0)	0.0111[1]
Ascites	A	32 (57.1)	19 (33.9)	11 (19.6)	
	B	2 (28.6)	1 (14.3)	0 (0)	0.0115[1]
Child grade	A	28 (73.7)	17 (44.7)	11 (28.9)	
	B	6 (24.0)	3 (12.0)	0 (0)	0.0132[1]
Okuda stage	A	22 (75.9)	14 (48.3)	10 (34.5)	
	B	12 (35.3)	6 (17.6)	1 (2.9)	0.0150[1]
TACE (No. of times)	A	3 (27.2)	2 (18.2)	1 (9.1)	
	B	13 (50.0)	7 (26.9)	3 (11.5)	
	C	13 (65.0)	8 (40.0)	5 (25.0)	
	D	5 (83.3)	3 (50.0)	2 (33.3)	0.1719[2]
PEA (No. of times)	A	1 (33.3)	0 (0)	0 (0)	
	B	2 (33.3)	1 (16.7)	0 (0)	
	C	16 (48.5)	10 (32.3)	5 (16.1)	
	D	15 (65.2)	9 (39.1)	6 (26.1)	< 0.0001[2]
Total ethanol dose	A	0 (0)	0 (0)	0 (0)	
	B	2 (33.3)	1 (16.7)	0 (0)	
	C	15 (51.7)	9 (31.0)	5 (16.1)	
	D	17 (73.9)	10 (43.5)	6 (26.1)	< 0.0001[2]

[1]*P* value: B *vs* A; [2]*P* value: D *vs* A. AFP: α-fetoprotein; TACE: Transcatheter arterial chemoembolization; PEA: Percutaneous ethanol ablation.

Table 4 Significant factors predicting survival found using Cox proportional hazards model

Variables	Hazard ratio	*P* value
No. of lesions		
Multiple *vs* single solitary lesions	2.626	0.001
Tumor margin		
Not clear *vs* clear	2.439	0.000
Total ethanol dose		
30-60 mL *vs* 10-30 mL	0.386	0.000
60-90 mL *vs* 10-30 mL	0.202	0.000
> 90 mL *vs* 10-30 mL	0.116	0.000

over 90 mL total ethanol dose. Statistically significant difference was found between the high-dose group and low-dose group ($P < 0.0001$), (Figure 1D).

Side effects

Fever, abdominal pain, nausea and vomiting occurred in most of the patients after TACE and PEA. These symptoms were self-limiting in almost all the patients, lasting less than one week. A slight increase in serum bilirubin (37 cases), elevated serum transaminase level (59 cases), ascites (6 cases), leucopenia (15 cases) and thrombocytopenia (11 cases) were associated with the combined therapy. These side effects were transitory or easily controlled with medication in most of the patients. Two patients died of variceal bleeding because of the increased portal vein pressure caused by deterioration of liver cirrhosis after repeated TACE-PEA, which had an impact on liver function.

DISCUSSION

The rationale for combined therapy of TACE and PEA relies on the fact that after TACE, tumor blood supply is markedly decreased and intratumoral septa are usually destroyed as a result of the necrosis induced by the procedure. These histopathologic changes make subsequent PEA treatment easier as they can provide enhanced ethanol diffusion within the tumor. Consequently, treatment with PEA is facilitated by the TACE-derived fibrous wall around the lesion, which favors a better retention of the injected ethanol within the tumor[11-15]. Tanaka *et al*[14] first reported the effectiveness of TACE combined with PEA for large (> 3.0 cm in diameter) primary HCC compared with that of TACE alone. His study found that a partial

response of the tumor was seen in only 10% of the patients, and the 1-, 2-, and 3-year survival rates were 68%, 37% and 0%, respectively with TACE alone, and histologic examinations showed that TACE alone caused complete necrosis in only 20% of the tumors. In contrast, PEA combined with TACE significantly increased the partial response rate (45%), prolonged the 1-, 2-, and 3-year survival rates (100%, 85% and 85%), and achieved complete histologic necrosis in 83% of the tumors. Dohmen *et al*[15] proved that the combined TACE and PEA treatment had a lower incidence of local recurrence than TACE alone which resulted in an increased survival of the patients with unresectable large HCC.

Ethanol in PEA diffused within the cells, causing immediate dehydration of cytoplasmic proteins with consequent coagulation necrosis followed by fibrosis, and entered the circulation, inducing necrosis of endothelial cells and platelet aggregation with consequent thrombosis of small vessels followed by ischemia of the neoplastic tissues. Advantages of PEA were[16-18]: no remarkable damage to the remaining parenchyma, being safe, easy to be repeated when new lesions appear, low in cost, easy to operate, and possessing good long-term results. PEA can be carried out either in patients with HCC who have a poor liver function or in elderly patients (age $\geqslant$ 70 years)[19,20]. Our results proved that higher doses of ethanol can be injected, which can achieve complete and homogeneous perfusion even in large lesions.

It is necessary to analyze prognostic factors in a large number of patients in sufficient detail and to evaluate the result of each method of treatment between groups with similar prognostic factors. Our study showed that only the number of tumors, tumor margin and the total ethanol dose were independent factors predicting survival. Although various prognostic factors have been reported[21-23], no conclusion has been drawn as to which factor is significant. In this study, the significant factors for better prognosis included the number of tumors, tumor margin and the total ethanol dose. The prognostic factors identified in this study suggested that, therapeutic results in patients with solitary tumors and clear tumor margin treated at a higher total ethanol dose should be better than those in patients with multiple tumors, without clear tumor margin treated at a lower total ethanol dose. It is worth noting the tumor margin is one of the important prognostic factors. It is determined based on hepatobiliary phase images and represents the growth pattern of tumor to some extent. The tumor margin imaging can predict microscopic portal vein invasion, intrahepatic metastasis and early recurrence after hepatectomy in HCC patients[24].

Ebara *et al*[25] and Vilana *et al*[26] proposed tumors < 30 mm in size and < 3 in number as indications for PEA, mainly because of technical limitation such as the inability to inject an effective volume of ethanol into the whole area of the tumor. Our results suggested that some tumors > 50 mm in size could be treated by PEA because the therapeutic results of PEA were also good for large HCC patients with solitary tumors and clear tumor margin at a higher total ethanol dose after TACE.

Long-term survival rates of PEA-treated patients are similar to those obtained in matched patients undergoing partial hepatectomy[27,28]. However, the long-term prognosis remains disappointing because of the high recurrence rate among patients with HCC after PEA, especially in those with multiple lesions, cirrhosis and a high level of AFP and those without a clear tumor margin and peritumoral capsule[29,30]. In fact, histological examination of HCC after PEA reveals that residual tumor tissues remain in portions isolated by septa or with extracapsular or intracapsular invasion. It has been demonstrated that the high vascularity of HCC promotes an early wash-out of injected ethanol, so that PEA for patients with hypervascular tumors may be less effective than for patients with hypovascular tumors[31,32].

COMMENTS

Background

The incidence of large hepatocellular carcinoma (HCC) is increasing in China and HCC has a poor prognosis due to its rapid infiltration and complicating liver cirrhosis. The results in this study indicated that combined transcatheter arterial chemoembolization (TACE) with percutaneous ethanol ablation (PEA) is a promising therapeutic approach for large unresectable HCC.

Research frontiers

The authors analyzed the prognostic factors in a large number of patients in detail and evaluated the result of each method of treatment between groups with similar prognostic factors. This study showed that the number of tumors, tumor margin and the total ethanol dose were independent factors predicting survival.

Innovations and breakthroughs

This is the first study to report that the number of tumors, tumor margin and the total ethanol dose were independent factors predicting survival of large HCC. The combined TACE and PEA therapy is a promising approach for large unresectable HCC.

Applications

By understanding the independent prognostic factors, this study may represent a future strategy in the treatment of patients with large unresectable HCC.

Terminology

TACE has become one of the most popular approaches of non-surgical treatment, with good results in reducing the tumor size of HCC and improving the survival of the patients. PEA is facilitated by the TACE-derived fibrous wall around the lesion, which favors a better retention of the injected ethanol within the tumor.

Peer review

This is a constructive study to report that the number of tumors, tumor margin and the total ethanol dose were independent factors predicting survival of large HCC, which is expected to improve the therapeutic effects for large unresectable HCC.

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BRIEF ARTICLE

Lactobacillus species shift in distal esophagus of high-fat-diet-fed rats

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Abstract

AIM: To analyze the microbiota shift in the distal esophagus of Sprague-Dawley rats fed a high-fat diet.

METHODS: Twenty Sprague-Dawley rats were divided into high-fat diet and normal control groups of 10 rats each. The composition of microbiota in the mucosa from the distal esophagus was analyzed based on selective culture. A variety of *Lactobacillus* species were identified by molecular biological techniques. Bacterial DNA from *Lactobacillus* colonies was extracted, and 16S rDNA was amplified by PCR using bacterial universal primers. The amplified 16S rDNA products were separated by denaturing gradient gel electrophoresis (DGGE). Every single band was purified from the gel and sent to be sequenced.

RESULTS: Based on mucosal bacterial culturing in the distal esophagus, *Staphylococcus aureus* was absent, and total anaerobes and *Lactobacillus* species were decreased significantly in the high-fat diet group compared with the normal control group ($P < 0.01$). Detailed DGGE analysis on the composition of *Lactobacillus* species in the distal esophagus revealed that *Lactobacillus crispatus*, *Lactobacillus gasseri* (*L. gasseri*) and *Lactobacillus reuteri* (*L. reuteri*) comprised the *Lactobacillus* species in the high-fat diet group, while the composition of *Lactobacillus* species in the normal control group consisted of *L. gasseri*, *Lactobacillus jensenii* and *L. reuteri*.

CONCLUSION: High-fat diet led to a mucosal microflora shift in the distal esophagus in rats, especially the composition of *Lactobacillus* species.

Key words: Obesity; *Lactobacillus*; Sprague-Dawley rats; Distal esophagus; Denaturing gradient gel electrophoresis

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most common clinical disorders, and its incidence

has been steadily increasing around the world in recent years[1-4]. Numerous studies have demonstrated that obesity is a risk factor for GERD. The incidence of GERD and its complications in subjects who are overweight or obese is higher than in those with normal weight[1-4]. With obesity gradually increasing worldwide, obesity-associated GERD is becoming a more complicated clinical problem. Unfortunately, to date, the pathogenesis of obesity-associated GERD has not been fully understood. Thus, the usual management of GERD is merely to inhibit acid production and improve motility of the upper gastrointestinal tract to ameliorate symptoms, which can seriously affect quality of life[5-8].

It is well accepted that GERD is mainly due to abnormality of the gastroesophageal junction or a decrease in esophageal clearance capacity. Current data have demonstrated that the association of obesity with GERD is mainly related to changes in lower esophageal sphincter motility or a pressure imbalance between the distal esophagus and gastric fundus[1-8]. However, the detailed mechanism needs to be further elucidated.

Recent studies have found that the composition of intestinal flora in obese individuals differs from that in normal individuals. Bacterial diversity, either different composition or quantity of bacteria in the gastrointestinal tract, is associated with gastrointestinal smooth muscle motility[9-13]. In clinical practice, many bacterial agents have been used for treatment of diarrhea, irritable bowel syndrome and other gastrointestinal motility diseases, and have achieved a positive response. Based upon the published findings, it can be estimated that microflora in the distal esophagus, to some extent, might play a role in regulating smooth muscle motility. However, the features of colonization and functionality of the microflora in the distal esophagus remain obscure. Some evidence has shown that the esophageal bacteria are transitory, and other studies have demonstrated a complex, residential microflora in the distal esophagus. Several studies also have confirmed the presence of a residential bacterial population in the distal esophagus in patients with esophageal-reflux-related disorders[14,15]. More detailed research has shown a significant alteration in the number of lactobacilli in the intestine of obese rats[11,16-19]. Thus, it is reasonable that obesity, a passive risk factor for GERD, might alter the composition of microflora in the distal esophagus, and lead to the formation of GERD.

Therefore, we hypothesize that potential variations in microbial composition in the distal esophagus of obese individuals might influence the distal esophageal motility and lead to GERD. Thus, we focused on the variation in microflora, especially *Lactobacillus* species, in the distal esophagus in obese Sprague-Dawley rats induced by high-fat diet, compared with normal rats, to illustrate the role of obesity or high-fat diet in the microfloral composition shift.

MATERIALS AND METHODS

Animals

Twenty-five healthy male Sprague-Dawley rats (purchased from the Experimental Animal Co., Hayes Lake, Hunan, China) aged 3 wk, weighing 50-70 g, were used. The animal experiments were approved by the Animal Experiment Ethics Committee of Central South University, Changsha, China.

Table 1 Comparison of the components of each diet: high-fat diet and common diet (per 0.1 kg)

Diet	Protein (g)	Fat (g)	Carbohydrate (g)	Energy (KJ)
Common diet	17.53	6.08	59.98	1250
High-fat diet	16.52	25.17	56.66	1810

Establishment of animal model

Sprague-Dawley rats were housed individually in cages at constant room temperature of 18-22℃, 50% humidity, in a 12-h light/dark cycle, and had free access to common diet and water. After 1 wk of adaptive feeding, five rats were killed and the other 20 were randomly divided into two groups of 10. One group was fed a high-fat diet (Dongchuang Nursery, Hunan, China; Table 1) for 6 wk, and the normal control group was fed a common diet. The daily diet was sterilized by Co^{60} irradiation and water by autoclave before feeding to the rats. Rats were killed at the end of 7 wk. Body weight, body length and feed consumption were recorded weekly and daily[20-22].

Histopathological examination of esophageal mucosa

The esophagus of each rat was removed and dissected longitudinally. Esophageal specimens were obtained from 1.5 cm above the gastroesophageal junction. Then 0.5 cm of each longitudinal strip was fixed in 10% formalin-buffered saline, embedded in paraffin, and processed for histopathological analysis. Two sections were cut from each paraffin block and stained with hematoxylin-eosin (HE) for evaluation of inflammation.

Determination of serum lipid levels

After fasting for 10 h, venous blood was obtained at the end of 7 wk. Three hundred microliters of serum for each sample was extracted and stored at -20℃. Serum triglyceride and total cholesterol were detected by GPO-PAP and CHOD-PAP methods, respectively.

Bacterial culture

The esophagus of each rat was removed and dissected longitudinally. Esophageal mucosa samples were obtained from 1 cm above the gastroesophageal junction. After being weighed on an electronic balance, the samples were homogenized immediately, and the homogenates were diluted at 10^{-7} in sterile normal saline. Ten microliters was spread thoroughly over the surfaces of specific medium plates. The media utilized were as follows: Mannitol Salt Agar for *Staphylococcus aureus* (*S. aureus*); Actinomyces Agar for *Actinomyces*; TTC Agar for *Enterococcus*; KF Streptococcus Agar for *Streptococcus*; EMB Agar for *Enterobacter*; Anaerobic Agar for total anaerobes; Clostridium Agar for

Clostridium; PY Agar for *Clostridium perfringens*; LBS Agar for *Lactobacillus* and *Lactobacillus crispatus* (*L. crispatus*); and BL Agar for *Bifidobacterium*. All aerobic plates were incubated at 37℃ for 24 h, followed by incubation for 12 h at room temperature. The LBS plates were incubated in a candle-jar atmosphere of 10% CO_2. Anaerobes were grown in an anaerobic jar at 37℃ for 72 h. The number of colonies from each plate was recorded.

Amplification of Lactobacillus 16S rDNA for denaturing gradient gel electrophoresis

DNA from *Lactobacillus* cultures was extracted according to the protocol of BioTeke Corporation (Beijing, China) and stored at -20℃. Universal bacterial primers (Invitrogen Biotechnology, Shanghai, China) HAD1-GC (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGGACTCCTACGGGAGGCAGCAGT-3') and HAD2(5'-GTATTACCGCGGCTGCTGGCAC-3')[23] were used to amplify V2 to V3 regions of the bacterial 16S rDNA. PCR reactions were run at 95℃ for 5 min, followed by 38 cycles of amplification at 94℃ for 40 s, 52℃ for 40 s, and 72℃ for 50 s, and a 7-min extension at 72℃.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed with the DCode™ Universal Detection System (Bio-Rad) that utilized 16 cm × 16 cm × 1 cm gels. Eight percent polyacrylamide gels were prepared and run with 1 × TAE buffer (2 mol/mL Tris base, 1 mol/mL glacial acetic acid, and 50 mmol/mL EDTA) diluted from 50 × TAE buffer (Sigma, Beijing, China). The denaturing gradient was formed with two 8% acrylamide (acrylamide/bis, 37.5:1) stock solutions (Bio-Rad). The gels contained a 30%-70% gradient of urea and formamide that increased in the direction of electrophoresis. A 100% denaturing solution contained 40% (v/v) formamide and 7.0 mol/mL urea. The electrophoresis was conducted with a constant voltage of 110 V at 60℃ for 12 h. Gels were stained with ethidium bromide solution (5 μg/mL) for 30 min, washed with deionized water, and viewed by UV transillumination (Bio-Rad).

Amplification and sequencing of the 16S V2-V3 region

Single predominant bands of the DGGE gel were selected by cutting with a sterile scalpel, and added to 50 μL deionized sterile water (Fermentas Life Sciences, Shenzhen, China). The gel pieces were placed at 4℃ for 24 h, centrifuged at 12000 × *g* for 10 min, and the supernatants were used as templates for PCR amplification. Universal bacterial primers (Invitrogen Biotechnology) HAD1(5'-TCCTACGGGAGGCAGCAGT-3') and HAD2(5'-GTATTACCGCGGCTGCTGGCAC-3')[23] were used for amplification. For each PCR amplification, 5 μL template was added to 45 μL PCR reaction mixture (Fermentas Life Sciences) that contained 5 μL 10 × PCR buffer, 5 μL 25 mmol/L $MgCl_2$, 1.5 μL 10 mmol/L dNTPs, 1.5 μL 100 ng/μL each primer, and 2.5 U Taq DNA polymerase. Reactions were run at 95℃ for 5 min, followed by 36 cycles of amplification at 94℃ for 30 s, 56℃ for 40 s, and 72℃ for 40 s, and a 10-min extension at 72℃. Wizard PCR Preps DNA Purification System (Promega, Beijing, China) were used to purify the PCR products. The purified products were sent to Beijing Genomics Institute of Technology for sequencing. Blast sequences were obtained through GenBank BLAST searches (http://www.ncbi.nlm.nih.gov/blast).

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 15.0. Results were expressed as measurement and enumeration data. Data are presented as means and SDs. For statistical comparisons, the *t* test and χ^2 test were performed and $P < 0.05$ was considered statistically significant.

RESULTS

High-fat diet resulted in elevated body weight and serum lipid with no significant histological change in vivo

There was no significant change in body weight in the high-fat diet and normal control groups at the end of the first week. Since the second week, the body weight and Lee's index increased weekly (Figure 1A and B). Accordingly, serum triglyceride and total cholesterol in the high-fat diet group were significantly increased compared with the normal control group (Figure 1C). HE staining of esophageal mucosa showed no musculature damage or mucosal injury in the distal esophagus in the high-fat diet or normal control group (Figure 1D).

High-fat diet led to microfloral shift in distal esophagus based on bacterial culture

Bacteria in the distal esophagus were cultured on selective media. In the high-fat diet group, *S. aureus* was absent, and the numbers of total anaerobes and lactobacilli were reduced significantly in the distal esophagus compared with the normal control group. There was no obvious difference between the common and adaptive feeding groups for composition of cultivable bacteria in the distal esophagus of Sprague-Dawley rats (Table 2).

Determination of Lactobacillus species shift in distal esophagus of high-fat diet-fed rats based on DGGE and sequencing

16S rDNA of cultivable *Lactobacillus* from the high-fat and common diet groups was amplified by PCR using universal bacteria primers HAD1-GC and HAD2 (Figure 2A). The amplified products of 16S rDNA were separated by DGGE. The two groups shared dramatically different bands, with bands A, C, D and E in the high-fat diet group, and bands B, C, D and E in the common diet group (Figure 2B). The purified bands were sequenced and BLASTed online with the V2-V3 region. The composition of *Lactobacillus* species in the distal esophagus in the common diet group was *Lactobacillus gasseri* (*L. gasseri*), *Lac-*

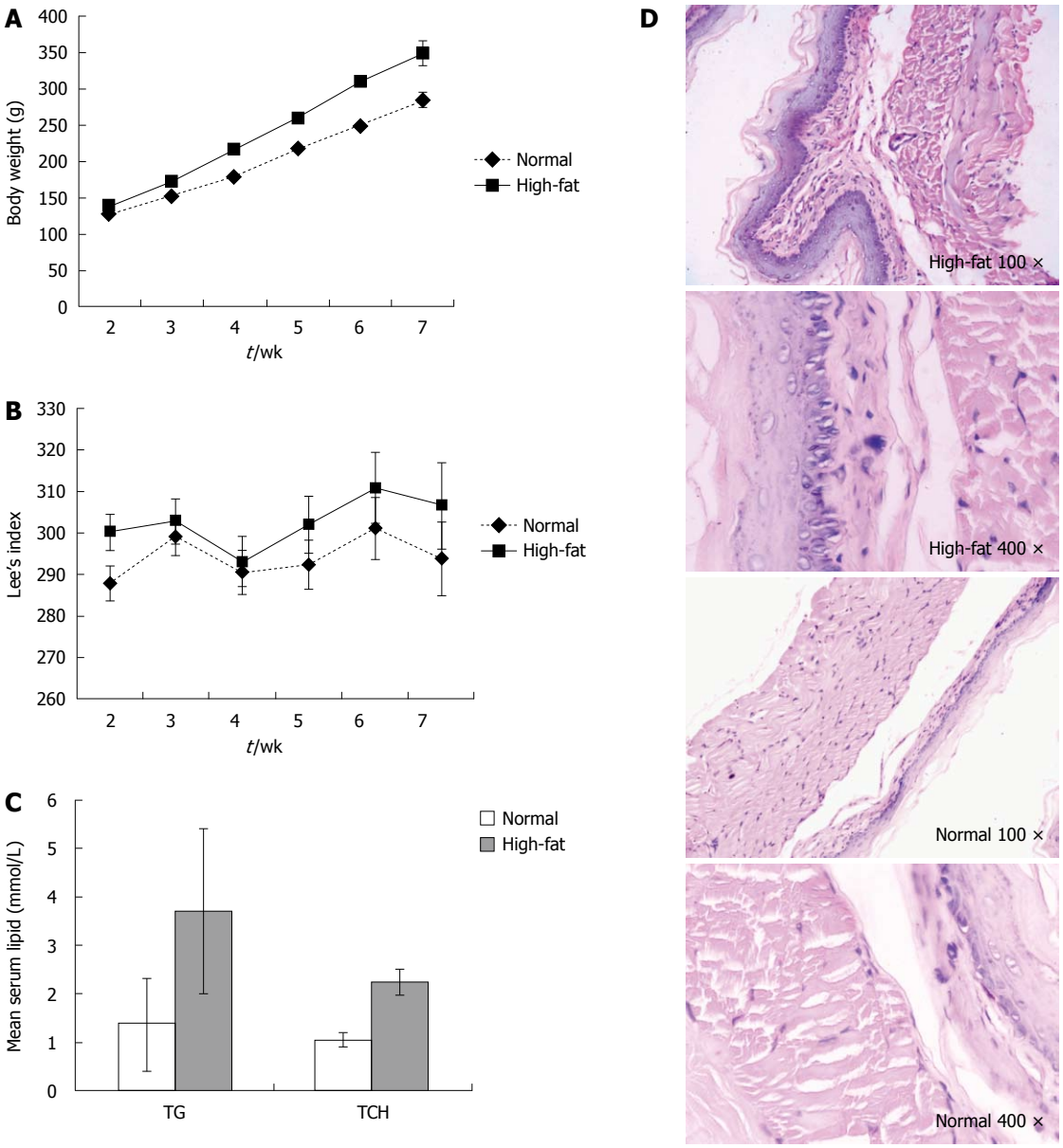


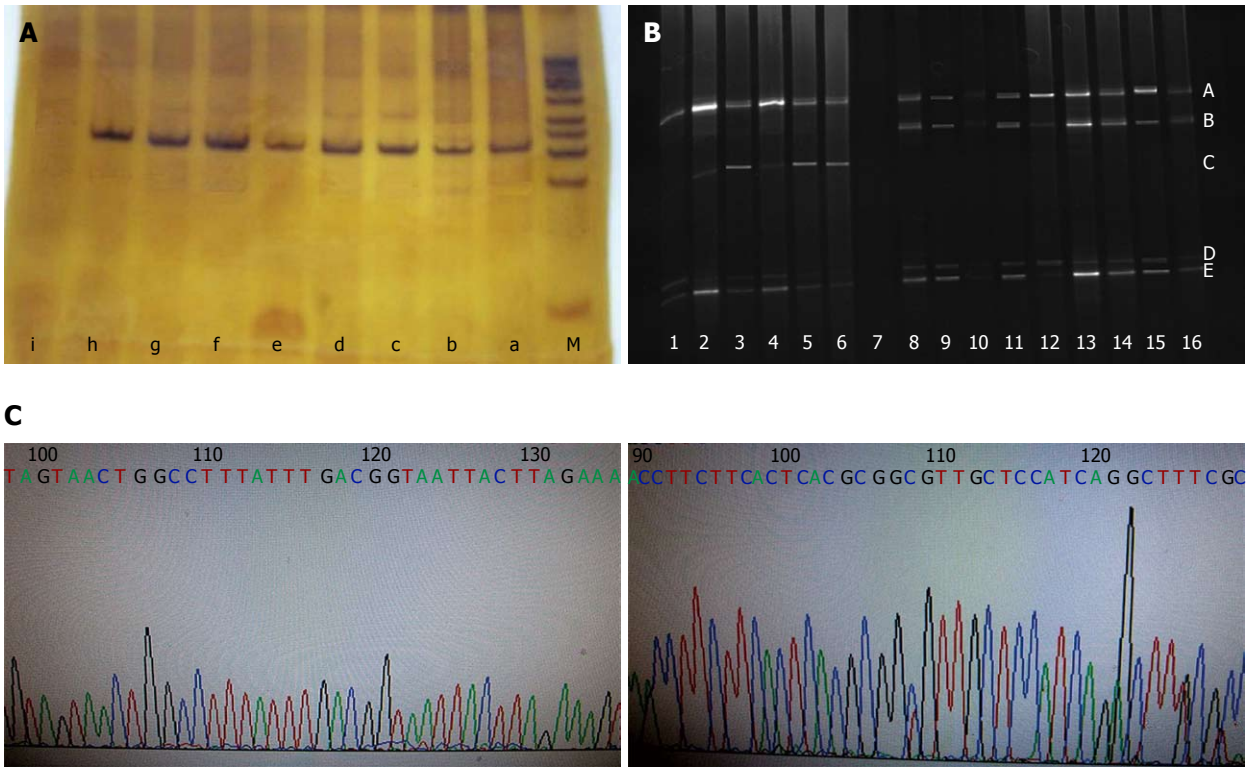
Figure 1 No significant change in distal esophageal mucosa in Sprague-Dawley rats fed a high-fat diet. A: Body weight changes of age- and sex-matched rats in the high-fat diet and normal control groups (n = 10 each); B: Lees index changes for the rats in (A) (n = 10); C: Mean serum lipid for the rats in (A); D: Histological analysis of representative distal esophagus from the rats in (A) (Original magnification, hematoxylin-eosin staining, 100 × or 400 ×).

tobacillus jensenii (*L. jensenii*) and *Lactobacillus reuteri* (*L. reuteri*), whereas the composition shifted to *L. crispatus*, *L. gasseri* and *L. reuteri* in the high-fat diet group (Figure 2C and D).

DISCUSSION

Accumulating evidence has shown that obesity is a risk factor in the development of GERD[1-8,24,25]. However, is not well established how obesity affects the incidence of GERD. According to the published experimental data, no musculature damage has been reported in the distal esophagus of obese rats or mice. However, different components of the microbiota have been identified between obese and lean animals/humans. Based on this evidence, the purpose of this study was to analyze the microbiota composition and focus on how the shift in microflora occurred in the distal esophagus of rats fed a high-fat diet.

To date, the role of microbiota in the distal esophagus under conditions of weight gain remains obscure. Some studies have shown that high-fat/high-sugar chow diet-fed, germ-free mice gain significantly less weight than their control littermates. The absence of microbiota has a protective role against weight gain in mice that consume a Western-style diet. Thus, body weight gain could be associated with a shift in the microbiota in mice fed a western-style diet. On the other hand, current research has demonstrated that there is a complex residential microbiota shift in the distal esophagus of patients with esophageal-reflux-related disorders, such as GERD, reflux esophagitis and Barrett's esophagus[14,15,26]. The possible explanations are that the reflux content might contain gastric bac-



D

Code	Sequence length (bp)	The most similar bacteria (species)	%Identity
A	253	Lactobacillus gasseri 224-1 (gb|ADFT01000001.1|)	98
B	250	Lactobacillus jensenii SJ-7A-US (gb|ACQD01000066.1|)	98
C	168	Lactobacillus crispatus 214-1 (gb|ADGR01000076.1|)	96
D	259	Lactobacillus reuteri MM2-3 (gb|ACLB01000138.1|)	99
E	231	Lactobacillus reuteri MM2-3 (gb|ACLB01000138.1|)	98

Figure 2 Determination of *Lactobacillus* species shift in distal esophagus of high-fat diet-fed Sprague-Dawley rats based on the combination of denaturing gradient gel electrophoresis and sequencing. A: 16S rDNA of cultivable *Lactobacillus* was amplified by polymerase chain reaction using universal bacteria primers HAD1-GC and HAD2 in the high-fat diet and common diet groups. a: *Lactobacillus crispatus* (*L. crispatus*) for positive control, b-d: High-fat diet group; e-h: Common diet group; i: Negative control; M: DNA marker; B: Amplified products of 16S rDNA were separated by denaturing gradient gel electrophoresis (DGGE). Lanes 1-6: High-fat diet group; lane 7: Negative control; lanes 8-16: Normal diet group. The bands were marked with A, B, C, D and E, respectively; C: Purified bands were purified and sequenced; D: BLASTed online with V2-V3 region. Bands A, B, C, D and E represented *Lactobacillus gasseri*, *Lactobacillus jensenii*, *L. crispatus*, *Lactobacillus reuteri*, and *Lactobacillus reuteri*, respectively.

Table 2 Analysis of cultivable microbiota of distal esophagus from high-fat diet group and common diet group (n = 10) (mean ± SE, lg CFU/g)

	Adaptive group (n = 5)	High-fat diet (n = 10)	Common diet (n = 10)
Total aerobic bacteria	4.92 ± 0.83	3.81 ± 1.83	4.45 ± 1.46
S. aureus	4.80 ± 1.42	0	3.80 ± 2.83[a]
Actinomycetes	4.52 ± 1.61	4.58 ± 0.47	4.12 ± 1.51
Enterococcus	3.21 ± 1.64	2.02 ± 1.82	2.89 ± 2.47
Streptococcus	4.14 ± 1.91	2.82 ± 3.55	3.74 ± 2.19
Enterobacter	3.22 ± 1.47	1.45 ± 2.91	3.05 ± 1.32
Total anaerobes	4.61 ± 0.42	2.11 ± 0.80[a]	4.48 ± 0.38[a]
Clostridium	4.47 ± 1.67	5.28 ± 1.60	4.77 ± 1.86
Clostridium perfringens	4.97 ± 1.49	3.59 ± 2.49	5.11 ± 1.58
Lactobacillus	5.31 ± 1.62	2.44 ± 0.97	5.44 ± 1.54[a]
Bifidobacterium	4.24 ± 2.97	2.53 ± 2.68	4.32 ± 3.21

[a]$P < 0.05$, indicates high-fat diet group *vs* common group. *S. aureus*: *Staphylococcus aureus*.

teria or damage the esophageal mucosa and lead to an abnormal inhabitation niche for the distal esophageal microbiota. Thus, the abnormal interactions among distal esophageal residential microbiota, gastric bacteria, and distal esophageal mucosa could lead to a composition shift in the microbiota in the distal esophagus.

Based on analysis of cultivable microbiota of the distal esophagus, we found a certain microbiota compositional shift in the distal esophagus of obese rats. The major microbiota shift in the distal esophagus involved the loss of *S. aureus* and decrease in anaerobes and *Lactobacillus* species. The focus in this research was diet-associated obesity, with integrated esophageal mucosal changes, and no obvious histological changes. This means that the microbiota shift in the distal esophagus was not due to the reflux of gastric content or alteration of the inhabitable niche of microbiota in the distal esophagus. Thus, a possible explanation for this microbiota shift in mice fed a western-style diet, compared with their control littermates, could be the

components of high-energy materials in the distal esophageal mucosa. These high-energy components might alter the mucosal microenvironment and help the local bacteria to colonize the distal esophageal mucosa.

GERD is an obvious motility disorder of the distal esophagus. Recent research has shown that several *Lactobacillus* or other bacterial species can alter the motility of smooth muscle in the distal esophagus[9-12]. It has been shown that different composition of *Lactobacillus* species can lead to different influences on smooth muscle motility in the distal esophagus[9-12]. However, the details are still unclear. Some evidence has shown increased smooth muscle contraction in certain GERD cases, whereas other studies have shown opposite results. The probable reason is that analysis of the shift in *Lactobacillus* was restricted to the level of the *Lactobacillus* genus. Fortunately, with the development of modern molecular techniques, analysis of *Lactobacillus* at the species level has become possible.

In our study, the combination of bacterial culture and DGGE was used to analyze the composition of *Lactobacillus* in the distal esophagus. It was clearly demonstrated that three cultivable *Lactobacillus* species, *L. gasseri*, *L. jensenii* and *L. reuteri*, colonized the distal esophagus in normal control rats. However, the composition of *Lactobacillus* species was shifted to *L. crispatus*, *L. gasseri* and *L. reuteri* in the distal esophagus in high-fat diet-fed rats. Four *Lactobacillus* species constituted a specific group in the distal esophagus of normal control rats as well as in obese rats. To our surprise, *L. jensenii* disappeared from the mucosa of the distal esophagus, which was replaced by *L. crispatus* in obese rats. It is possible that the disappearance of *L. jensenii* and re-colonization with *L. crispatus* results in alteration of smooth muscle motility in the distal esophagus. Therefore, this could probably be involved in the etiology of GERD, because of the variations in cultivable *Lactobacillus* species in the distal esophagus in obese rats. Another reasonable explanation is that different *Lactobacillus* species could have different metabolic characteristics, and influence the inhabitation niche of the microbiota in the distal esophagus. Yet another possibility is that the composition of cultivable *Lactobacillus* species in the distal esophagus of obese rats could be beneficial in preventing esophageal reflux.

Clearly, many unresolved questions remain to be elucidated. Our research will next focus on the effects of the composition shift in *Lactobacillus* on smooth muscle motility in the distal esophagus. Then, we will distinguish between the promoting or inhibitory properties of ingredients or metabolites of different *Lactobacillus* species. Therefore, the data could result in potential therapeutic targets in GERD.

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COMMENTS

Background

Obesity is a risk factor for gastroesophageal reflux disease (GERD), but how obesity affects the incidence of GERD is not well established. Current research has demonstrated that there is a complex residential microbiota shift in distal esophagus of esophageal-reflux-related disorders in humans. The different components of the microbiota are identified between obese animals/humans and lean animals/humans. Thus, it is reasonable that obesity, a passive risk factor of GERD, might alter the composition of microflora in the distal esophagus, and lead to development of GERD.

Research frontiers

Bacterial diversity is associated with gastrointestinal smooth muscle motility, and is also identified between obese animals/humans and lean animals/humans. However, the microbiota shift in the distal esophagus in obesity has not been fully addressed. In this study, we demonstrated the composition of cultivable *Lactobacillus* species shift in the distal esophagus of obese rats.

Innovations and breakthroughs

Published data show that different constitution of *Lactobacillus* species may exert different influences on smooth muscle motility in the distal esophagus. However, the details are still unclear. Analysis on the shift in *Lactobacillus* is restrained at the genus level. This study analyzed the composition of *Lactobacillus* in the distal esophagus, and found that the composition of cultivable *Lactobacillus* species shifted in the distal esophagus in obese rats. Therefore, potential variations in microbial composition in the distal esophagus in obesity may influence the distal esophageal motility and lead to GERD.

Applications

This study proved that the composition of cultivable *Lactobacillus* species shifted in the distal esophagus in obese rats, therefore, it may represent a further study between the microbial composition shift in the distal esophagus and GERD. This could lead to the development of a potential therapeutic target in GERD.

Terminology

GERD is a more serious form of gastroesophageal reflux, which is common. Persistent reflux that occurs more than twice weekly is considered GERD, and it can affect people of all ages. The main symptom of GERD in adults is frequent heartburn, also called acid indigestion - burning-type pain in the lower part of the mid-chest, behind the breast bone, and in the mid-abdomen.

Peer review

This study considers the investigation of microbiota composition in the distal esophagus of high-fat-diet-fed rats. The authors hypothesize that potential variations in microbial composition in the distal esophagus of obese individuals may influence distal esophageal motility and lead to GERD. In this study, the composition of *Lactobacillus* spp. was analyzed using the combination of bacterial culturing, denaturing gradient gel electrophoresis and sequencing in a rat model. The interesting and important finding of this study was the fact that high-fat diet led to a shift in mucosal microflora (especially *Lactobacillus* species) in the distal esophagus in rats, which resulted in alteration of smooth muscle motility. This study makes an additional contribution to studies of the etiology of GERD.

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BRIEF ARTICLE

Hemihepatic *versus* total hepatic inflow occlusion during hepatectomy: A systematic review and meta-analysis

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Abstract

AIM: To evaluate the clinical outcomes of patients undergoing hepatectomy with hemihepatic vascular occlusion (HHO) compared with total hepatic inflow occlusion (THO).

METHODS: Randomized controlled trials (RCTs) comparing hemihepatic vascular occlusion and total hepatic inflow occlusion were included by a systematic literature search. Two authors independently assessed the trials for inclusion and extracted the data. A meta-analysis was conducted to estimate blood loss, transfusion requirement, and liver injury based on the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Either the fixed effects model or random effects model was used.

RESULTS: Four RCTs including 338 patients met the predefined inclusion criteria. A total of 167 patients were treated with THO and 171 with HHO. Meta-analysis of AST levels on postoperative day 1 indicated higher levels in the THO group with weighted mean difference (WMD) 342.27; 95% confidence intervals (CI) 217.28-467.26; P = 0.00001; I^2 = 16%. Meta-analysis showed no significant difference between THO group and HHO group on blood loss, transfusion requirement, mortality, morbidity, operating time, ischemic duration, hospital stay, ALT levels on postoperative day 1, 3 and 7 and AST levels on postoperative day 3 and 7.

CONCLUSION: Hemihepatic vascular occlusion does not offer satisfying benefit to the patients undergoing hepatic resection. However, they have less liver injury after liver resections.

Key words: Inflow occlusion; Hemihepatic; Vascular occlusion; Hepatectomy; Pringle maneuver

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INTRODUCTION

Liver resection is performed mainly for benign and malignant liver tumors, especially for hepatocellular carcinoma. It is a potential curative treatment option in patients with early stage carcinoma[1]. Intraoperative bleeding is a main concern during liver resections, and mortality and morbidity are clearly correlated with the amount of blood loss

and the subsequent blood transfusions[2]. Many methods of hepatic vascular control have been introduced to control intraoperative blood loss. In 1908, Pringle applied inflow vascular occlusion technique (the Pringle maneuver) at the hepatic hilar for the first time. It is a technique of total compression of the hepatoduodenal ligament and the most commonly used and relatively easy method for controlling afferent blood flow[3]. However, the Pringle maneuver also carries the risk of global ischemic damage to the liver and intestinal congestion, especially in patients with chronic liver diseases, the degree of which is likely to be accentuated by a prolonged period of vascular inflow occlusion[4,5]. In 1987, Bismuth and Makuuchi proposed a hemihepatic vascular occlusion (HHO) technique to reduce the severity of visceral congestion and total liver ischemia, especially for the remaining liver[6,7]. By this method, visceral congestion is considered to be limited, because considerable portal blood flow is preserved and only portions of the liver are rendered anoxic[8]. The technique with occlusion of vessels supplying the hemiliver containing the tumor, has been suggested to reduce intraoperative bleeding and postoperative liver functional disturbances because of the interruption of blood flow to the liver[9]. But, portal vein and artery dissection to perform selective clamping is time consuming and may result in another blood loss[10]. Many prospective randomized controlled trials (RCTs) and retrospective clinical trials have evaluated the feasibility, safety and efficacy of HHO and total hepatic inflow occlusion (THO), however, the clinical significance between the two vascular control methods remain inconsistent. So, the optimal method of vascular control during hepatic resection continues to be debated.

Up to now, a meta-analysis including all available RCTs is still insufficient. We conducted a systematic review and meta-analysis to evaluate the feasibility, safety and efficacy of HHO and THO in patients undergoing hepatectomy.

MATERIALS AND METHODS

Systematic literature search

A systematic literature search was independently conducted by two authors. They systematically searched the Medline, Embase, Science Citation Index, PubMed and CNKI (China National Knowledge Infrastructure Whole Article Database). The following keywords were used: hemihepatic vascular occlusion, hemihepatic occlusion, selective inflow occlusion, selective clamping or selective portal clamping. The literature search was performed with restriction in languages of English or Chinese and types of randomized controlled trial or controlled clinical trial. The last search was done on November 2, 2010.

Inclusion and exclusion criteria

Type of studies: Only RCTs were considered for this review. Quasi-randomized studies, cohort studies, and case-control studies were excluded.

Type of participants: Patients who were about to undergo selective liver resection for benign or malignant liver tumor were included, irrespective of age, gender, cirrhosis, tumor size and nodule numbers. Trials in which patients required contralateral hepatic resection or had distant metastasis or synchronous malignancy in other organs were excluded in the study.

Types of interventions: We included trials comparing total hepatic inflow occlusion with hemihepatic vascular occlusion in hepatectomy, irrespective of ischemic preconditioning before vascular occlusion. Trials only comparing other types of vascular occlusion were excluded.

Type of outcome measures: Primary outcomes: Operative blood loss, biochemical markers of liver injury, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and transfusion requirement. Secondary outcomes: Peri-operative mortality, peri-operative morbidity, operating time , ischemic duration and hospital stay.

Selection of studies

Two authors identified and evaluated independently the trials for inclusion in form of abstracts or full text if necessary. Any disagreement in study selection and data extraction was resolved by discussion.

Data extraction

Two authors extracted the data on a standard form that included population characteristics (sex, age, percentage of major liver resections, methods of ischemic preconditioning and the presence of chronic liver disease) the co-interventions and information on the outcome measures in each trial.

Quality assessment

We assessed the methodological quality of the trials independently. The assessment was made based on sample size calculation; sequence generation; allocation concealment; whether blinding method was adopted for the participants of patients and those who performed the trial and evaluate the outcome; efficacy of randomization; deviations, withdrawals and dropouts; and definition of outcome parameters[11,12].

Statistical analysis

We pooled the synchronized extraction results as estimates of overall therapeutic effects in a meta-analysis using Review Manager Version 5.0 for Windows. The estimated effect measures were odds ratio (OR) for dichotomous data and weighted mean difference (WMD) for continuous data, both reported with 95% confidence intervals (CI). We checked all results for clinical and statistical heterogeneity. Clinical heterogeneity was evaluated based on the study populations and interventions, definition of outcome measures, concomitant treatment, and perioperative management. Heterogeneity was determined by Chi-squared test. P value of 0.10 was considered significant difference and I^2 values were used for the evaluation of statistical heterogeneity (I^2 of 50% or more indicating presence of heterogeneity)[13]. We used a fixed-effects model to synthesize

Table 1 Characteristics of randomized controlled trials comparing total hepatic inflow occlusion with hemihepatic vascular occlusion

Author (yr)	Design	Sample size (*n*)	THO (*n*)	HHO (*n*)	Journal	Comparison
Figueras *et al* (2005)	RCT	80	39	41	*Annals of Surgery*	Complete *vs* selective portal triad clamping
Wu *et al* (2002)	RCT	58	28	30	*Arch Surg*	Hemihepatic *vs* total hepatic occlusion techniques
Yuan *et al* (2010)	RCT	120	60	60	*The American Journal of Surgery*	Pringle maneuver *vs* hemihepatic vascular occlusion
Liang *et al* (2009)	RCT	80	40	40	*Hepato-Gastroen-terology*	Continuous hemihepatic with intermittent total hepatic inflow occlusion
Total	--	338	167	171	--	--

THO: Total hepatic inflow occlusion; HHO: Hemihepatic vascular occlusion; RCT: Randomized controlled trials.

Table 2 Characteristics of patients in randomized controlled trials comparing total hepatic inflow occlusion with hemihepatic vascular occlusion

Author (yr)	Age (mean yr) THO/HHO	Sex (male:female) THO/HHO	Cirrhosis (*n*:N) THO/HHO	Ischaemic preconditioning	Resection margin (≤ 1 segments:≥ 2 segments) THO/HHO	Diseases HCC: Others THO/HHO
Figueras *et al* (2005)	61.8/62	31:8/28:13	18:39/21:41	IC	25:14/29:12	16:23/17:24
Wu *et al* (2002)	57.5/53.2	23:5/25:5	28:28/30:30	IC	5:23/7:23	25:3/26:4
Yuan *et al* (2010)	48.6/49.3	46:14/41:19	39:60/35:60	IC if transaction time > 30 min or CC	5:55/5:55	44:16/43:17
Liang *et al* (2009)	49.4/49.55	27:13/31:9	17:40/19:40	IC or CC	6:34/10:30	20:20/21:19
Total	--	127:40/125:46	102:167/105:171	--	41:126/51:120	105:62/107:64

IC: Intermittent clamping; CC: Continuous clamping; HCC: Hepatocellular carcinoma; N: The number of all patients in one trial; *n*: The number of patients with cirrhosis; THO: Total hepatic inflow occlusion; HHO: Hemihepatic vascular occlusion.

Table 3 Outcomes of randomized controlled trials comparing total hepatic inflow occlusion with hemihepatic vascular occlusion

Author (yr)	Operative time (min) THO/HHO	Ischemic duration (min) THO/HHO	Blood loss (mL) THO/HHO	Transfusion requirements THO/HHO	Complications total (*n*) THO/HHO	In-hospital stay (d) THO/HHO	In-hospital death (*n*) THO/HHO
Figueras *et al* (2005)	207 ± 48/219 ± 45	41 ± 14/47 ± 18	671 ± 533/735 ±397	4:39/6:41	15:39/ 12:41	9.38 ± 4.9/8.15 ± 3.8	0:39/1:41
Wu *et al* (2002)	409 ± 19.2/ 399 ± 15.6	96.0 ± 10.9/ 94.2 ± 9.9	1685 ± 170/ 1159 ± 221	12:28/5:30	8:28/10:30	14.8 ± 1.4/16.4 ± 1.4	0:28/0:30
Yuan *et al* (2010)	114.2 ± 37.2/ 133.5 ± 44.6	16.6 ± 8.7/ 14.9 ± 4.5	339.5 ± 205.1/ 354.4 ± 240.3	6:60/4:60	19:60/12:60	13.7 ± 5.2/10.2 ±4.1	1:60/0:60
Liang *et al* (2009)	203.98 ± 38.36/ 236.15 ± 49.2	40.17 ± 13.30/ 42.38 ± 12.79	569.8 ± 285.56/ 649.35 ± 279.05	14:40/15:40	8:40/9:40	9.85 ± 3.55/10.12 ± 2.41	0:40/0:40
Total	--	--	--	36:167/30:171	50:167/43:171	--	1:167/1:171

THO: Total hepatic inflow occlusion; HHO: Hemihepatic vascular occlusion.

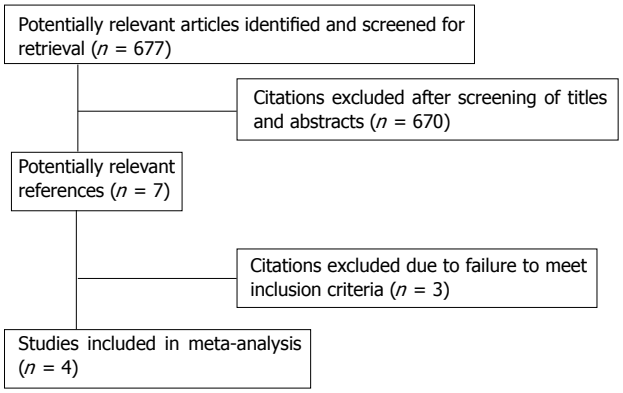

Figure 1 Reference flow chart.

data when heterogeneity was absent, otherwise a random-effects model would be used. Data were presented as forest plot and funnel plot was used to assess publication bias.

RESULTS

We searched a total of 677 references published between 2002 and 2010. Four RCTs[14-17] including 338 patients met the predefined inclusion criteria (Figure 1). All the trials (Table 1) compared HHO (*n* = 171) with THO (*n* = 167). Three trials enrolled cirrhotic and non-cirrhotic patients[14,16,17] and one trial enrolled only cirrhotic patients[15]. In all trials, both major (> 2 segments) and minor (≤ 1 segments) hepatic resections were performed, but one trial exclusively included patients undergoing complex central liver resections. Tables 2-4 summarize the baseline characteristics and outcomes of the trials. The potential bias of included trials are shown in Table 5. Only one of the trials reported the blinding methods used and the generation of allocation sequence[16].

Effects of interventions

Blood loss. Information on intraoperative blood loss was

Table 4 Postoperative aspartate aminotransferase and alanine aminotransferase levels of patients in randomized controlled trials comparing total hepatic inflow occlusion with hemihepatic vascular occlusion

Author (yr)	AST (U/L) Day 1 THO/HHO	AST (U/L) Day 3 THO/HHO	AST (U/L) Day 7 THO/HHO	ALT (U/L) Day 1 THO/HHO	ALT (U/L) Day 3 THO/HHO	ALT (U/L) Day 7 THO/HHO
Wu *et al* (2002)	420 ± 790/290 ± 770	180 ± 320/190 ± 510	50 ± 40/30 ± 20	370 ± 490/480 ± 510	330 ± 320/320 ± 270	90 ± 20/70 ± 20
Yuan *et al* (2010)	812.6 ± 475.3/ 447.6 ± 210.3	423.7 ± 265.4/ 207.5 ± 79.3	143.6 ± 87.5/ 64.2 ± 29.4	1013.6 ± 654.4/ 369.4 ± 347.2	592.2 ± 416.4/ 218.4 ± 185.3	172.4 ± 125.8/ 79.6 ± 55.3
Figueras *et al* (2005)	NS	NS	NS	402 ± 258/372 ± 234	NS	NS

THO: Total hepatic inflow occlusion; HHO: Hemihepatic vascular occlusion.

Table 5 Assessment the methodological quality of included studies

Author (yr)	Sample size calculation	Generation of allocation sequence	Allocation concealment	Deviations, withdrawals and dropouts	Efficacy of randomization	Blinding	Definition of outcome parameters
Figueras *et al* (2005)	Yes	No description	Sealed envelope	Yes	Yes	No description	Yes
Wu *et al* (2002)	No description	No description	Sealed envelope	No description	Yes	No description	Yes
Yuan *et al* (2010)	Yes	Yes	Sealed envelope	Yes	Yes	Single-blinded	No description
Liang *et al* (2009)	No description	No description	No description	Yes	Yes	No description	Yes

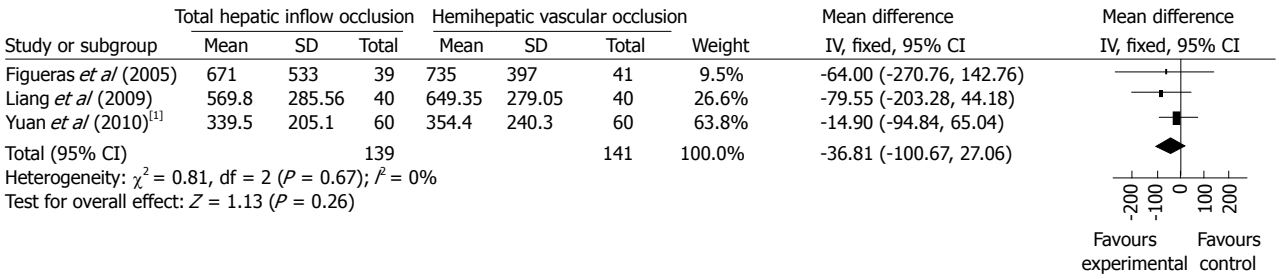

Figure 2 Meta-analysis of blood loss in randomized controlled trials comparing total hepatic inflow occlusion with hemihepatic vascular occlusion. [1]Blood loss.

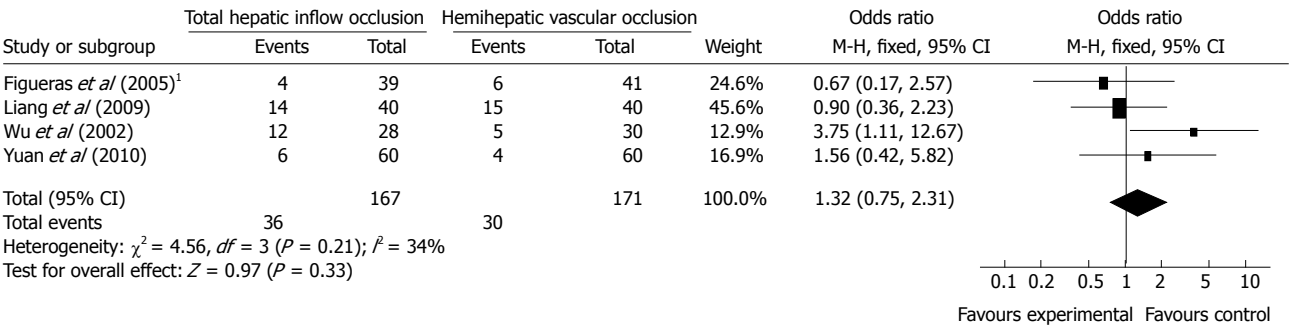

Figure 3 Meta-analysis of aspartate aminotransferase levels on postoperative 1st d. [1]Transfusion requirements (*n*).

available in all analyzed trials. The trial by Wu *et al*[15] reported significantly more blood loss in patients of both groups. Statistical heterogeneity was presented and $P = 0.000\,001$. Funnel plot to evaluate publication bias for outcome of blood loss demonstrated a strong asymmetry, suggesting the existence of severe publication bias. Clinical heterogeneity analysis found that complex central liver resections were performed on cirrhotic patients, and the cut surface area was wider and would increase intraoperative blood loss. Meta-analysis of the other three trials showed no significant difference between THO group and HHO group (WMD -36.81; 95% CI -100.67 to 27.06, $P = 0.26$, $I^2 = 0\%$) (Figure 2).

Transfusion requirement. All trials reported the number of patients who needed transfusion in both groups. Funnel plot did not demonstrate a strong asymmetry. Meta-analysis (Figure 3) indicated no difference in postoperative transfusion requirement between the groups (OR 1.32 95% CI 0.75-2.31, $P = 0.33$, $I^2 = 34\%$). Since there was no uniform definition of the average transfusion volume in the trials, we did not compare the transfusion volume in the study.

Biochemical markers of liver injury. All the four tri-

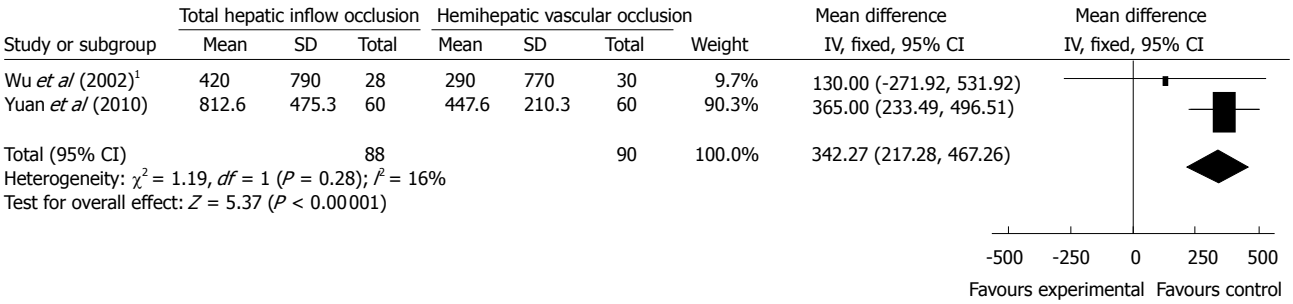

Figure 4 Meta-analysis of aspartate aminotransferase levels on postoperative Day 1. [1]Aspartate aminotransferase (D1).

als provided AST and ALT levels on postoperative days. However, data on AST and ALT were available in only two studies. We did not draw funnel plots to examine the potential publication bias in this review, because the number of the included trials was small. Wu *et al*[15] provided the data of ALT and AST levels on postoperative days 1, 3, 5 and 7, and Yuan *et al*[16] gave the information on postoperative days 1, 3 and 7. The ALT levels on postoperative day 1 in Figueras's study[14] were also available. Meta-analysis of ALT levels on postoperative days 1, 3 and 7 showed no significant difference between the two groups (WMD on day 1/191.03, 95% CI -239.04 to 621.10, $P = 0.38$, $I^2 = 94\%$; WMD on day 3/192.86, 95% CI -163.66 to 549.37, $P = 0.29$, $I^2 = 94\%$, and WMD on day 7/54.43, 95% CI -16.81 to 125.67, $P = 0.13$, $I^2 = 94\%$). Meta-analysis of AST levels on postoperative days 3 and 7 in the two studies showed no significant difference between THO group and HHO group (WMD on day 3/127.52, 95% CI -88.92 to 343.96, $P = 0.25$, $I^2 = 73\%$; WMD on day 7/49.10, 95% CI -9.1 to 107.3, $P = 0.10$, $I^2 = 94\%$). Meta-analysis of AST levels on postoperative day 1 indicated higher postoperative AST levels in the THO group (WMD 342.27; 95% CI 217.28 to 467.26; $P = 0.00001$, $I^2 = 16\%$) (Figure 4).

Peri-operative mortality and morbidity. Four studies provided data on peri-operative mortality and morbidity. In total, two patients died in the four trials. Both died from liver failure, one in THO group and the other in HHO group. Meta-analysis of these studies revealed neither of the two groups showed superiority in overall morbidity (OR 1.28, 95% CI 0.79-2.07, $P = 0.31$, $I^2 = 0\%$) and mortality (OR 1.03, 95% CI 0.14-7.44, $P = 0.98$, $I^2 = 0\%$). Meta-analysis of bile leak (OR 0.92, 95% CI 0.35 -2.44, $P = 0.87$, $I^2 = 0\%$) and hepatic insufficiency (OR 1.02, 95% CI 0.29 - 3.60, $P = 0.97$, $I^2 = 35\%$) showed no statistically significant difference.

Operating time, ischemic duration and hospital stay. There was no statistically significant difference in operating time (WMD -12.44, 95% CI -32.88 to 8.00, $P = 0.23$, $I^2 = 86\%$) between the two groups, also in hospital stay (WMD 0.63, 95% CI -1.60 to 2.85, $P = 0.58$, $I^2 = 91\%$) and in ischemic duration (WMD 0.61, 95% CI -1.40 to 2.61, $P = 0.55$, $I^2 = 43\%$)

DISCUSSION

The key points in hepatectomy are to control intraoperative bleeding and prevent postoperative complications such as liver failure and bile leakage[18]. Intraoperative blood loss has been shown to significantly influence the short-term prognosis of patients undergoing liver resection[19,20]. Hemihepatic vascular clamping selectively interrupts the arterial and venous inflow to the right or left hemiliver and therefore avoids both splanchnic blood stasis and ischemia or ischemia-reperfusion injury to the whole liver[9,21]. A retrospective study[22] indicated that the average bleeding volume and transfusion requirements were less in hemihepatic vascular occlusion group compared with Pringle maneuver group. But, other retrospective studies[8,23] showed no difference between the two groups. Our meta-analysis showed no significant difference in blood loss and transfusion requirements between the two groups. Three[14,16,17] of the four trials in the review showed no difference in the amount of hemorrhage and blood transfusion requirements, but one study[15] reported that the amount of operative blood loss and the incidence of blood transfusion were significantly higher in group THO patients (1685 mL *vs* 1159 mL, $P = 0.049$) and the volume of blood loss was much higher than in other studies. It could be explained by the fact that the patients in the study had cirrhosis and underwent complex central liver resections, while other trials included both cirrhotic and non-cirrhotic patients. The procedures presented herein were more difficult and time-consuming than conventional major hepatectomy and transected plane was also wider[15,24-26]. Both factors induced massive bleeding and difficulties in hemostasia.

Liver injury due to ischemia and subsequent reperfusion are major concerns in inflow vascular occlusion[27-29] and are usually monitored after surgery by measuring aminotransferase levels[30]. We found no significant difference on ALT levels on postoperative days 1, 3 and 7 in the two groups, also on AST levels on postoperative days 3 and 7. Three RCTs[14,15,17] and one retrospective study[18] drew the same conclusion. Theoretically, the blood flow in one lobe of the liver in group HHO is preserved and the liver function damage may be less than that in group THO[31].Yuan *et al*[16] indicated that the Pringle maneuver group was associated with a significantly higher peak in ALT and AST levels ($P = 0.01$). Meta-analysis showed that AST levels on postoperative day 1 were also higher in the THO group (WMD 342.27, 95% CI 217.28-467.26, $P = 0.00001$, $I^2 = 16\%$). Chau *et al*[23] concluded that patients subjected to HHO responded better than those subjected to the Prin-

gle maneuver in terms of earlier recovery of postoperative liver function. Therefore, HHO resulted in less liver injury and was advantageous in the recovery of postoperative liver function.

Unfortunately, only two trials in our analysis included data on ALT and AST levels. There were no significant differences in patients' general characteristics, resection margin, and ratio of cirrhotic to non-cirrhotic patients ($P = 0.05$). However, intermittent clamping was used in the trial by Wu *et al*[15], whereas Yuan *et al*[16] did continuous clamping if transaction time was ≤ 30min, otherwise intermittent clamping would be used. A RCT[32] comparing intermittent portal triad clamping with continuous clamping showed no statistically significant differences, although the peak AST level was lower in the intermittent portal triad clamping. Belghiti *et al*[33] suggested that in chronic patients, the transaminase levels were significantly higher in the continuous portal triad clamping than in the intermittent portal triad clamping. Cirrhotic liver and pre-existing liver were less able to tolerate ischemia than normal liver in clinical observations or animal experiments[28,34,35]. The proportion of chronic patients in the two RCTs were 100% and 61.7% respectively, which may influence the ALT and AST levels in HHO and THO groups and account for the lack of difference between the two groups on postoperative days 3 and 7. Due to the limited number and non-available data in the trials, no subgroup analysis was performed in patients with cirrhosis, which is known to increase the sensitivity of the livers to ischemia[30].

There was one death in Figueras' trials[14] in HHO group as a result of hepatic insufficiency in a patient with hepatitis C virus (HCV) cirrhosis . His blood loss during the operation was 2120 mL and 5 units of red blood cell transfusion were required. Yuan *et al*[16] reported one patient in the Pringle maneuver group who died of liver failure on the 26th d after a right hepatectomy. The total mortality was 0.51% and total peri-operative morbidity was 27.51%. But no statistically significant difference was found in the peri-operative mortality, peri-operative morbidity, operating time, ischemic duration and hospital stay. Complications included ascites, bile leak, hepatic insufficiency, portal thrombosis, pleural effusion, wound infection, hemorrhage and so on. Meta-analysis of bile leak and hepatic insufficiency showed no significant difference between THO group and HHO group.

This review has some limitations. First, our literature search might have not detected all relevant evidences and the number of RCTs included in this review is small. Second, incomplete reporting of important methodological issues, such as sample size calculation, randomization process and blinding assessment of trial quality, might raise doubts on the adequate power of these studies[36]. Third, the heterogeneity of the patients in the included trials may influence the conclusions as some trials included major and complex central liver resections and some included normal and cirrhotic livers.

In conclusion, the current evidence shows no advantage of hemihepatic vascular occlusion over the total hepatic inflow occlusion in terms of blood loss, transfusion requirement, mortality and morbidity, operating time and hospital stay. However, HHO results in less liver injury after liver resections. Further trials are required to assess optimal technique of hepatic vascular control for the patients hepatectomy especially for the patients with chronic cirrhosis.

COMMENTS

Background

Possibility of life-threatening hemorrhage always exists in patients with liver resection, so liver vascular control to reduce blood loss is important. Since the Pringle maneuver technique was successfully applied by Pringle in 1908, many methods of hepatic vascular control have been introduced to accelerate the development of hepatic surgery. Bismuth and Makuuchi proposed the hemihepatic vascular occlusion technique, which attracted much attention among surgeons.

Research frontiers

Both Pringle maneuver and hemihepatic vascular occlusion techniques can reduce blood loss during transaction of the hepatic parenchyma but Pringle maneuver produces ischemic injury to the remaining liver and intestinal congestion. Hemihepatic vascular occlusion technique has become very popular in recent years, because it is thought to limit visceral congestion and can protect the remaining liver. Many studies including randomized controlled trials (RCTs) have been designed to evaluate the safety, feasibility and efficiency of the two methods.

Innovations and breakthroughs

The authors searched and assessed all the RCTs comparing the two techniques and drew a conclusion by a systematic review and meta-analysis. They found that hemihepatic vascular occlusion did not offer benefit to the patients except for reducing ischemic liver injury after liver resections.

Applications

Hemihepatic vascular occlusion technique should be recommended for hepatectomy to reduce peri-operative blood loss and protect the remaining liver after the surgery.

Terminology

Hemihepatic vascular occlusion is a method which selectively interrupts the arterial and portal inflow to the part of the liver (right or left hemiliver) ipsilateral to the lesion that requires resection. It can be achieved after placing a curved renal pedicle clamp across the right or left portal structures. And Pringle maneuver involves compression of the hepatoduodenal ligament to interrupt all arterial and portal inflow to the whole liver.

Peer review

The manuscript is methodologically well designed and is concise in its data and conclusion. However, it should be subjected to linguistic revision to improve several mistakes in grammar and style.

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CASE REPORT

Cure of alopecia areata after eradication of *Helicobacter pylori*: A new association?

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Abstract

Alopecia areata is a disease of the hair follicles, with strong evidence supporting autoimmune etiology. Alopecia areata is frequently associated with immune-mediated diseases with skin manifestations such as psoriasis and lichen planus, or without skin manifestations such as autoimmune thyroiditis and idiopathic thrombocytopenic purpura. *Helicobacter pylori* (*H. pylori*) infection is present in around 50% of the world's population and has been associated with a variety of immune-mediated extra-digestive disorders including autoimmune thyroiditis, idiopathic thrombocytopenic purpura, and psoriasis. A case of a 43-year old man with an 8-mo history of alopecia areata of the scalp and beard is presented. The patient was being treated by a dermatologist and had psychiatric support, without any improvement. He had a history of dyspepsia and the urea breath test confirmed *H. pylori* infection. The patient went into remission from alopecia areata after *H. pylori* eradication. If such an association is confirmed by epidemiological studies designed for this purpose, new therapeutic options could be available for these patients, especially in areas where infection with *H. pylori* is highly prevalent.

INTRODUCTION

Alopecia areata is a disease of the hair follicles, with strong evidence supporting an autoimmune origin[1], although the exact pathogenesis of the disease is not clear. Alopecia areata has a frequency ranging from 0.7% to 3.8% in patients attending dermatology clinics, affects both sexes[2], and a familial occurrence is often reported[3,4]. The pattern of hair loss can vary and can affect any part of the body. Alopecia areata frequently occurs in association with other autoimmune diseases, including autoimmune thyroiditis[5], psoriasis[6-8] and Sjögren syndrome[9], among others.

Helicobacter pylori (*H. pylori*) is a microaerophilic Gram-negative bacterium that colonizes the gastric mucosa[10] and is present in around 50% of the world's population[11], with varying prevalence rates between 7% in the Czech Republic and 87% in a South African population[12]. In the case of Medellín, Colombia, prevalence of *H. pylori* infection in children under 12 years is 60.9%[13] and in adults, it is 77.2%[14]. *H. pylori* infection has been associated with the pathogenesis of gastric disorders such as gastritis, duodenal and gastric ulcers, gastric cancer, mucosa-associated lymphoid tissue lymphoma[10], and a variety of

extra-digestive disorders, many of them clearly identified as immune-mediated[15], such as idiopathic thrombocytopenic purpura[16,17], autoimmune thyroiditis[18,19], Sjögren's syndrome[20,21], rosacea[22] and psoriasis[23,24].

A case of a 43-year-old man with patchy alopecia areata and *H. pylori* infection is presented. The patient had hair regrowth after bacterial eradication.

CASE REPORT

A 43-year-old man presented with an 8-mo history of patchy hair loss in the scalp and beard (Figure 1A-C). He had consulted a dermatologist who prescribed 0.25% desoximetasone and 5% minoxidil, according to the guidelines for the management of alopecia[25], and had psychiatric support with escitalopram 5 mg/d, without any response other than progression of the condition.

The patient had a history of dyspepsia, therefore, he underwent analysis to determine *H. pylori* status. Urea breath test (^{13}C-UBT) (6.95 $\delta^{13}CO_2$; negative, < 1)[26], and *H. pylori* IgG antibodies (IgG index: 52.4; negative, < 9) were positive. Subsequent laboratory evaluation included normal values of ultrasensitive thyroid stimulating hormone, free thyroxine and free tri-iodothyronine; and negative antinuclear, antithyroid peroxidase and intrinsic factor antibodies. The patient was prescribed first line *H. pylori* eradication with proton pump inhibitor (omeprazole) 20 mg twice daily, amoxicillin 1000 mg twice daily, and clarithromycin 500 mg twice daily for 14 d, according to recommendations from the Maastricht Ⅲ Consensus Report[27], and was followed photographically every 2 wk. He was instructed not to take or apply any medications for alopecia areata. *H. pylori* eradication was confirmed 6 wk after treatment with a negative result of the ^{13}C-UBT (0.81 $\delta^{13}CO_2$).

Figure 1 shows the photographic sequence of the lesions before and after *H. pylori* eradication. From week 4, there was evidence of hair regrowth in the scalp and beard (Figure 1D-F). To date, the patient continues in complete remission from alopecia areata, as shown in Figure 1M-O.

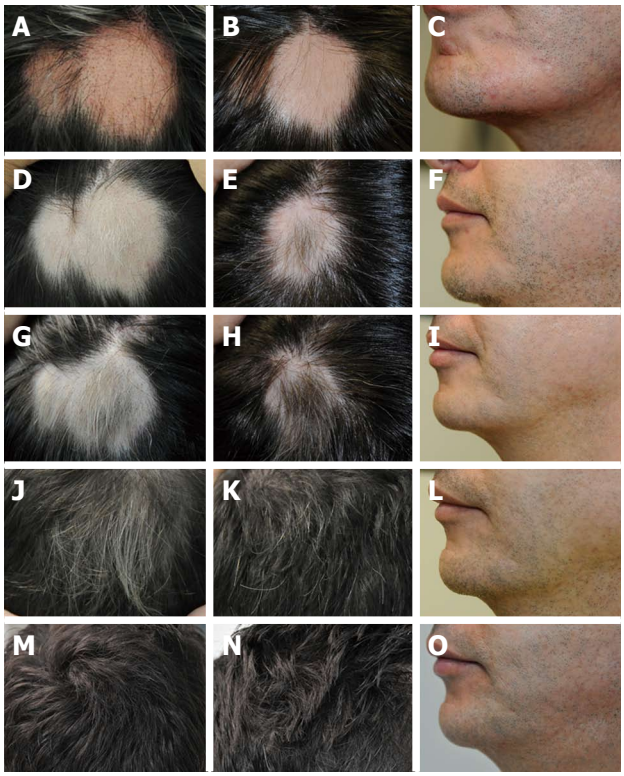

Figure 1 Photographic sequence of lesions before and after *Helicobacter pylori* eradication. A-C: Alopecia areata of the scalp (A and B) and beard (C) at baseline visit (week 0) before *Helicobacter pylori* (*H. pylori*) eradication. Positive ^{13}C-UBT (6.95 $\delta^{13}CO_2$); D-F: Evidence of hair regrowth at week 4; G-I: Hair regrowth at week 8. Negative ^{13}C-UBT (0.81 $\delta^{13}CO_2$); J-L: Hair regrowth at week 16; M-O: Hair regrowth at week 44. Negative ^{13}C-UBT (0.67 $\delta^{13}CO_2$).

DISCUSSION

H. pylori infection has been associated with numerous immune and non-immune disorders including dermatological conditions, such as chronic urticaria[28-30], rosacea[22,28,31-39], psoriasis[23,24], Schönlein-Henoch purpura[40-46], Behçet's disease[47,48], prurigo nodularis[49], chronic cutaneous pruritus[50], progressive systemic sclerosis[51-54], Sjögren's syndrome[20,21,55-57], and Sweet's syndrome[58]; many of them improving or going into remission after eradication of *H. pylori* infection[24,30,49,59-61]. Several mechanisms have been suggested to mediate the systemic effects of *H. pylori* infection, including the development of antigen-antibody complexes and cross-reactive antibodies (by molecular mimicry)[61-63], where antibodies developed against *H. pylori* cross-react with autoantigens to cause tissue damage, as has been reported in atrophic gastritis[62,64], chronic gastritis[65-67], chronic idiopathic thrombocytopenic purpura[16,17,68-70], Hashimoto's thyroiditis[19], atherosclerosis[71], arterial hypertension[72], unstable angina pectoris[73], ischemic heart disease[74,75], Alzheimer's disease[76], systemic sclerosis[77,78], central serous chorioretinopathy[79], iron deficiency[80,81], autoimmune pancreatitis[82-86], and chronic urticaria[87].

Alopecia areata has been described to be of autoimmune origin[88], with the presence of inflammatory cells around and within the human hair follicles. Alopecia areata has been associated with other autoimmune disorders including thyroid disease[89-93], psoriasis[6,7], and celiac disease[94-97]; conditions that have also been associated with *H. pylori* infection.

In the literature, there is ample evidence to suggest an association between *H. pylori* and alopecia areata that could explain the cure in this patient after eradication of infection. There is concurrent alopecia areata with immune diseases that are also concurrent with *H. pylori* infection. There are three different scenarios: immune-mediated skin diseases associated with *H. pylori* infection and alopecia areata, including psoriasis[6,7,23,24,98-103] and lichen planus[101,104-109]; immune-mediated non-skin conditions associated with *H. pylori* infection and alopecia areata, including autoimmune thyroiditis[18,19,110-115], celiac disease[94-97,116-118], idiopathic thrombocytopenic purpura[119,120], and autoimmune pancreatitis[82,84,85,121-124]; and laboratory findings that show the immunological nature of the conditions that are found in *H. pylori*-infected patients as well as in alopecia areata patients, including parietal cell antibodies[117,125-127] and thyroid antibodies[90,128].

After reviewing the medical literature, an association between *H. pylori* infection and alopecia areata has not been clearly demonstrated; only three reports have explored such association and had different results[129-131]. Abdel Hafez *et al*[131] have compared 31 patients with alopecia areata with 24 healthy controls and have found no significant difference in the *H. pylori* status, as determined by an antigen stool test. Rigopoulos *et al*[130] have compared *H. pylori* seroprevalence in 30 patients with alopecia areata and 30 healthy controls, and found no significant difference between the groups, whereas Tosti *et al*[129] have found, in a group of 68 patients with alopecia areata, that the seroprevalence of *H. pylori* infection was higher than in matched controls. It is of note that the presence of IgG antibodies against *H. pylori* does not confirm current infection and is only an indicator of previous exposure to the bacterium[132]. However, none of the studies tried to eradicate the infection and evaluate posterior hair regrowth.

Here, I have described the case of one patient who had patchy hair loss of the scalp and beard. The patient's condition started to improve within 4 wk of completing *H. pylori* eradication (Figure 1D-F). By week 16 (Figure 1J-L), the patient had completely reversed the hair loss, and by week 44 (Figure 1M-O), he remained *H. pylori*-negative and completely cured of alopecia areata. Although prior studies have only reported the prevalence of *H. pylori* infection in alopecia areata patients, to the best of my knowledge, this is the first documented case of reversed hair loss after *H. pylori* eradication.

There have been a few early studies in which antibiotic treatment was used in an attempt to cure alopecia areata, but in no case was there information on whether the patients were infected with *H. pylori*. Dapsone was used unsuccessfully[133,134]. There was one case of a 13-year-old girl with multiple autoimmune diseases who was successfully treated for alopecia areata with co-trimoxazole, a drug with antibiotic properties and immunomodulatory effects that could have been responsible for hair regrowth. Finally, there was one case in the literature describing the occurrence of alopecia areata after antibiotic treatment with rifampicin[135]. However, further case-control studies could be useful to rule out this possibility completely.

Hence, a common denominator in various autoimmune diseases is *H. pylori* infection; therefore, *H. pylori* status could be determined in several autoimmune conditions, and if positive, eradication treatment could follow as an initial step. More studies are needed to clarify the reality of the proposed association.

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Gut-liver axis plays a role in hepatocarcinogenesis of patients with Crohn's disease

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Abstract

The development of hepatocellular carcinoma (HCC) is attributed to several factors, including chronic viral infection, alcohol consumption, exposure to aflatoxin B1 and metabolic disorders. Several recent reports have shown that HCC can occur in patients with long-standing Crohn's disease (CD) in the absence of other underlying high-risk liver diseases. There may be an association between CD and hepatocarcinogenesis, however, the precise mechanism for this requires further investigations.

Key words: Inflammatory bowel disease; Crohn's disease; Hepatocellular carcinoma; Intestinal flora; Azathioprine; Enterohepatic circulation

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TO THE EDITOR

We have read with great interest the article by Ishida *et al*[1], which was recently published in *World J Gastroenterology*, issue No. 25, 2010. The authors reported a case of hepatocellular carcinoma (HCC) that occurred in a patient with Crohn's disease (CD) in the absence of chronic hepatitis or liver cirrhosis. This suggested that Azathioprine treatment could be related to hepatocarcinogenesis in CD patients. Upon reading this interesting case report, we wondered whether the risk factor for the development of HCC in the setting of CD was CD itself or its treatment.

Azathioprine is currently the most common immunosuppressive drug for the treatment of inflammatory bowel disease (IBD), particularly for maintaining the remission of the patients with a complex clinical course. There is little doubt that, once this drug is indicated, its treatment should be continued for an extended period of time. Whether or not immunosuppressive therapy increases the risk of malignancy in IBD patients is controversial. Although the prolonged use of Azathioprine is considered theoretically to increase the occurrence of cancer, studies aimed at elucidating the risk of neoplasia in IBD patients treated with Azathioprine have concluded that Azathioprine does not substantially increase the risk of cancer development[2-4]. A global consensus about the association between immunosuppressants and malignancies has suggested a favorable risk/benefit ratio in the long-term use of Azathioprine[5].

Although the causes of IBD remain incompletely understood, the prevailing consensus is that the intestinal flora drives an unmitigated intestinal immune response and inflammation in the genetically susceptible host. CD is considered to be a systemic disorder that often involves multiple organs including the gastrointestinal tract. A meta-analysis[6] that assessed the relative risk of all types of cancers occurring outside the gastrointestinal tract found an increased risk in CD patients; and a potential correlation between long-standing CD and the development of HCC may therefore exist. A recent study[7]

revealed an intimate cross-talk between gut microbes, the lower bowel and liver in the evolution of HCC, and demonstrated that gut microbes could promote HCC. Several mechanisms could be involved. Firstly, bacteria may alter the colonic mucosal integrity and/or receptor activation, permitting the passive or facilitated the entry of harmful bacteria or their products into the circulation. Secondly, the microbial colonization of the bowel may invoke the release of numerous cytokines from the intestine and/or mesenteric lymph nodes that act upon the liver. Finally, intestinal bacteria may disrupt enterohepatic feedback loops, such as those associated with bile acid recirculation.

Recently, enterohepatic *Helicobacter* species, such as *H. hepaticus*, *H. bilis*, *Helicobacter sp. flexispira* and *H. cinaedi*, which belong to a rare phyla of luminal flora, have been identified in the lower intestinal and biliary tract of animals, and their overgrowth may cause chronic inflammatory bowel and liver diseases in rodents, poultry and primates. These bacteria have also been implicated in gastroenteritis, cholecystitis and certain liver diseases, including HCC in humans[8-11]. Several clinical observations have indicated that the modulation of the gut-liver axis using probiotics may play a therapeutic role, especially in the pathophysiological conditions where intestinal microflora may be involved as a cofactor of chronic liver damage.

In summary, it is possible that several factors related to CD may directly or indirectly affect the development of HCC in patients with CD. We consider that altered gut microbes would more likely disrupt enterohepatic homeostasis and promote the development of liver cancer than medication. A study of cumulative cases and further researches may unmask the intestinal bacteria that are associated with the increased risk of HCC in humans. Such microbes may represent attractive therapeutic targets.

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Events Calendar 2011

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

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An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/…"; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$; CONCLUSION (no more than 26 words).

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be

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Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

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

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

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]

No author given

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

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

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

Books

Personal author(s)

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

Chapter in a book (list all authors)

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

Author(s) and editor(s)

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

Conference proceedings

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

Conference paper

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

Electronic journal (list all authors)

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

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